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The increased severity in patients presenting to hospital with diarrhea in Dhaka, Bangladesh since the emergence of the hybrid strain of *Vibrio cholerae* O1 is not unique to cholera patients

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SUMMARY

Background: A hybrid strain of *Vibrio cholerae* O1 El Tor that expresses a classical cholera toxin (CT) emerged in 2001. This hybrid variant rapidly replaced the previous El Tor strain around the world. The global emergence of this variant coincided with anecdotal reports that cholera patients were presenting with more severe dehydration and disease in many locations.
Methods: A comparison was made of the severity of disease before and after the emergence of the hybrid strain in cholera patients attending an icddr,b hospital in Dhaka, Bangladesh.
Results: It was found that cholera patients presented with more severe dehydration and severe disease in the later period. However, this was also true for all non-cholera patients as well. In addition, in sub-analyses of patients who presented with rotavirus and enterotoxigenic *Escherichia coli* (ETEC), similar results were found. Comparing the two periods for differences in patient characteristics, nutritional status, vaccination status, and income, no plausible cause for patients presenting with more severe disease was identified in the later period.
Conclusions: As a shift in severity for both cholera and non-cholera was observed, these results indicate that the altered El Tor strain cannot fully explain the difference in cholera severity before and after 2001.

1. Introduction

Cholera is a life-threatening dehydrating diarrheal disease that results from the ingestion of toxigenic serogroups of *Vibrio cholerae*. *V. cholerae* is differentiated serologically by the O antigen of its lipopolysaccharide (LPS). The vast majority of human cholera is caused by the O1 and O139 serogroups, which carry the genes for cholera toxin (CT).[1] The O1 serogroup of *V. cholerae* is further classified into two biotypes (classical and El Tor) and two major serotypes (Inaba and Ogawa).[2] Classical and El Tor strains differ from each other in both phenotypic and genetic characteristics, as well as by small differences in the type of CT produced. Although classical strains are thought to have caused the first six cholera pandemics between 1817 and 1923, and continued to cause endemic and epidemic disease after that, the El Tor biotype emerged in 1905 and is the causative agent of the current seventh cholera pandemic that started in 1961 and continues today. El Tor biotype strains have now fully replaced the earlier classical strains, and the last classical strain of *V. cholerae* O1 recorded by the International Centre for Diarrheal Disease Research (icddr,b) was identified in 1992.[1,3,4]

Cholera in Bangladesh occurs both as an endemic disease, with seasonal peaks before and after the monsoons, and in epidemics that often take place during or following frequent floods, droughts, and cyclones that occur in the country.[5] Several factors have previously been associated with a higher risk of cholera and cholera with severe dehydration, including periods of flooding, malnutrition, retinol deficiency, partial and pre-existing immunity, and the presence of blood group O and other genetic risks factors.[5–12]

It has been suggested that cholera outbreaks have become more frequent and severe over the past 10–15 years,[5,11,13,14] with higher

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morbidity. One postulated explanation for the increasing severity of disease is the emergence in the year 2001 of strains of *V. cholerae* O1 El Tor that have acquired CT genes of the classical biotype, but that are otherwise similar to previous seventh pandemic El Tor strains.[14] These strains have been referred to as 'altered El Tor biotype strains'.[15] The relationship between altered El Tor biotype strains and more severe disease has been proposed because cholera caused by classical strains of *V. cholerae* O1 has often been more severe than that caused by El Tor strains when both biotypes have co-circulated;[2,4] this suggests that the differences in CT genes might relate to disease severity. However, careful documentation to show that cholera has been more severe in recent years than in previous years, and an exploration of the possible explanations for this change in severity, has not, to the authors' knowledge, been undertaken previously. To address this, a systematic analysis of the surveillance system that is in place at icddr,b and records the causes of diarrhea seen, disease severity, and a variety of patient data, was performed in the present study.

In Bangladesh, altered strains of El Tor *V. cholerae* O1 producing CT of the classical type were first identified in 2001,[15,16] and the molecular analysis of *V. cholerae* strains isolated from patients attending icddr,b since 2001 has shown that 100% of the strains have this hybrid or altered phenotype.[17] Therefore the time periods selected for this study were a 4-year interval after the emergence of the hybrid strains (2005–2008) and a 4-year interval before the emergence of the altered El Tor strains (1997–2000). One major flood occurred in each of these study periods (1998 and 2007).

This comparison allows examination of the potential role of the altered El Tor biotype strains in cholera severity. To determine whether any possible association was cholera-specific, similar analyses were also performed for 'all non-cholera patients', and sub-analyses were performed for other common causes of watery diarrhea, i.e., rotavirus and enterotoxigenic *Escherichia coli* (ETEC).

2. Materials and methods

2.1. Study design

This study was conducted at the Clinical Research and Service Centre (CRSC) of the International Centre for Diarrheal Disease Research (icddr,b) in Dhaka, Bangladesh. The hospital cares for approximately 125 000 patients annually, including 10 000–20 000 cholera patients. A surveillance system exists at the icddr,b that systematically samples children and adults with diarrheal illnesses. Since 1996, every 50th patient has been enrolled in the systematic surveillance system. Patients entered into the surveillance system and their family members are interviewed by health workers who collect demographic, socioeconomic, and clinical data. A physician documents the clinical condition, including dehydration status, as per World Health Organization (WHO) guidelines,[18,19] and a stool or rectal swab sample is collected for microbiological evaluation. All demographic, microbiological, treatment, and outcome data are recorded systematically and entered into a database; this database was used for the present study. During the earlier time period of this severity study, 1997–2000, an estimated 9940 patients were entered into the surveillance system, whereas in the more recent time period, 2005– 2008, details of 9237 patients were entered.

2.2. Microbiological evaluation

As part of the surveillance system, stool or rectal swab specimens were evaluated for enteric pathogens including *V. cholerae*, ETEC, *Salmonella spp*, *Shigella spp*, *Campylobacter jejuni*, and rotavirus, using standard techniques described previously.[20–23] For the isolation of *V. cholerae*, specimens were cultured on taurocholate–tellurite–gelatin agar plates. Specimens were also enriched in alkaline peptone water for 4 h and then cultured.[24] Specific monoclonal antibodies were used on colonies of *V. cholerae* to detect *V. cholerae* O1 and O139 serogroups, as well as to differentiate between Ogawa and Inaba serotypes of *V. cholerae* O1.[23,25,26] Every 100th strain of *V. cholerae* O1 was typed by PCR to distinguish the altered El Tor variant from the original El Tor biotype. Quantification of CT production was not carried out.[15] For the detection of ETEC, specimens were cultured overnight on MacConkey agar plates;[22,27] six freshly isolated lactose-fermenting *E. coli* colonies were isolated and genes for heat-labile or heat-stable enterotoxin were detected by PCR, as described previously.[28] Stool samples were also examined by direct microscopy to detect enteric parasites.[7]

2.3. Assessing clinical severity

As part of the surveillance system at the icddr,b hospital, the dehydration status of the patient on presentation was determined by Dhaka method based on WHO criteria.[42] The assessment was based on clinical evaluation and examinations of the level of consciousness, eyes, and tongue, the presence of thirst, a skin-pinch test, and radial pulse; these assessments of the patient were performed by trained health personnel. Criteria for assessing hydration status were consistent throughout the study periods.

The nutritional status of children was expressed in terms of the number of standard deviations of an anthropometric index, including height-for-age, weight-for-age, and weight-for-height expressed as a '*Z*-score', in relation to the new WHO growth standards.[29] The anthropometric measurements used were HAZ (height-for-age *Z*-score), WAZ (weight-for-age *Z*-score), and WHZ (weight-for-height *Z*-score). *Z*-scores of <-2 standard deviations (SD) were considered as indicating a moderate to severe degree of being stunted, underweight, or wasted, respectively. A *Z*-score of <-3 SD was considered evidence of severe malnutrition in all categories.

2.4. Vaccination status of children

The icddr,b surveillance system records the clinical history, including the immunization status of children. In the case of a child, the parents or female caregivers are interviewed, and a trained research assistant collects this information using a predefined and pre-tested surveillance questionnaire; responses are entered into an electronic database. From this database, information was extracted for 669 children aged <2 years attending the hospital during the two time periods (1997–2000 vs. 2005–2008). With regard to the recorded vaccination status, this was obtained mostly from the interview of parents/female caregivers; EPI health cards were reviewed in about 2% of cases.

2.5. Statistical analysis

Statistical analyses were performed using SPSS v. 12.0 software (SPSS Inc., Chicago, IL, USA). Differences in proportions of cases were assessed by Pearson's Chi-square analysis. The strength of associations was assessed by calculation of the odds ratio (OR) with 95% confidence intervals (CIs) using Epi Info version 3.4 (Epi Info 2002; US Centers for Disease Control and Prevention). Comparison between two means was performed using the independent samples *t*-test; when data were not normally distributed, the Mann–Whitney *U*-test was used. Statistical significance was defined as a two-tailed *p*-value of <0.05.

3. Results

3.1. Patients entered into the hospital surveillance system and comparison of pathogens isolated between the two time periods

The icddr,b hospital surveillance data from a 4-year period before the first isolation of altered El Tor variant strains of *V. cholerae* O1 (1997–2000) were compared to data from a later 4-year period during which 100% of isolates of *V. cholerae* O1 were of the altered El Tor variant (2005–2009) (Table 1). Flooding is a regular occurrence in Dhaka, and it has previously been shown that the cholera burden and severity increase during flooding.[13] The two study periods were chosen to cover the periods pre- and post-emergence of the hybrid strain of *V. cholerae* and were matched in number of years; each period included a single flood.

Overall, the proportion of pathogens was comparable in the two periods, with a slight decrease in proportion of cholera patients in the second period (28% vs. 25%). Serotypes of cholera fluctuate routinely. Ogawa strains predominated in the earlier period, but Ogawa and Inaba strains were more equally represented in the later time period. Although *V. cholerae* O139 strains were present in the earlier time period, they were not detected in the later time period. The second most common pathogen isolated in both periods was rotavirus (24% and 22%, respectively). The rate of isolation of several other diarrheal pathogens from patients decreased slightly between the two time periods, including ETEC, rotavirus, *Shigella spp*, *Salmonella spp*, and *Entamoeba histolytica* (Table 1). In the subset of children under 5 years of age (data not shown), the frequency of detection of rotavirus increased a small but significant amount between the two time periods (39% vs. 43%; $p < 0.001$).

3.2. Comparison of disease severity in patients with cholera and other pathogens between the two time periods

The age and sex of patients with cholera was not significantly different between the two time periods (Table 2). Cholera patients admitted in the later time period had evidence of more severe disease on admission, as reflected by a larger percentage having more than 6 stools a day, more patients having severe dehydration on presentation, more patients requiring intravenous fluids, and patients staying slightly longer in the hospital before being discharged home. The increased severity of diarrheal disease was seen in both adults and children less than 5 years of age.

The severity of disease for all non-cholera patients seen in the two time periods (7182 for the period 1997–2000 and 6923 for the period 2005–2008; Table 3) was similarly assessed, as well as the severity in subsets of patients presenting with ETEC (1243 for the period 1997–2000 and 617 for the period 2005–2008) and rotavirus (2361 for the period 1997–2000 and 2009 for the period 2005–2008) (data not shown). Importantly, it was found that the non-cholera patients as a group also presented with more severe dehydration and more severe disease in the later period (Fig. 1). In addition, in the sub-analyses of patients who presented with rotavirus and ETEC, other common causes of dehydrating diarrhea in Bangladesh, similar results were found: patients with these pathogens also presented with more severe disease and severe dehydration in the later time period than in the earlier time period (data not shown).

3.3. Correlation of cholera severity with malnutrition in children

The severity of cholera related more to indices of weight than height, and was even more prominent in undernourished and wasted patients in the later time period (64% vs. 71%; 64% vs. 75%) (**Supplementary Material**, Table S1A). Malnourished patients admitted with diarrhea due to pathogens other than *V. cholerae* also had more frequent severe dehydration, similar to those patients with cholera (**Supplementary Material**, Table S1B).

Table 1
Total patients admitted to the icddr,b hospital and included in the surveillance system during the two periods studied, and pathogens identified

	Total patients in surveillance system		
	1997–2000 (n = 9940)	2005–2008 (n = 9237)	
Pathogens isolated	No. (% of total)	No. (% of total)	p-Value
Vibrio cholerae (total)	2758 (28.0)	(25.0)	0.16
V. cholerae O1[a]	2266 (23.0)	2314 (25.0)	<0.001
Ogawa	1940 (20.0)	1336 (14.0)	<0.001
Inaba	326 (3.3)	978 (11.0)	<0,001
V. cholerae O139	492 (4.9)	0 (0)	
Rotavirus	2361 (24.0)	2009 (22.0)	<0.001
ETEC	1243 (13.0)	617 (6.7)	<0.001
Shigella	549 (5.5)	300 (3.0)	<0.001
Salmonella	136 (1.4)	96 (1.0)	0.03
Giardia	178 (1.8)	157 (1.7)	0.23
Entamoeba histolytica	125 (1.3)	80 (0.9)	<0.001
Others	1840 (19.0)	2017 (21.0)	<0.001

ETEC, enterotoxigenic *Escherichia coli*.
[a] From 2001 onwards, all *V. cholerae* O1 strains isolated at the icddr,b were of the altered El Tor biotype; prior to that, all were of the original El Tor biotype.

Table 2
Severity of disease in patients admitted with cholera to the icddr,b hospital during the two periods studied: all cholera patients

	1997–2000 (n = 2758)	2005–2008 (n = 2314)	p-Value[a]
	No. (% of total)	No. (% of total)	
Severe dehydration	1315 (48)	1635 (71)	<0.001
IV fluids required	1877 (68)	1851 (80)	<0.001
>6 stools/day	2531 (92)	2189 (95)	<0.001
>10 vomit/day	426 (15)	380 (16)	0.344
Length of hospital stay in hours, median[b]	24	27	<0.001
Fever >37.7 °C	55 (2.0)	15 (0.7)	<0.001
Age, years, median	21	20	
Sex, male	1521 (55)	1314 (57)	0.242

IV, intravenous.
[a] p-values were derived from Chi-square tests.
[b] Mann–Whitney U-test, for continuous variables.

Table 3
Severity of disease in patients admitted with non-cholera to the icddr,b hospital during the two periods studied: all non-cholera patients

	1997–2000 (n = 7182)	2005–2008 (n = 6923)	p-Value[a]
	No. (% of total)	No. (% of total)	
Severe dehydration	554 (8)	1347 (20)	<0.001
IV fluids required	1047 (15)	1728 (26)	<0.001
>6 stools/day	6315 (89)	6287 (92)	<0.001
>10 vomit/day	415 (6)	443 (6)	0.344
Length of hospital stay in hours, median[b]	8	10	<0.001
Fever >37.7 °C	576 (8)	347 (5)	<0.001
Age, years, median	21	20	
Sex, male	4247 (60)	4112 (60)	0.242

IV, intravenous.
[a] p-values were derived from Chi-square tests.
[b] Mann–Whitney U-test, for continuous variables.

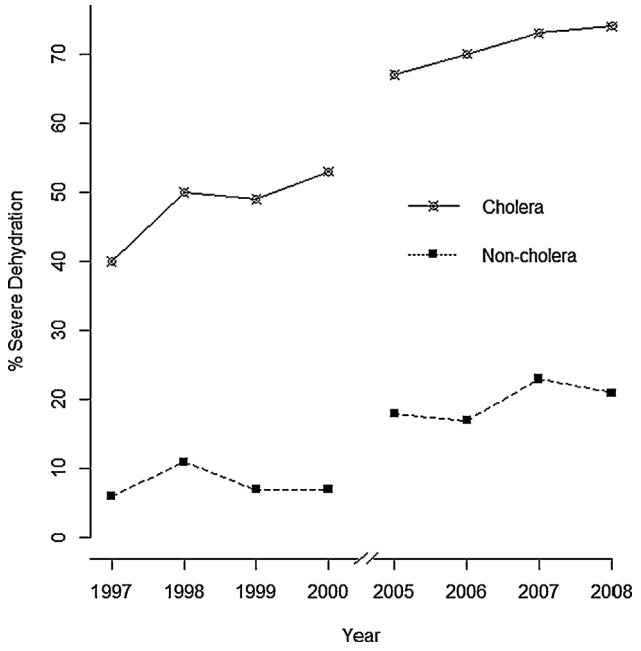

Figure 1. Severity of disease in cholera and non-cholera patients admitted to the icddr,b hospital by year.

3.4. Changes in nutritional and vaccination status of children between the two time periods

Malnutrition indicators as well as the vaccination status of children were compared between the two time periods (**Supplementary Material**, Table S2A). Despite the increasing severity of cholera seen in the later time period, children were less malnourished in the later period (severely stunted 23% vs. 14%; severely undernourished 31% vs. 25%; $p = 0.03-<0.001$) and were more likely to have received childhood vaccines (DPT 69% vs. 81%; polio 69% vs. 81%; $p = 0.001-<0.001$) (**Supplementary Material**, Table S2A). This finding was also observed in the non-cholera cohort (**Supplementary Material**, Table S2B). A similar finding was observed in the rotavirus cohort, but no trend towards improved nutritional status was detected in the ETEC cohort (data not shown).

3.5. Changes in health-seeking behavior of patients with cholera between the two time periods

Other possible explanations for the observation of more severe dehydration in patients presenting with cholera in the later time period might include the following: (1) a change in health-seeking behavior, such as individuals self-treating less severe disease at home with antibiotics and ORS (oral rehydration salts) and coming to care later if that failed; (2) a change in the socioeconomic background of the patient, which could influence a range of variables related to disease severity; and (3) perhaps the need to travel longer distances to reach care in the later period, leading to patients arriving more severely ill. Although there was a slight but significant increase in the use of ORS at home before presentation in the population overall, there was no difference in the use of ORS in children under 5 years of age who also presented with increased cholera severity in the later time period (**Supplementary Material**, Table S3A). Children in the later period took antibiotics at home before presentation less frequently, although there was no difference in the population overall. There was also no difference in patients having diarrhea at home for more than a day before presentation, and no difference in the mean distance to the hospital between the two time periods. Finally, patients in the later period were less likely to come from a poorer family, consistent with the overall improvement in economic conditions in Bangladesh over the 12-year period of the study. Comparative analyses of the non-cholera cohort overall, and the subsets with rotavirus and ETEC specifically, also showed an increased use of ORS and decreased use of home antibiotics in the later period (**Supplementary Material**, Table S3B). Non-cholera patients, overall, and rotavirus diarrheal patients traveled further in the later period, although ETEC patients traveled a shorter distance.

4. Discussion

These data confirm earlier reports that cholera patients seen in more recent years have suffered an increased severity of diarrheal disease, with higher numbers of stools on presentation, more severe dehydration, longer hospital stays, and the requirement for more IV fluids, compared to patients seen prior to 2000.[5,14,15,30] Importantly, it was also found that all non-cholera diarrhea patients and patients with diarrhea caused by rotavirus or ETEC were also more likely to present with more severe diseases in the later period compared to the earlier period. In order to try to understand this phenomenon, a number of possible contributors were analyzed, but it was not possible to identify an obvious correlate. Better nutritional status, better vaccination rates (suggesting improved engagement with the health care system), improved socioeconomic status, improved use of ORS, similar duration of illness, and similar distance traveled to seek care were found in the later period, consistent with the overall improvements in health indicators seen in Bangladesh in recent years.[31] As such, there is currently no obvious explanation for the observation that all diarrheal patients presenting for care at the icddr,b hospital seemed to have more severe disease. A shift in severity for both cholera and non-cholera was observed, and these results indicate that the altered El Tor strain cannot fully explain the difference in cholera severity before and after 2001.

Cholera severity has been associated with a range of host and environmental factors, including the timely use of rehydration, access to care, pre-existing immunity, blood group, and other host genetic factors.[6,7] Severe dehydration from cholera is seen more commonly in children who are malnourished.[32] Previous studies have suggested that a possible synergy between the depressed absorption of carbohydrate, protein, and fat by intestinal mucosal cells, lower lactase activity, and increased secretion produced by CT[33,34] might also contribute to an increased severity of cholera.

The relative prevalence of the two serotypes of *V. cholerae* O1, Ogawa and Inaba, fluctuates from season to season and from one year to another,[35] and usually one or the other of the serotypes is responsible for the majority of cases in any geographic area.[36,37] In the present study, there was a difference in the relative predominance of the two serotypes: *V. cholerae* O1 Ogawa strains predominated in the earlier period, but the serotypes were more evenly distributed in the later time period. It has previously been observed that cholera caused by the Ogawa serotype is associated with more severe dehydration.[38] Since Ogawa strains were less predominant in the later period, this difference in circulating serotypes does not appear to be a major determinant of the increased severity seen.

The biotype (classical versus El Tor) of *V. cholerae* can also affect disease severity, with cholera caused by classical biotype organisms being considered more severe.[2,3] There are a number of genotypic and phenotypic differences between the biotypes,[39] so the reason for this altered severity is not understood; however, it is possible that the differences between the classical and El Tor CT molecules themselves may contribute. Indeed, this is the reason that has been proposed to explain the more severe disease seen with hybrid strains of El Tor *V. cholerae* expressing classical-type

CT. To the authors' knowledge, there is a paucity of experimental data to support this hypothesis.

The reason that classical CT might cause more severe disease compared to El Tor CT is uncertain. The B subunit of the classical-type toxin differs from the El Tor CTB at the 18th and 47th amino acid residue positions (classical CT: His-18 and Thr-47; El Tor CT: Tyr-18 and Ile-47). The B subunit mediates attachment to the ganglioside GM1 on eukaryotic cell surfaces. The A subunit of the El Tor and classical CT toxins are identical in amino acid sequence.[40] In vitro, CT expression by El Tor strains is characteristically assessed using AKI medium, growth at 37 °C and in relative anaerobiosis; under these conditions, El Tor altered variant strains produce 20 times more CT than prototypic El Tor strains, an amount equivalent to that produced by classical strains.[39,41] Classical strains of *V. cholerae* are associated with more fluid accumulation in the rabbit ileal loop model than prototypic El Tor strains, but altered El Tor strains cause fluid secretion equivalent to that of classical strains.[39] The authors are not aware of data on the effect of altered El Tor CT in CHO cells or cyclic adenosine monophosphate (cAMP) induction in vitro. As such, whether hybrid strains of El Tor *V. cholerae* expressing classical CT can cause more severe disease in humans is possible but uncertain, and the data presented in the present report would suggest that increasing severity in cholera patients cannot be ascribed solely to the emergence of the hybrid organisms.

This study has a number of significant limitations, and could not address a number of potential influences. This was a retrospective analysis of surveillance data, and a shift in reporting bias between the two periods cannot be excluded, although staff at the icddr,b adhere to standard clinical operating procedures that characterize the assessment of dehydration by WHO criteria[42] and the microbiological analysis did not change during the study period. In addition, changes in population not discernible in the analysis are possible, although the catchment area of the icddr,b remained constant and there was no large in or out migration during the study periods. This study could also not exclude the possibility that the increasing severity might be related to differing levels of pre-existing immunity against cholera in the population; however, the percentage of patients presenting with cholera in both periods was the same (suggesting a similar population burden during the two periods). Similarly, in other studies by the present team, no change in the baseline vibriocidal antibody titer of cholera patients presenting to the icddr,b during these periods was found, an observation consistent with a stable burden of cholera in the community.[23,43,44] Blood group and other host factors can also affect cholera severity, and these data are not collected as part of the surveillance system, so it is not possible to comment on their potential impact, although no major population change occurred between the two time periods. No direct comparison was performed of the full microbiological characteristics, including full genomic sequences of the circulating strains during the two periods, although a previous analysis has shown that the older and hybrid strains are largely identical except for differences in CT.[3,4]

In summary, although the present data cannot exclude the possibility that the recently emerged variant El Tor *V. cholerae* O1 can cause more severe disease, the results of this study suggest that other yet undefined factors may be driving the observed shift in when and how patients with cholera and other dehydrating diarrheal illnesses present in Dhaka, Bangladesh.

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Ethics statement: The surveillance system and data analyses were approved by the Ethics Review Committee of the icddr,b.

Conflict of interest: No conflict of interest to declare.

Author contributions: FC, ET, FQ, AF, and SC contributed to the study design. FC, FQ, AF, and AIK contributed to the implementation and supervision of the study. FC, AK, FQ, ET, and AF analyzed the data and took responsibility for the accuracy of the data analysis. All authors participated in the writing of the manuscript and had access to the data in the study. All authors saw and approved the final version of the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijid.2015.09.007.

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