Impact factor 5.9

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Special edition:

Impact of anthropogenic changes to water on human pathogens

July 2016

Featuring

- Outbreak of unusual *Salmonella enterica* serovar Typhimurium monophasic variant 1,4 [5],12:i:-, Italy, June 2013 to September 2014
- Carbapenem-resistant isolates of *Acinetobacter baumannii* in a municipal wastewater treatment plant, Croatia, 2014

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A note from the editors: impact of anthropogenic changes to water on human pathogens

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Citation style for this article:
Eurosurveillance editorial team. A note from the editors: impact of anthropogenic changes to water on human pathogens. Euro Surveill. 2016;21(15):pii=30200. DOI: http://dx.doi.org/10.2807/1560-7917.ES.2016.21.15.30200

Article submitted on 14 April 2016 / accepted on 14 April 2016 / published on 14 April 2016

Water safety is vital, and the quality of water important in terms of public health. One of the objectives of the Water Quality and Health Strategy of the World Health Organization (WHO) for the 2013 to 2020 period, is to obtain 'the most rigorous and relevant evidence regarding water quality and health' [1]. In terms of infectious diseases, water can transport pathogens in the environment through different steps of the water cycle [2,3]. In the water, some physical or chemical parameters, e.g. temperature, pH, salinity, organic matter, may affect the survival of the pathogens [4]. Substances intended to control pathogens or insects (antimicrobials, antivirals and pesticides) can occur in water subsequent to their use [5,6] or manufacturing process [7,8]. This may paradoxically induce pathogen or vector resistance to these substances [9-12]. Moreover, water can also bear a number of pathogens with resistance acquired through other pathways [13]. Exposure of people to waterborne pathogens may occur by drinking or swallowing water, inhaling aerosolised droplets and contact with water through bathing and recreation [14,15]. Some pathogens may also be disseminated by water further in the environment (e.g. in the soil and air) potentially allowing human exposure. Consuming foods, grown on/in or irrigated with pathogen-contaminated water may also lead to infection [16,17].

As a number of changes to water, e.g. canalisation, temperature, nutrient enrichment, addition of pest-control or antimicrobial/viral substances, and pathogen contamination, result from human activities, it is relevant to understand their impact on infectious disease epidemiology. To provide some examples relevant for European public health, and to present issues related to the detection and identification of cases of waterborne outbreaks and the proof of anthropogenic change to water as the cause, we issued a call for papers [18]. Subsequent to this, we now publish five articles, through which a number of issues arise and which can be summarised as follows.

The challenges of outbreaks potentially caused by microbial contamination of water are first illustrated in a report from Italy, where an outbreak of monophasic *Salmonella* Typhimurium 1,4 [5],12:i:- with sole resistance to nalidixic acid is described [19]. Attempts to determine the source of this outbreak led to extensive environmental investigations. While its cause could not be ascertained, a number of surface water samples in the outbreak area, including of water used for growing fruit and vegetables, were positive for the outbreak strain. Moreover some water samples from local sewage treatment plants also tested positive, thus leading to the hypothesis that wastewater may have contaminated irrigation water [19]. The epidemiological investigation was complicated and the origin of the outbreak strain and how this strain acquired its resistance to nalidixic acid remain unresolved. The study reinforces the value of detecting waterborne outbreaks early.

Generally, water may become contaminated from a non-point source, such as the runoff of water from manure in agricultural fields, or from a point discharge, such as a hospital wastewater outlet or a sewage treatment plant. The issue of clinical wastewater harbouring microorganisms resistant to antimicrobials, and its subsequent effect on sewage and freshwater is important for public health, particularly if resistant bacteria introduced in the water can not only survive but also grow in wastewater. *Acinetobacter baumanni* for example, is considered a nosocomial pathogen, but its ecology is as of yet not fully understood and the observation of community outbreaks has made environmental niches suspect. A study from Croatia finds multiresistant *A. baumannii* strains in both influent and effluent water to a sewage treatment plant in Zagreb, indicating that such strains can evade the treatment process. The study shows moreover that isolated strains can survive and grow in effluent sewage water up to 50 days, posing a potential risk for further dissemination in the recipient river to the plant [20].

As a risk exists for surface water to become contaminated by wastewater pathogens, there is relevance in fully assessing its safety for further human use. A study from Serbia conducted during the bathing season reveals adenovirus and rotavirus genetic materials in recreational waters of the Danube, along popular public beaches in addition to faecal contamination. As the presence of viruses could not necessarily be predicted by the amount of bacteria measured in the water via routine quality control, the authors conclude that viral indicators may be helpful for further assessing the risks posed by water, in particular in areas where the sewer network is insufficient or inadequate [21].

Which panels of viruses could serve as relevant indicators of water quality in certain circumstances would need further investigations, as this may depend in part on their infectivity doses and persistence in environmental water. Also this might require to know what potential viruses contaminate the water to begin with, possibly first requiring agnostic screening techniques. In this regard, the development and implementation of assays that can be used for the surveillance of the whole population of viruses in water samples can be of interest. In this special issue, a methodology combining tangential flow filtration of sewage combined with deep sequencing, without the need for cell culture, is presented as an agnostic approach to survey viruses in sewage. The use of this methodology is proposed for the surveillance of poliovirus, but broader applications, including creating new viral sequence databases for retrospective analysis of presently unknown human viruses that may be discovered in the future are suggested [22].

Should it be a priori known what viruses likely contaminate water in an area, defining more specific tools to confirm their presence may be considered. Moreover in terms of further risk assessment, and as also discussed in the Serbian study in this issue [21], assays to determine the presence of infectious virus might also be of value.

As illustrated by some of the above studies [19,21], adequate management of wastewater is crucial. Indeed, water contaminated by wastewater can subsequently cumulate in larger water bodies such as lakes or the ocean. There, its impact may be less clear, as not only pathogens, but supportive nutrients may be carried by the wastewater. In combination with meteorological factors such as temperature, this may lead to the sporadic or intermittent occurrence of 'exotic' or 'unusual' pathogens in some areas [4]. An article from the Netherlands describes three cases of *Vibrio cholera* non-O1 serogroup (VCNO) bacteraemia reported in the country. Cases had been prior exposed to fish and/or had contact with surface water. The Dutch study includes a review of the literature to identify sources and risk factors for bacteraemia [23].

In conclusion, this special issue provides some insights into the importance of surveillance of pathogens in the water [19-23] and outbreaks or cases caused by waterborne pathogens [19,21,23]. Wider studies could help further refine criteria for assessing water treatment processes. Through pollution of ground water with antimicrobials and multi-resistant bacteria, waterborne outbreaks of multi-resistant bacteria are likely to become more frequent in the future. The special issue illustrates that addressing the problems due to anthropogenic changes to water on the epidemiology of human pathogens will require a multi-disciplinary approach.

References

1. World Health Organization (WHO). Water Quality and Health Strategy 2013-2020. Geneva: WHO; 2013. Available from: http://www.who.int/water_sanitation_health/publications/2013/water_quality_strategy/en/
2. World Health Organization (WHO). Guidelines for drinking water quality.3rd edition. Geneva: WHO; 2008. Chapter 11, Microbial fact sheets. Available from: http://www.who.int/water_sanitation_health/dwq/GDW11rev1and2.pdf
3. Huckele S, Track T. Risk management of emerging compounds and pathogens in the water cycle (RiSKWa).Environmental Sciences Europe.2013;25:1. DOI: 10.1186%2F2190-4715-25-1
4. Hunter PR. Climate change and waterborne and vector-borne disease.J Appl Microbiol. 2003;94(s1) Suppl;37S-46S. DOI: 10.1046/j.1365-2672.94.s1.5.x PMID: 12675935
5. Sura S, Degenhardt D, Cessna AJ, Larney FJ, Olson AF, McAllister TA. Transport of three veterinary antimicrobials from feedlot pens via simulated rainfall runoff.Sci Total Environ. 2015;521-522:191-9. DOI: 10.1016/j.scitotenv.2015.03.080 PMID: 25839178
6. Chee-Sanford JC, Mackie RI, Koike S, Krapac IG, Lin YF, Yannarell AC, et al. Fate and transport of antibiotic residues and antibiotic resistance genes following land application of manure waste. J Environ Qual. 2009;38(3):1086-108. DOI: 10.2134/jeq2008.0128 PMID: 19398507
7. Marathe NP, Regina VR, Walujkar SA, Charan SS, Moore ER, Larsson DG, et al. A treatment plant receiving waste water from multiple bulk drug manufacturers is a reservoir for highly multi-drug resistant integron-bearing bacteria. PLoS One. 2013;8(10):e77310. DOI: 10.1371/journal.pone.0077310 PMID: 24204801
8. Larsson DG, de Pedro C, Paxeus N. Effluent from drug manufactures contains extremely high levels of pharmaceuticals.J Hazard Mater. 2007;148(3):751-5. Available from: DOI: 10.1016/j.jhazmat.2007.07.008 PMID: 17706342
9. Kümmerer K. Antibiotics in the aquatic environment--a review--part I. Chemosphere. 2009;75(4):417-34. DOI: http://dx.doi.org/10.1016/j.chemosphere.2008.11.086
10. Söderström H, Järhult JD, Olsen B, Lindberg RH, Tanaka H, Fick J. Detection of the antiviral drug oseltamivir in aquatic environments.PLoS One. 2009;4(6):e6064. DOI: 10.1371/journal.pone.0006064
11. Berglund B. Environmental dissemination of antibiotic resistance genes and correlation to anthropogenic contamination with antibiotics.Infect Ecol Epidemiol. 2015;5:28564.
12. Brogdon WG, McAllister JC. Insecticide resistance and vector control.Emerg Infect Dis. 1998;4(4):605-13. DOI: 10.3201/eid0404.980410 PMID: 9866736
13. Walsh TR, Weeks J, Livermore DM, Toleman MA. Dissemination of NDM-1 positive bacteria in the New Delhi environment and its implications for human health: an environmental point prevalence study.Lancet Infect Dis. 2011;11(5):355-62. DOI: 10.1016/S1473-3099(11)70059-7 PMID: 21478057
14. Pandey PK, Kass PH, Soupir ML, Biswas S, Singh VP. Contamination of water resources by pathogenic bacteria.AMB Express. 2014;4(1):51. DOI: 10.1186/s13568-014-0051-x PMID: 25006540
15. World Health Organization (WHO). Bartram J, Chartier Y, Lee JV, Pond K, Surman-Lee S, Editors. Legionella and the prevention of legionellosis. Geneva: World Health Organization (WHO); 2007. Available from: http://www.who.int/water_sanitation_health/emerging/legionella.pdf

16. Centers for Disease Control and Prevention (CDC). Water Contamination. Atlanta: CDC; [Accessed 12 Apr 2016]. Available from: http://www.cdc.gov/healthywater/other/agricultural/contamination.html
17. Food and Agriculture Organization of the United Nations (FAO). Quality control of wastewater for irrigated crop production. Rome: FAO; 1997. Chapter 2 - Health risks associated with wastewater use. Available from: http://www.fao.org/docrep/w5367e/w5367e04.htm
18. Eurosurveillance editorial team., Call for papers for a special issue on impact of anthropogenic changes to water on human pathogens. Euro Surveill. 2015;20(2):21010.
19. Cito F, Baldinelli F, Calistri P, Di Giannatale E, Scavia G, Orsini M, et al. Outbreak of unusual Salmonella enterica serovar Typhimurium monophasic variant 1,4 [5],12:i:-, Italy, June 2013 to September 2014. Euro Surveill. 2016;21(15):30194. DOI: 10.2807/1560-7917.ES.2016.21.15.30194
20. Hrenovic J, Goic-Barisic I, Kazazic S, Kovacic A, Ganjto M, Tonkic M. Carbapenem-resistant isolates of Acinetobacter baumannii in a municipal wastewater treatment plant, Croatia, 2014.Euro Surveill. 2016;21(15):30195. DOI: 10.2807/1560-7917.ES.2016.21.15.30195
21. Jovanović Galović A, Bijelović S, Milošević V, Hrnjaković Cvjetkovic I, Popović M, Kovačević G, et al. Testing for viral material in water of public bathing areas of the Danube during summer, Vojvodina, Serbia, 2014. Euro Surveill. 2016;21(15):30196. DOI: 10.2807/1560-7917.ES.2016.21.15.30196
22. Furtak V, Roivainen M, Mirochnichenko O, Zagorodnyaya T, Laassri M, Zaidic SZ, et al. Environmental surveillance of viruses by tangential flow filtration and metagenomic reconstruction. Euro Surveill. 2016;21(15):30193. DOI: 10.2807/1560-7917.ES.2016.21.15.30193
23. Engel MF, Muijsken MA, Mooi-Kokenberg E, Kuijper EJ, van Westerloo DJ. Vibrio cholerae non-O1 bacteraemia: description of three cases in the Netherlands and a literature review. Euro Surveill. 2016;21(15):30197. DOI: 10.2807/1560-7917.ES.2016.21.15.30197

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Outbreak of unusual *Salmonella enterica* serovar Typhimurium monophasic variant 1,4 [5],12:i:-, Italy, June 2013 to September 2014

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Citation style for this article:
Cito F, Baldinelli F, Calistri P, Di Giannatale E, Scavia G, Orsini M, Iannetti S, Sacchini L, Mangone I, Candeloro L, Conte A, Ippoliti C, Morelli D, Migliorati G, Barile NB, Marfoglia C, Salucci S, Cammà C, Marcacci M, Ancora M, Dionisi AM, Owczartek S, Luzzi I, on behalf of the outbreak investigation group. Outbreak of unusual Salmonella enterica serovar Typhimurium monophasic variant 1,4 [5],12:i:-, Italy, June 2013 to September 2014. Euro Surveill. 2016;21(15):pii=30194. DOI: http://dx.doi.org/10.2807/1560-7917.ES.2016.21.15.30194

Article submitted on 15 April 2015 / accepted on 14 April 2016 / published on 14 April 2016

Monophasic variant of *Salmonella enterica* subspecies *enterica* serovar Typhimurium (monophasic *S.* Typhimurium), with antigenic structure 1,4,[5],12:i:-, appears to be of increasing importance in Europe. In Italy, monophasic *S.* Typhimurium represented the third most frequent *Salmonella* serovar isolated from human cases between 2004 and 2008. From June 2013 to October 2014, a total of 206 human cases of salmonellosis were identified in Abruzzo region (Central Italy). Obtained clinical isolates characterised showed *S.* Typhimurium 1,4,[5],12:i:- with sole resistance to nalidixic acid, which had never been observed in Italy in monophasic S. Typhimurium, neither in humans nor in animals or foods. Epidemiological, microbiological and environmental investigations were conducted to try to identify the outbreak source. Cases were interviewed using a standardised questionnaire and microbiological tests were performed on human as well as environmental samples, including samples from fruit and vegetables, pigs, and surface water. Investigation results did not identify the final vehicle of human infection, although a link between the human cases and the contamination of irrigation water channels was suggested.

Introduction

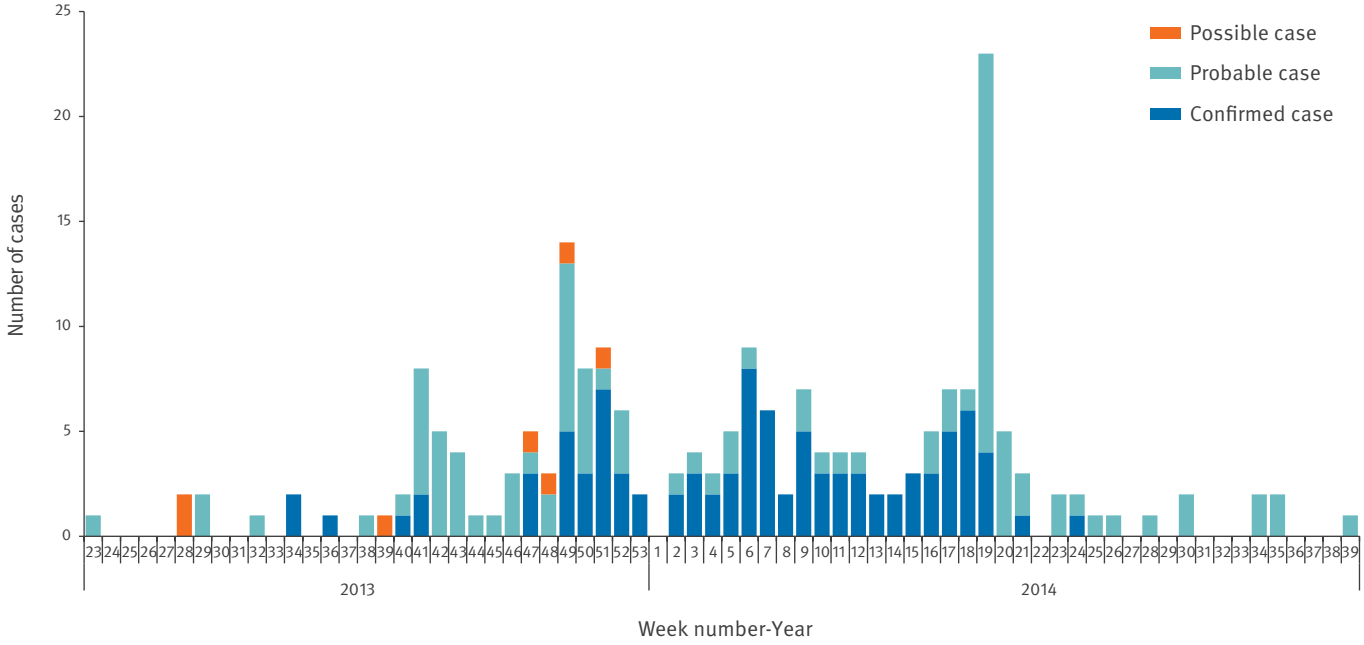
Monophasic variant of *Salmonella enterica* subspecies *enterica* serovar Typhimurium (monophasic *S.* Typhimurium), with antigenic structure 1,4 [5],12:i:-, is considered an emergent pathogen in many European countries [1]. It accounted for 4.6%, 7.2% and 8.6% of total cases of salmonellosis reported to the European Surveillance System (TESSy) in 2011, 2012 and 2013 respectively [2], and ranked third among the *Salmonella* serovars identified in humans in the European Union (EU) during this period.

Human infection is usually acquired through the consumption of contaminated food and several food-borne outbreaks caused by this serovar have been reported in Canada, Europe and the United States (US) [1-3]. Fresh beef and pork account for the major sources of infection, but dried sausages and frozen chicken pies have also been implicated in food-borne outbreaks, as well as vegetables and juices. The majority of monophasic *S.* Typhimurium isolates detected in live animals originates from pigs [3]. Human outbreaks potentially related to environmental sources, including water, have been also observed [4-8].

In Italy, the laboratory-based surveillance network for enteric pathogens EnterNet Italia [9] provides information on the microbiological characteristics of *Salmonella* spp. strains isolated from humans. The data are gathered through a network of regional reference laboratories which characterise the strains isolated from peripheral diagnostic laboratories. The EnterNet Italia network is coordinated by the National Reference Laboratory for *Salmonella* infection in humans of the Istituto Superiore di Sanità (ISS).

Figure 1

Number of confirmed, probable and possible monophasic *Salmonella* Typhimurium outbreak cases based on symptom onset week[a], Italy, 2 June 2013–27 September 2014 (n = 204)[b]



[a] For 57 cases symptom onset date was not known and specimen date was used.

[b] Although a total of 206 cases were part of the outbreak, only 204 are depicted in this figure because for two confirmed cases the onset day and the specimen date were missing information.

According to EnterNet data, monophasic *S.* Typhimurium represented the third most frequent *Salmonella* serovar isolated from clinical samples in the country between 2004 and 2008 [10], ranking second in 2009 [11]. It accounted for 2.95 isolates per 100,000 population/year between 1980 and 2011, with particularly high isolation rates in children aged one to five years [12]. Strains characterised by resistance to ampicillin, streptomycin, sulfafurazole, and tetracycline (ASSuT) (with or without additional resistances) represented 75% of all the monophasic *S.* Typhimurium isolates from either 2008 or 2009. Forty-eight per cent of strains belonged to DT193 and 13% to U302. The most common pulsed field gel electrophoresis (PFGE) profiles were STMXB 00131 (47%) and STMXB 0079 (37%) [3].

In October 2013 a significant increase of human cases of salmonellosis in L'Aquila province (Abruzzo region, Central Italy) was reported to EnterNet Italia. In the period between June and October 2013 only, ca 30 salmonellosis cases, corresponding to 9.9 cases per 100,000 population [13], were observed in the L'Aquila province, clearly excessing levels of previous recent years. For example, between 2005 and 2009, the average number of salmonellosis cases notified in the province was equal to nine per year, with a maximum value of 30 annual cases in 2005 [14]. The vast majority of cases observed in L'Aquila province during 2013 and 2014 was due to monophasic *S.* Typhimurium. Isolates from patients' stool samples were indistinguishable by traditional typing methods, such as phage type, PFGE and multilocus-variable number tandem repeat Analysis (MLVA) and were solely resistant to nalidixic acid, which had never been observed in Italy in monophasic *S.* Typhimurium neither in humans nor in animals or foods [15]. According to TESSy, monophasic *S.* Typhimurium with this peculiar antimicrobial profile had also rarely been reported in the EU.

Here we present the results of the epidemiological, environmental and molecular investigations carried out in the Abruzzo region in 2013–2014. The aims of these investigations were to gather relevant information on exposures and to identify the potential sources of infection so as to allow adequate control measures.

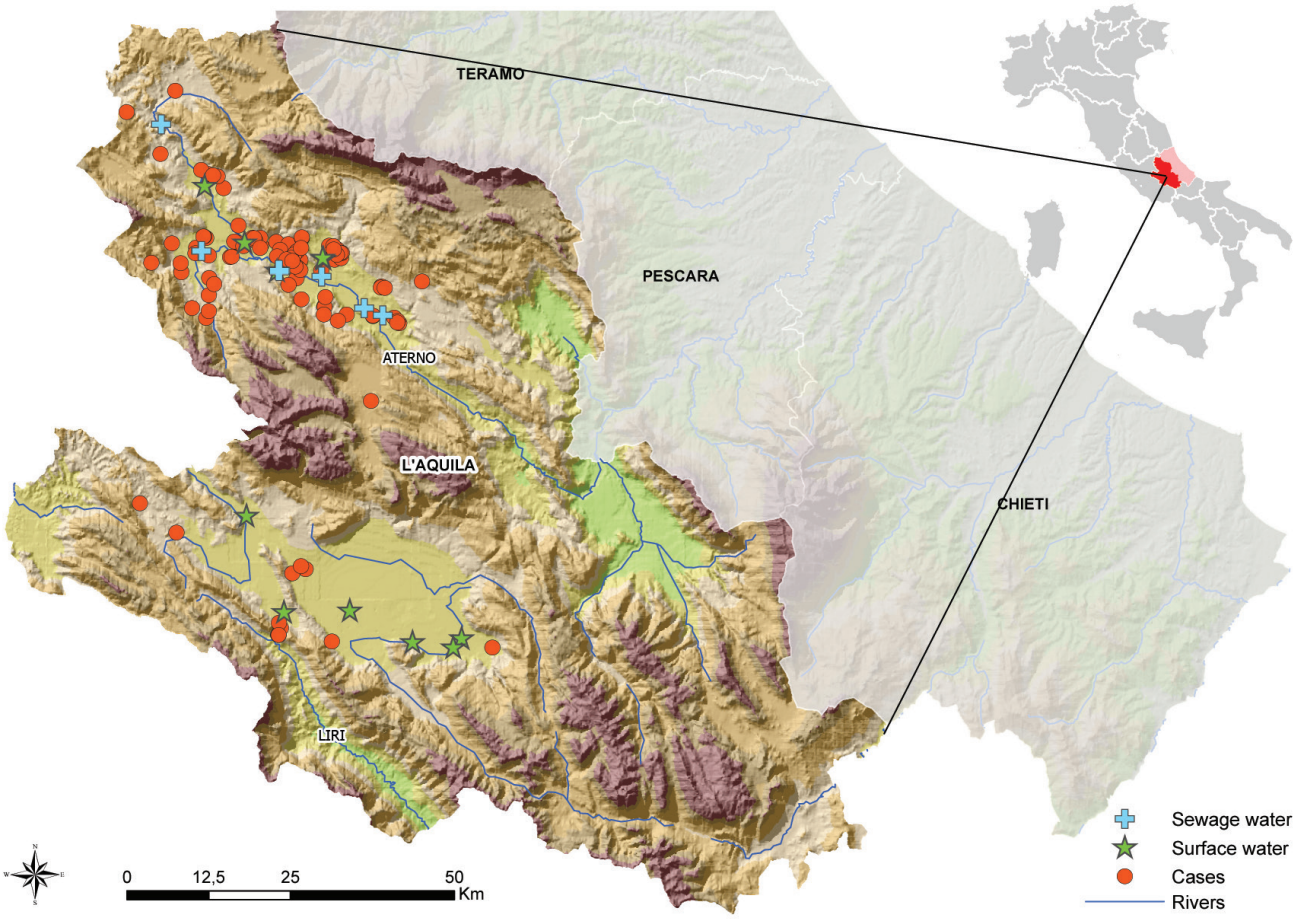
Methods

Case ascertainment and case definition

Following the observation of the significant increase of human cases of salmonellosis in L'Aquila province (Abruzzo region, Central Italy), an alert was sent to all public health services of Abruzzo region in October 2013. Prospective and retrospective active finding of cases was conducted for the period from January 2013 to October 2014, involving hospitals in Abruzzo as well as microbiological laboratories in the whole Italy through the EnterNet Italia surveillance network.

Figure 2

Geographical distribution of outbreak cases (n = 115)[a] and of surface and sewage water samples (n = 21) with a monophasic *Salmonella* Typhimurium outbreak strain isolate, L'Aquila province, Italy, April–October 2014



[a] Although there were a total of 179 outbreak cases in the Abruzzo region, only 115 are depicted in this figure because for 64 cases the residential address was missing information.

The outbreak strain was defined as: 'monophasic *S.* Typhimurium resistant to nalidixic acid, with a PFGE profile equal to the reference type XBAI.0027 and MLVA 3-14-11-NA-211 encoded by the molecular surveillance service (MSS) of the European Centre for Disease Prevention and Control (ECDC)'.

Definitions of possible, probable and confirmed cases were established on the basis of phenotypic and genotypic characteristics of the *Salmonella* isolates, and the existence of a possible epidemiological link with Abruzzo region (people living in Abruzzo or reporting to have travelled to Abruzzo in the seven day-period before the disease onset). A confirmed case was a patient epidemiologically linked to Abruzzo, who was laboratory confirmed with the outbreak strain from January 2013 onwards. A probable case was a patient epidemiologically linked to Abruzzo, who was laboratory confirmed with *Salmonella* group B from June 2013 onwards. A possible case was a patient with gastroenteritis epidemiologically linked to Abruzzo from June 2013 onwards, but who was not laboratory confirmed with *Salmonella*.

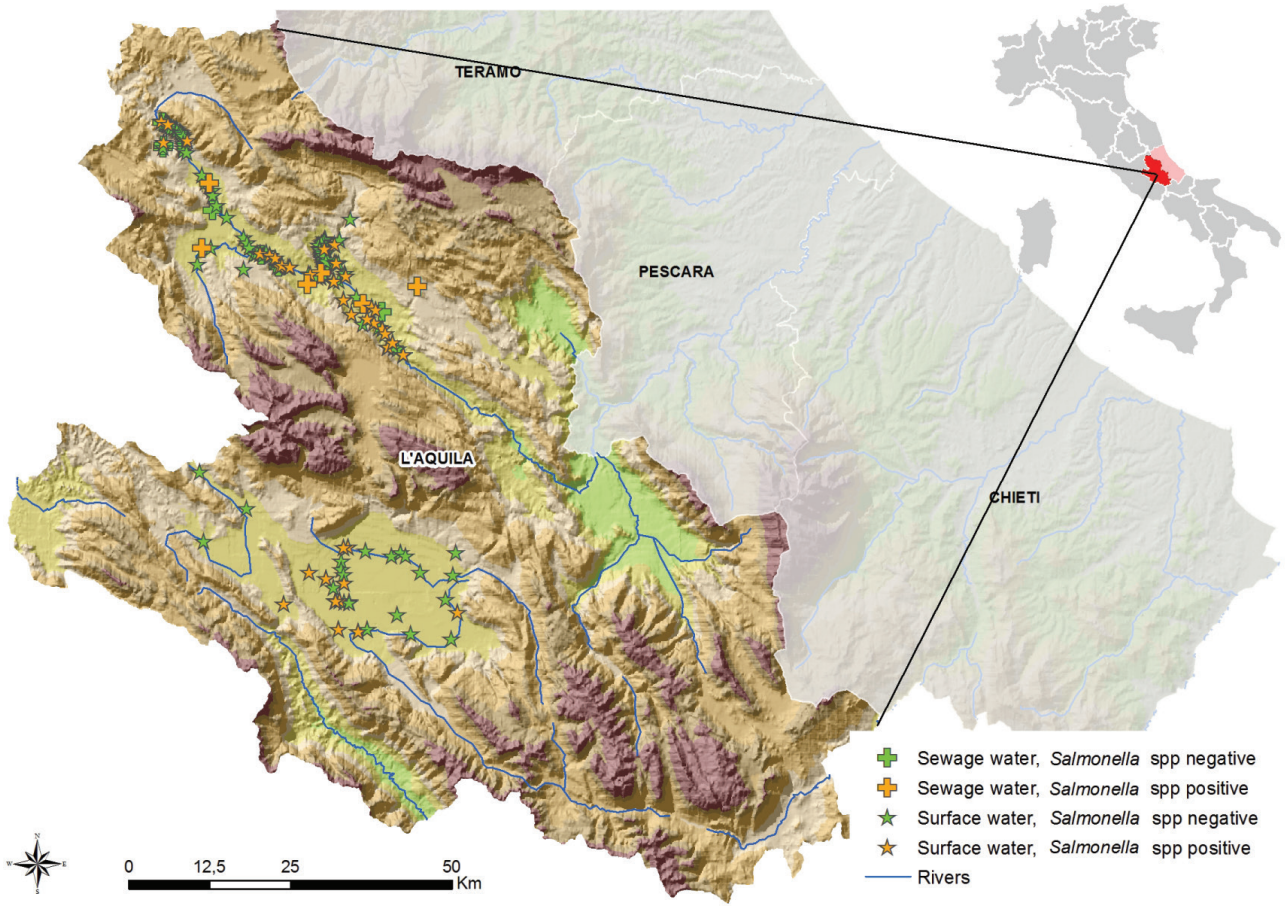
An international alert was also launched through the European Epidemic Intelligence Information System (EPIS) in April 2014.

Epidemiological investigations

From October 2013, cases were requested to respond to a standardised questionnaire. This was administered by a face to face interview of either the patients or, if they were younger than 18 years-old, their parents. Until the end of March 2014 the questionnaire was generic and included only information on demographic data and presence of clinical symptoms. Starting from April 2014, a more elaborate version was used to also collect information on the occurrence, duration and severity of clinical symptoms, healthcare seeking behaviours, and exposures possibly presenting a risk for *Salmonella* infection in the seven days before symptoms onset. These included exposure to possible animal reservoirs, contact with people with gastrointestinal illness, as

Figure 3

Geographical distribution and microbiological testing results of surface and sewage water samples in the L'Aquila province, Italy, April–October 2014 (n = 302)



well as travel, details on food and beverages consumed including their purchase places, and the consumption of meals out of home. Cases were also asked about the consumption of local handmade food products of animal and vegetable origins.

The implementation of a case–control study was originally considered but could not be realised due several difficulties which are further discussed.

Data from the questionnaires were entered into a database using Epi Info 7 (Centers for Disease Control and Prevention, Atlanta, United States (US)). Data management and analysis were performed using Stata 12 (Stata Corporation, Texas, US). Data derived from the 2011 demographic census performed by the Italian National Institute of Statistics (ISTAT) were used to calculate the attack rates [16]. The possible existence of a relationship between the age of cases and both the duration of clinical symptoms and disease severity was assessed by the calculation of Kendall's rank correlation coefficient.

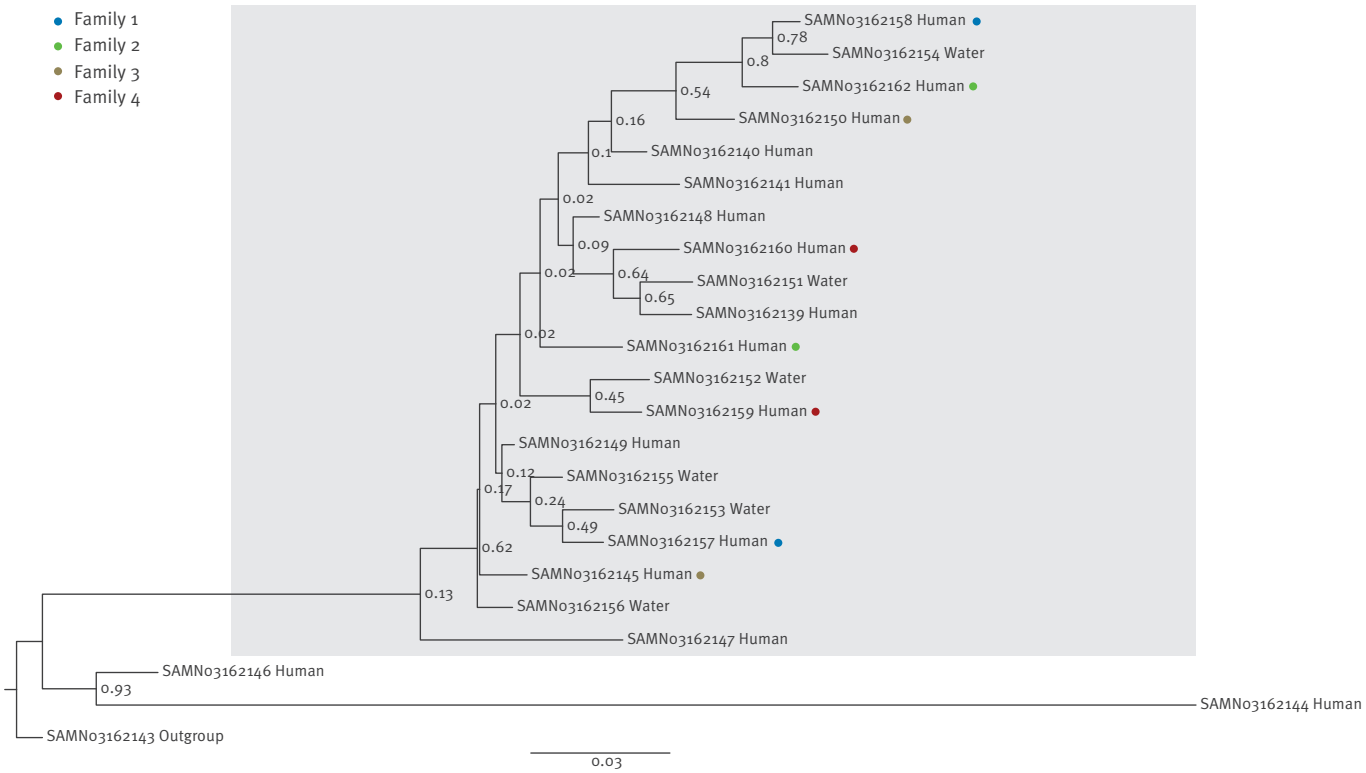
Environmental investigation

Given the temporal and spatial extension of the outbreak, a possible implication of environmental sources was suspected. Accordingly, the regional health authorities established a microbiological monitoring plan in April 2014. This included sampling of all commercial pig herds of L'Aquila province (faecal, dust, drinking water and feed samples were taken in each herd), and the examination of ileocaecal lymph nodes taken from swine at slaughterhouses to assess the status of *Salmonella* infection in pigs bred and slaughtered in the province. In addition, locally produced fruit and vegetables, mainly fresh vegetables grown in open fields and intended to be consumed raw, were directly collected in a subset of producing farms. These farms were selected on the basis of the results of surface water sampling, which was performed as further described. In each cultivated field at least eight samples of fruit and vegetables, with the minimum weight of 2 kg and chosen to be representative of the whole cultivated surface, were taken. Overall, 23 pig herds, 11 swine slaughterhouses and 17 fruit and vegetable-producing farms in L'Aquila province were investigated.

The monitoring plan also concerned surface water. Channels used for irrigation (including at farm sites) were sampled as well as surface water from three main catchment basins of the Tevere, Liri-Garigliano and Aterno rivers and their tributaries. Nine sewage water

Figure 4

Maximum likelihood relationship of monophasic *Salmonella* Typhimurium whole genome sequences, obtained from environmental water and clinical samples during an outbreak of monophasic *Salmonella* Typhimurium, L'Aquila province, Italy, April–October 2014



Bootstrap values are indicated at the tree nodes. The scale bar denotes the estimated number of nt substitutions per site.

treatment plants were additionally subjected to microbiological controls on fluids entering and leaving the plants. The sampling programme was performed until the end of October 2014.

Daily data on rainfall covering a period from September 2013 to June 2014 [17] were obtained from the Centre of Excellence for the Remote Sensing and Forecast of Severe Weather (CETEMPS) of L'Aquila University and a preliminary analysis was conducted to assess the possible association between rainfalls and the occurrence of salmonellosis cases, using the Pearson's correlation coefficient. The correlation was evaluated through a cross-correlation approach by using two different time lags: the time shift between rain and cases detection and a time window used to average the quantities of rain along a period of time before the occurrence of cases. Data were aggregated using five days interval since this was considered the best temporal aggregation to summarise and analyse data on rainfall and on the occurrence of salmonellosis case. Only cases occurring in the L'Aquila municipality and in the neighbouring municipalities were included in the analysis, since available data on rainfall referred to that area. The analysis was performed using the R free package [18].

Characterisation of strains

Biochemical identification of isolates was conducted using the automated Vitek 2 system (Biomerieux, Marcy l'Etoile, France). The isolates were serotyped with commercial antisera (Statens Serum Institut, Copenhagen, Denmark) according to the Kauffmann–White scheme by slide agglutination. After having identified the somatic antigen and the phase-1 flagellar antigen, if the second one was negative, the phase inversion method was used to allow the expression of the second flagellar phase. Strains were assigned to *Salmonella* serovar Typhimurium monophasic variant 1,4 [5],12:i:- on the basis of a multiplex polymerase chain reaction (PCR) [19]. Antibiotic susceptibility of isolates was evaluated by disk diffusion method [20]. Phage typing was performed using *S.* Typhimurium phages according to the method described by Anderson et al. [21]. Strains were typed by PFGE and MLVA according to the ECDC Laboratory standard operating procedures [22,23].

The genomes of 20 respective strains matching the outbreak strain definition and isolated from human (n = 14) and environment samples (n = 6; five from sewage plants and one from surface water) were fully sequenced. They were chosen on the basis of geographical location, timeline isolation and biochemical properties. Moreover most of the strains chosen to be

Table

Results of the environmental investigations on samples taken in pig herds, farms, slaughterhouses, sewage treatment plants and from surface waters in L'Aquila province, Italy, April–October 2014

Sampling place	Sample type	Number	Samples positive for the outbreak strain of monophasic *Salmonella* Typhimurium
Pig herds	Dust	26	0
	Faeces	41	0
	Feed	34	0
	Drinking water	22	0
Farms	Bulb vegetables	9	0
	Root vegetables	13	0
	Leaf vegetables	9	0
	Fruits	6	0
	Irrigation water	4	1
Slaughterhouses	Swine ileocaecal lymph nodes	32	0
Sewage treatment plants	Effluent water	111	9
Rivers and water channels	Surface water	191	11
Total	–	**498**	**21**

fully sequenced were isolated from cases belonging to the same families, whereby cases belonging to a family lived in the same household. Three additional isolates, two unrelated outbreak monophasic *S.* Typhimurium isolates (differing from outbreak strain for antimicrobial resistance patterns) and a further strain isolated five years prior, were also sequenced and added as controls and outgroup respectively. Whole genome sequencing was performed by a Personal Genome Machine (PGM) Ion torrent platform. After quality trimming, retained reads were in the 40 to 350 nt length range, exhibiting an average phred score of 30 and providing an estimated coverage spanning from 18x to 45x for the selected isolates. Trimmed reads were submitted to the Sequence Read Archive (SRA) repository [24] under the Bioproject accession number SRP049581 and were used to build a single nucleotide polymorphism (SNP) reference-based matrix using the US Food and Drug Administration (FDA) SNP-pipeline programme [25]. The phylogenetic tree was built using the Tamura–Nei model in molecular evolutionary genetics analysis (MEGA)6 [26]. Confidence of branch points was estimated by Fast bootstrap analysis with 500 replicates.

Results

Outbreak description

A total of 206 cases (98 confirmed, 101 probable, 7 possible) were identified in Italy between June 2013 and September 2014 (Figure 1). No cases possibly linked to this outbreak were reported from the 14 countries (including the US) reacting to the EPIS alert.

Of the 206 cases, the place of residence was unknown for 19. Among those with available information, only eight cases were detected outside the Abruzzo region. In this region with a total of 179 cases, most (n = 146) were reported from the province of L'Aquila. The attack rate was 14 cases per 100,000 inhabitants in Abruzzo region (95% confidence interval (CI): 12–16), and 49 cases per 100,000 inhabitants in L'Aquila province (95% CI: 41–58). Information on patient's age was collected for 192 cases and the median value was 4 years (range: 0–91 years). The age of cases significantly differed (Fisher's test $p<0.01$) from that of the Abruzzo population, taken as 'control' population, and the greatest number of cases (n = 98; 51%) occurred in patients aged between one and four years (Pearson's chi-squared: 1,248; $p<0.01$). No significant difference was observed by sex (53% males and 47% females).

Data on clinical signs were complete for 90 patients and the mean duration of illness was seven days (range: 1–30 days). Diarrhoea was the most important symptom (89/90; 99%), followed by abdominal pain (57/85; 67%), weakness (22/39; 56%), bloody diarrhoea (19/46; 41%), vomiting (32/86; 37%), fever defined as >37.5°C (28/92; 30%), nausea (13/77; 17%), and diffuse pain (4/35; 11%). Among symptomatic cases, 46 (51%) were hospitalised. The median age of hospitalised patients was four years (range: 1–91 years). No significant correlation was observed between the age of cases and the duration of the illness (Kendall's coefficient = 0.03) or its severity (Kendall's coefficient = 0.05). A man aged >70 years died following the *S.* Typhimurium infection.

Epidemiological investigation

Of a total 112 cases approached for interviews, 106 took part in the questionnaires, corresponding to a participation rate of 95%. Of these, 51 cases answered to the first generic questionnaire (48%) and 55 to the following more elaborate version (51%). Not all questions on clinical signs, travel history, animal or ill person

exposure, and on the food and beverage consumption were properly answered by all interviewed cases, thus the number of respondents for each question must be taken into account.

During the week before disease onset, eight of 49 respondents (16%) reported to have travelled. These eight cases resided in L'Aquila and had travelled outside the Abruzzo region (however, no cases travelled to the same place) and 24 of 95 (25%) respondents reported contacts with an individual presenting gastrointestinal illness. Contacts with pets were reported by 31 of 90 cases (34%).

In the week prior to the onset of the disease, 38 of 49 cases had consumed food outside their home: in school canteens (20/41), in restaurants (10/49), in bars (13/44), in fast foods (7/45), or in other places (8/23). As far as school canteens are concerned, the investigation was extended to verify possible common food suppliers and no common places or epidemiological links were identified.

The investigation on food eating habits did not reveal the consumption of any particular food considered at risk for *Salmonella* infection, apart from eggs and vegetables. Most of the cases reported drinking tap or bottled water, respectively 41/66 (62%) and 56/76 (74%).

Environmental investigation

All samples taken among the pig herds (n = 123), the fruit and vegetables produced by farms (n = 37), and in the slaughterhouses (n = 32) from April to October 2014 tested negative for *Salmonella* (Table). Monophasic *S.* Typhimurium isolates, fitting the definition of outbreak strain, were detected in 11 of 191 samples of surface water, in one of four samples of irrigation water taken on farms, and in nine of 111 samples of sewage treatment plants taken in several geographical locations of L'Aquila province (Table).

The positive sample of irrigation water was taken from irrigation pipelines with water from the Vera river, which is tributary of the Aterno river, and from which surface water samples also tested positive. The samples of fruit and/or vegetables taken from the fields irrigated with the contaminated water were negative for *Salmonella*. Monophasic *S.* Typhimurium outbreak strain was detected in five sewage treatment plants localised on the Aterno river and in one sewage treatment plant localised on the Raio stream flowing into Aterno river (Figure 2). The geographical location of 21 water samples testing positive for monophasic *S.* Typhimurium with the same characteristics as the outbreak strain is shown in Figure 2.

In addition, three further surface water and five sewage samples resulted positive for monophasic *S.* Typhimurium, but showing different phenotypic characteristics than the outbreak strain, while another 50 samples (34 of surface waters and 16 of sewage) were positive for other *Salmonella* serovars. The geographical locations of 302 sewage and surface water samples, which were microbiologically analysed as well as their testing results are reported in Figure 3.

The analyses performed to explore the possible correlation between rainfalls and the occurrence of salmonellosis cases gave a significant value of the Pearson's coefficient (0.47) when an increase of the average value of rainfall over a 10 day period was considered and for a time shift between rain and cases detection of 20 days.

Characterisation of strains

Phylogenetic relationships among the selected isolates were investigated using the SNP matrix obtained from the whole genome sequencing. The single maximum parsimonious (MP) and maximum likelihood (ML) analyses resulted in a single tree with similar topology (Figure 4). The two strains not matching the definition of outbreak strain, clustered as a separate clade with high bootstrap support, and one of them differed from the antigenic profile (SAMN03162144), while the other exhibited a completely different antimicrobial resistance profile (SAMN03162146). In contrast, outbreak-related isolates were grouped in a well-separated clade from the other strains. However, genetic relationships among isolates (both from patients and water samples) inside the outbreak clade were supported by very low bootstrap values (range: 13–80%). Subclades with higher bootstrap values include both water and clinical-related strains.

Discussion

In the outbreak described here, cases were solely identified in Abruzzo region and occurred continuously and uninterruptedly from June 2013 to September 2014. The outbreak strain was repeatedly isolated in sewage treatment plants and surface water, including water used for irrigation in the region. A cross-correlation analysis between rain episodes and human cases moreover showed that an increased level of rainfalls (averaged over a 10 days period) preceded the onset of salmonellosis cases by ca 20 days. Whole genome sequencing phylogenetic analysis, albeit not robust, also indicated some relationships between clinical and water strains. To gain more information on whether outbreak strains could be of clonal origin, the phylogenetic distances among the outbreak isolates would need to be carefully evaluated taking into account the relative long period of time during which the human cases occurred and the subsequent higher probability of *Salmonella* population differentiation and heterogeneity.

Taken together, the results are in line with the hypothesis of a waterborne mechanism for the outbreak, and might suggest a possible relationship between heavy precipitations and the presence of *Salmonella* in surface waters, maybe via the flooding of sewage treatment plants, subsequently leading to contamination of irrigation waters. That floods, as result of intensive

precipitation events, bring pathogens from sewage water to surface water is a known phenomenon: heavy rainfalls in a relatively short time can cause sewer overflows to surface water and/or soil, thus increasing the risk of contaminating irrigation waters [27]. Here, the contamination of surface and irrigation waters by the outbreak strain could also have been due to problems with the treatment plants or to persisting damage inflicted to water pipelines of the sanitary sewer system of the city of L'Aquila and surrounding villages by the devastating earthquake in 2009.

In the environmental investigation, the apparent absence of the outbreak strain in pigs reared in the Abruzzo region and the negative microbiological results in fruits and vegetables, may suggest the existence of yet undetected infection reservoirs, such as wild animals. Nevertheless, given the time elapsed between the possible occurrence of an environmental contamination and the collection of samples, the results cannot definitely rule out pigs or vegetables as sources of infection. Fruits and vegetables may be generally colonised by a wide variety of microorganisms and *Salmonella* is usually associated to fresh produce [28]. The occurrence of *Salmonella* in whole and fresh-cut leafy greens has been reported [1] and outbreaks due to *S.* Typhimurium, which may survive for extended time periods in manure and manure-amended soils [29], have been associated with the consumption of fresh lettuce [30]. Rainfalls have also been proven to play a role in *Salmonella* dispersal and contamination of tomato plants in the field, especially during concentrated and relatively intense rain events and when plastic mulch was used [6].

Aside from contamination via animal manure for fertilization purposes, it is also well known that vegetables can become contaminated with pathogens by irrigation water [31]. In the US this was confirmed in repeated incidents: a multistate outbreak of *Salmonella* Newport infection associated with eating tomatoes in 2002 and 2005 [5], an *E. coli* O157:H7 outbreak associated with shredded lettuce in 2006 [32] and the outbreak caused by *Salmonella* Saintpaul, which was found in irrigation water and in Serrano peppers [33].

A ban of using surface water to irrigate produce and other crop due to the presence of *Salmonella* spp. in some areas of L'Aquila province was already in force since September 2013, before the peak of the outbreak. Unfortunately the ban was just applied to a limited area of L'Aquila province. Only starting from June 2014 did the local health authorities ban the use of surface water for crop irrigation on a larger area [34-36]. Sanitization interventions were subsequently performed in water cleaning plants and no more human cases were observed after September 2014. The role of banning the use of surface waters for irrigation in the cessation of the outbreak can nevertheless not be ascertained and the possibility of multiple transmission routes from the environment to humans, involving food-vehicles, cannot be excluded.

The observed higher incidence in patients aged between one and four years-old is not surprising in *Salmonella* infections, given the increased vulnerability of children and the elderly to this infection. On the other hand, this finding could suggest the implication of food vehicles normally consumed also by children or the contamination of drinking water.

A case–control study would have added more significant information on the possible source of this outbreak. Although originally considered and planned, this study proved impossible. Indeed, the uncertainties on the times of first exposures due to interventions occurring late in the outbreak progress made it difficult to find suitable controls. In addition, the realisation of such a study was hampered by the extended geographical area involved.

During the period of the outbreak there was also an increase of cases of *Salmonella* infections due to *Salmonella* strains different from the outbreak strain. Since laboratory diagnosis concerned solely *Salmonella* spp. no other pathogen was investigated. The characterization of all pathogens responsible for cases of gastroenteritis might have added more information on the possible source of this outbreak and possibly further supported the hypothesis of a waterborne mechanism.

The delays between generating hypotheses on the contamination routes and implementing the sampling programme and interviews of cases could also have affected the identification of a food vehicle. This confirms once again the importance of having an effective epidemiological surveillance system in place, to early identify all possible suspected clusters of infection and to quickly mobilise the human and diagnostic resources for a rapid identification of sources and vehicles of infection.

Outbreak investigation group

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Acknowledgement

The authors acknowledge the contribution of the Abruzzo Regional Veterinary Service and of all the personnel of the Istituto Zooprofilattico Abruzzo and Molise. This study has been partially funded by the 2010 targeted research project of Italian Ministry of Health RF-2010-2310899 titled 'Campylobacter, L. monocytogenes and Verocytotoxin producing E. coli (VTEC) in Italy: a molecular epidemiology approach to identify sources of infection and risk factors for human exposure' and by a grant of the Italian Ministry of Health, Centro Nazionale per la Prevenzione ed il Controllo delle Malattie (CCM) entitled 'Sorveglianza delle malattie trasmesse da alimenti e acqua (EnterNet): adeguamento del sistema italiano al quadro normativo europeo' 1616-2014.

Conflict of interest

None declared.

Authors' contributions

FC: coordination of outbreak investigation, designed the questionnaire, data analysis, drafted the article. FB: designed the questionnaire, fulfilled the databases, data analysis, drafted the article. PC: coordination of outbreak investigation, drafted the article. EDG, LS, AMD, SO, NBB, CM, SS: laboratory typing on human and environmental samples. GS: coordination of outbreak investigation, drafted the article. MO: phylogenetic tree building, drafted the article. SI: Contributed data into the databases. IM: sequencing data analysis. LC: data analysis. AC: GIS outbreak coordination data analysis. CI: GIS outbreak coordination, database management. DM, GM: coordination of outbreak investigation. CC, MM, MA: laboratory sequencing. IL: coordination of outbreak investigation, laboratory reporting of cases, drafted the article. GB, GB: laboratory work on human sample. FDP, MDG, MDL, GV: done the environmental investigation. MG, CT: done the case interviews. All the authors: reviewed and approved the article.

References

1. European Food Safety Authority, European Centre for Disease Prevention and Control,. The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2012.EFSA Journal.2014;12(2):3547.
2. European Food Safety Authority, European Centre for Disease Prevention and Control,. The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2013.EFSA Journal.2015;13(1):3991.
3. European Food Safety Authority Panel on Biological Hazards (BIOHAZ),. Scientific opinion on monitoring and assessment of the public health risk of "Salmonella Typhimurium-like" strains.EFSA Journal.2010;8:1826.
4. Angelo KM, Chu A, Anand M, Nguyen TA, Bottichio L, Wise M, et al. Outbreak of Salmonella Newport infections linked to cucumbers--United States, 2014. MMWR Morb Mortal Wkly Rep. 2015;64(6):144-7.PMID: 25695319
5. Greene SK, Daly ER, Talbot EA, Demma LJ, Holzbauer S, Patel NJ, et al. Recurrent multistate outbreak of Salmonella Newport associated with tomatoes from contaminated fields, 2005. Epidemiol Infect. 2008;136(2):157-65. DOI: 10.1017/S095026880700859X PMID: 17475091
6. Cevallos-Cevallos JM, Danyluk MD, Gu G, Vallad GE, van Bruggen AH. Dispersal of Salmonella Typhimurium by rain splash onto tomato plants.J Food Prot. 2012;75(3):472-9. DOI: 10.4315/0362-028X.JFP-11-399 PMID: 22410220
7. Kozlica J, Claudet AL, Solomon D, Dunn JR, Carpenter LR. Waterborne outbreak of Salmonella I 4,[5],12:i:-.Foodborne Pathog Dis. 2010;7(11):1431-3. DOI: 10.1089/fpd.2010.0556 PMID: 20617936
8. Ailes E, Budge P, Shankar M, Collier S, Brinton W, Cronquist A, et al. Economic and health impacts associated with a Salmonella Typhimurium drinking water outbreak-Alamosa, CO, 2008. PLoS One. 2013;8(3):e57439. DOI: 10.1371/journal.pone.0057439 PMID: 23526942
9. Surveillance program EnterNet Italia. Available from: http://www.iss.it/ente/
10. Dionisi AM, Graziani C, Lucarelli C, Filetici E, Villa L, Owczarek S, et al. Molecular characterization of multidrug-resistant strains of Salmonella enterica serotype Typhimurium and Monophasic variant (S. 4,[5],12:i:-) isolated from human infections in Italy. Foodborne Pathog Dis. 2009;6(6):711-7. DOI: 10.1089/fpd.2008.0240 PMID: 19580448
11. Istituto Superiore di Sanità (ISS). ENTERNET Salmonella. Sorveglianza delle infezioni da Salmonella. Dati Storici dal 2007 al 2009. Rome: ISS; 2010. Italian. Available from: http://www.iss.it/binary/salm/cont/4f_Dati_SALMONELLA_2007_2009.pdf
12. Graziani C, Mughini-Gras L, Owczarek S, Dionisi A, Luzzi I, Busani L. Distribution of Salmonella enterica isolates from human cases in Italy, 1980 to 2011.Euro Surveill. 2013;18(27):20519. DOI: 10.2807/1560-7917.ES2013.18.27.20519 PMID: 23870078
13. Italian National Institute of Statistics (ISTAT). Italian population census. ISTAT. Available from: http://demo.istat.it
14. Ministry of Health. Bollettino nazionale epidemiologico malattie infettive. Italian. Available from: http://www.salute.gov.it/portale/documentazione/p6_2_8_1_1.jsp?lingua=italiano&id=3
15. Barco L, Ramon E, Cortini E, Longo A, Dalla Pozza MC, Lettini AA, et al. Molecular characterization of Salmonella enterica serovar 4,[5],12:i:- DT193 ASSuT strains from two outbreaks in Italy. Foodborne Pathog Dis. 2014;11(2):138-44. DOI: 10.1089/fpd.2013.1626 PMID: 24328499
16. Italian National Statistician Institute (ISTAT). Censimento Popolazione. Italian. Available from: http://dati-censimentopopolazione.istat.it/
17. Centre of Excellence for the Remote Sensing and Forecast of Severe Weather (CETEMPS). Available from: http://cetemps.aquila.infn.it/index.php?option=com_wrapper&view=wrapper&Itemid=127
18. R Core Team. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing.2013. Available from: http://www.R-project.org/
19. Lim YH, Hirose K, Izumiya H, Arakawa E, Takahashi H, Terajima J, et al. Multiplex polymerase chain reaction assay for selective detection of Salmonella enterica serovar typhimurium. Jpn J Infect Dis. 2003;56(4):151-5.PMID: 14583637
20. Alessiani A, Sacchini L, Pontieri E, Gavini J, Di Giannatale E. Molecular typing of Salmonella enterica subspecies enterica serovar Typhimurium isolated in Abruzzo region (Italy) from 2008 to 2010.Vet Ital. 2014;50(1):31-9.PMID: 24715591
21. Anderson ES, Ward LR, Saxe MJ, de Sa JD. Bacteriophage-typing designations of Salmonella typhimurium.J Hyg (Lond). 1977;78(2):297-300. DOI: 10.1017/S0022172400056187 PMID: 321679
22. European Centre for Disease Prevention and Control (ECDC). Laboratory standard operating procedure for MLVA of Salmonella enterica serotype Typhimurium. Stockholm: ECDC; 2011.
23. Centre for Disease Prevention and Control (CDC). Standard Operating Procedure for PulseNet PFGE of Escherichia coli O157:H7, Escherichia coli non-O157 (STEC), Salmonella serotypes, Shigella sonnei and Shigella flexneri. PNL05. Atlanta: CDC; April 2013.
24. International Nucleotide Sequence Database Collaboration,Leinonen R, Sugawara H, Shumway M. The sequence read archive.Nucleic Acids Res. 2011;39(Database issue):D19-21. DOI: 10.1093/nar/gkq1019 PMID: 21062823
25. Pettengill JB, Luo Y, Davis S, Chen Y, Gonzalez-Escalona N, Ottesen A, et al. An evaluation of alternative methods for constructing phylogenies from whole genome sequence data: a case study with Salmonella. PeerJ. 2014;2:e620. DOI: 10.7717/peerj.620 PMID: 25332847
26. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0.Mol Biol Evol. 2013;30(12):2725-9. DOI: 10.1093/molbev/mst197 PMID: 24132122
27. Liu C, Hofstra N, Franz E. Impacts of climate change on the microbial safety of pre-harvest leafy green vegetables as indicated by Escherichia coli O157 and Salmonella spp. Int J Food Microbiol.2013;163(2-3):119-28. DOI: 10.1016/j.ijfoodmicro.2013.02.026 PMID: 23558195
28. Beuchat LR. Ecological factors influencing survival and growth of human pathogens on raw fruits and vegetables.Microbes Infect. 2002;4(4):413-23. DOI: 10.1016/S1286-4579(02)01555-1 PMID: 11932192
29. Himathongkham S, Bahari S, Riemann H, Cliver D. Survival of Escherichia coli O157:H7 and Salmonella typhimurium in cow manure and cow manure slurry.FEMS Microbiol Lett. 1999;178(2):251-7. DOI: 10.1111/j.1574-6968.1999.tb08684.x PMID: 10499275
30. Horby PW, O'Brien SJ, Adak GK, Graham C, Hawker JI, Hunter P, et al. A national outbreak of multi-resistant Salmonella enterica serovar Typhimurium definitive phage type (DT) 104 associated with consumption of lettuce. Epidemiol Infect. 2003;130(2):169-78. DOI: 10.1017/S0950268802008063 PMID: 12729184
31. Golberg D, Kroupitski Y, Belausov E, Pinto R, Sela S. Salmonella Typhimurium internalization is variable in leafy vegetables and fresh herbs.Int J Food Microbiol. 2011;145(1):250-7. DOI: 10.1016/j.ijfoodmicro.2010.12.031 PMID: 21262550
32. The California Food Emergency Response Team. Investigation of the Taco John's Escherichia coli O157:H7 outbreak associated with iceberg lettuce. California: US Food and Drug Administration (FDA) and California Food Emergency Response Team; 2008. Available from: http://www.cdph.

ca.gov/pubsforms/Documents/fdb%20eru%20IceLet%20TacoJohno22008.pdf
33. Centers for Disease Control and Prevention (CDC),. Outbreak of Salmonella serotype Saintpaul infections associated with multiple raw produce items--United States, 2008.MMWR Morb Mortal Wkly Rep. 2008;57(34):929-34.PMID: 18756191
34. Municipal Decree no. 70 of 12 June 2014. Available from: http://trasparenza.comune.laquila.gov.it/archivio28_provvedimenti_0_56240_725_1.html
35. Municipal Decree no. 74 of 7th July 2014. Available from: http://trasparenza.comune.laquila.gov.it/archivio28_provvedimenti_0_59005_725_1.html
36. Municipal Decree no. 80 of 4th August 2014. Available from: http://trasparenza.comune.laquila.gov.it/archivio28_provvedimenti_0_60959_725_1.html

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Research article

Carbapenem-resistant isolates of *Acinetobacter baumannii* in a municipal wastewater treatment plant, Croatia, 2014

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Citation style for this article:
Hrenovic J, Goic-Barisic I, Kazazic S, Kovacic A, Ganjto M, Tonkic M. Carbapenem-resistant isolates of Acinetobacter baumannii in a municipal wastewater treatment plant, Croatia, 2014. Euro Surveill. 2016;21(15):pii=30195. DOI: http://dx.doi.org/10.2807/1560-7917.ES.2016.21.15.30195

Article submitted on 05 February 2015 / accepted on 14 April 2016 / published on 14 April 2016

Acinetobacter baumannii **is an emerging hospital pathogen. Whereas *A. baumannii* isolated from patients or hospitals has been reported, there are few data regarding propagation of viable *A. baumannii* in the natural environment. This study investigates the occurrence and antimicrobial susceptibility of viable *A. baumannii* in municipal wastewater and its persistence through the wastewater treatment process. A total of 21 *A. baumannii* isolates were recovered at a secondary type of municipal wastewater treatment plant in Zagreb, Croatia: 15 from raw influent wastewater and six from final effluent. All isolates were carbapenem- and multidrug-resistant. Among 14 isolates tested for bla_{OXA} genes, all harboured the constitutive $bla_{OXA\text{-}51\text{-}like}$ gene, while the acquired $bla_{OXA\text{-}23\text{-}like}$ and $bla_{OXA\text{-}40\text{-}like}$ genes were found in 10 and three isolates respectively. Six *A. baumannii* isolates recovered from effluent wastewater multiplied and survived in sterilised effluent wastewater up to 50 days. These findings support the idea that multidrug-resistant *A. baumannii* can occur and have the ability to survive in the environment.**

Introduction

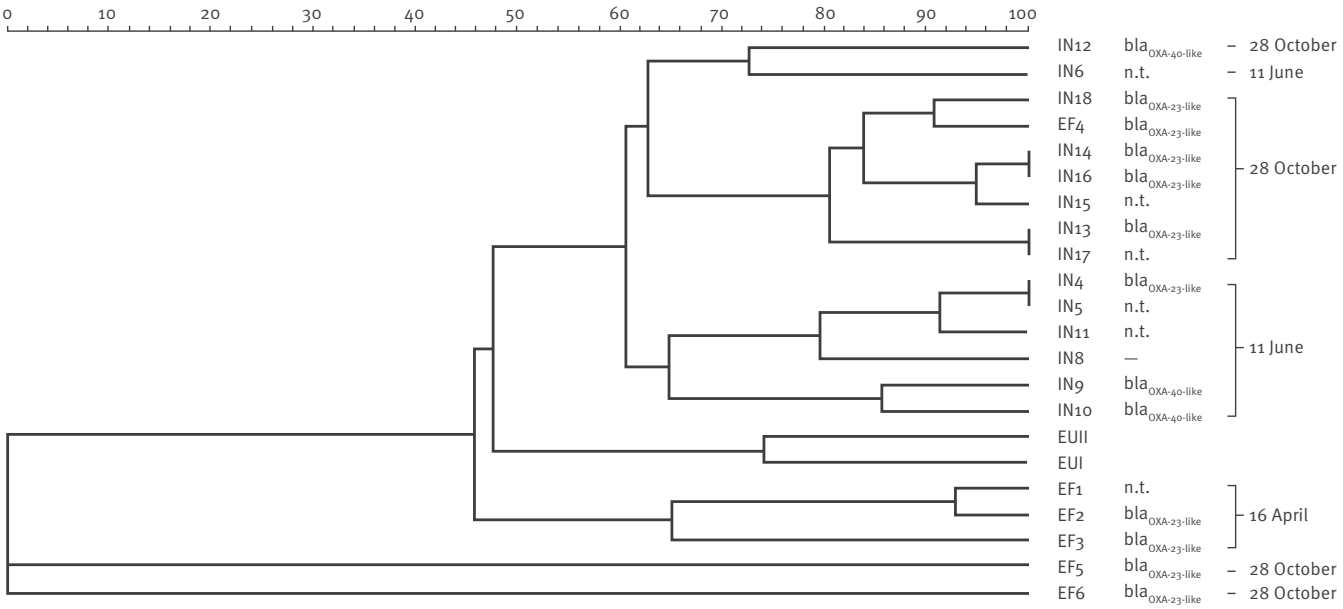
Over the last decade, hospital-acquired infections due to *Acinetobacter baumannii* have increased dramatically worldwide including in Croatia [1,2]. Difficulties caused by this pathogenin the hospital setting are exacerbated by its abilities to form biofilms on abiotic or biotic surfaces and to cope with different environmental conditions, including desiccation and disinfectants [3-5]. Non-susceptibility to commonly used antimicrobials [6] has also been observed, with carbapenem resistance becoming a global problem since 2000[7,8]. In Croatia, proportions of carbapenem-resistant *A. baumannii* strains in clinical samples increased drastically from 2008 to 2012 (10% to 73% of isolates respectively), with some individual hospitals recording a rate of 90% [2,9].

The most important mechanism of carbapenem resistance in *A. baumannii* involves OXA-type carbapenemases, which are encoded by bla_{OXA} lineage genes. Five main phylogenetic subgroups including OXA-23-like, OXA-40-like, OXA-51-like, OXA-58-like, and OXA-143-like have been recognised [10]. Aside from OXA-143-like strains, whose prevalences in certain areas remain to be determined [11], *A. baumannii* strains of all these subgroups are globally distributed. Carbapenemase genes in *A. baumanii* are moreover constitutive (e.g. OXA-51-like) or acquired (e.g. OXA-23-like, OXA-40-like, OXA-58-like). In Croatia, *A. baumannii* isolates with constitutive OXA-51-like carbapenemases have been described [8,12,13]. Moreover, since 2009, isolates with acquired OXA-40-like carbapenemases dominate in most hospitals [13,14], while isolates with acquired OXA-23-like and OXA-58-like enzymes are sporadically reported [14].

How *A. baumannii* is introduced into the hospital environment remains incompletely understood [5,15]. Acute community-acquired human infections have been reported [16] and suggest a source of the pathogen outside of the hospital. Even if such infections are mainly reported from tropical and subtropical areas and represent only a minor proportion of all *A. baumannii* infections worldwide, community-acquired infections underline the importance of searching for possible environmental reservoirs of the pathogen and their potential consequences. In nature, multidrug-resistant (MDR) *A. baumannii* strains have been isolated from hospital wastewaters [17,18]. Aside from reports of *A. baumannii* in wastewaters close to hospitals, only few

Figure 1

Pulsed-field gel electrophoresis dendogram based on *Apa*I-digested DNA from isolates of *Acinetobacter baumannii*, Zagreb, Croatia, 2014 (n=20 isolates)[a]



EF: Isolates derived from effluent wastewater; EUI: international clonal lineage I (RUH 2037); EUII: international clonal lineage II (RUH 134); IN: isolates derived from influent wastewater; n.t.: not tested for *bla*OXA genes; –: no acquired *bla*OXA-like gene.

The isolates' names according to their origin (influent or effluent wastewater), as well as the carbapenemase they harboured and date of collection are shown on the right hand side of the dendogram.

[a] A total of 21 A. *baumannii* isolates were obtained throughout the study but the *Apa*I digestion failed for one, hence 20 isolates were further analysed by pulsed-field gel electrophoresis and presented in the dendogram.

studies report detection elsewhere in the environment [19,20].

The aim of this study was to screen municipal wastewater for the presence of viable *A. baumannii*, characterise the recovered strains as well as investigate their potential for survival in the environment after passage through the wastewater treatment process.

Methods

Sampling and characterisation of wastewater

Wastewater was collected at the largest Croatian wastewater treatment plant of Zagreb. This plant (capacity 1,200,000 population equivalents) is designed for the secondary treatment of municipal wastewater, which originates from a combined sewage system of domestic, hospital, industrial and storm water. The central treatment plant receives wastewater from all nine clinical hospitals of Zagreb. In accordance with the national legislation, sanitary hospital wastewaters are released into the sewage system without pre-treatment, and only radioactive wastewaters and infective solid waste are disposed of separately. The sewage system of Zagreb is an underground network of sewer with different retention time of wastewater depending on the inhabitants' behaviours and stormwater runoff.

Composite 24h samples of influent and effluent wastewater were collected in June and October 2014 and April and October 2014, respectively. The physicochemical parameters of water samples were measured according to the Standard Methods for Examination of Water and Wastewater [21]. Temperature was measured online and average values of composite samples were calculated.

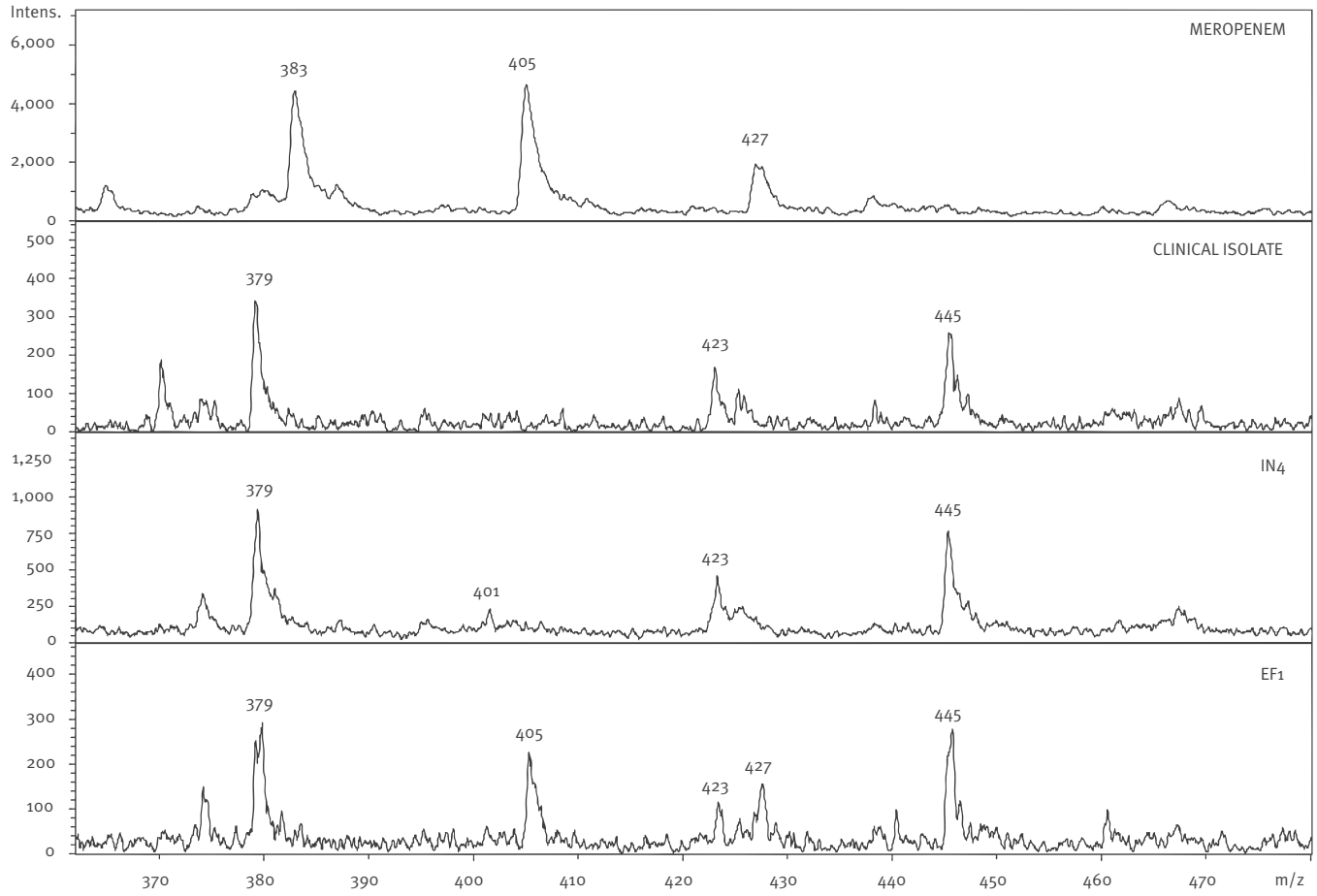
Bacteriological analyses

For bacteriological analyses, wastewater was aseptically sampled in sterile 1L glass bottles and transferred to the laboratory within 1h. The wastewater samples were concentrated on sterile membrane filters of pore size 0.45µm in triplicate both before and after dilution in sterile peptone water.

Intestinal enterococci were determined according to HRN ISO 7899–2 [22]. Membrane filters were incubated on Slanetz Bartley agar (Biolife) at 37 °C for 72h and subsequent confirmation of intestinal enterococci (i.e. colonies with dark brown halo) was done on bile esculin azide agar (Sigma-Aldrich) after incubation at 44 °C for 4h.

Figure 2

MALDI-TOF MS spectra of meropenem and its degradation products after 2.5h of contact with isolates of *Acinetobacter baumannii*



Intens.: intensity; MALDI-TOF MS: matrix-assisted laser desorption ionization-time of flight mass spectrometry.

The top panel entitled MEROPENEM represents the negative control solution with only meropenem and characteristic peaks indicating no degradation of this antibiotic (m/z 383 meropenem molecule; m/z 405 meropenem sodium salt; m/z 427 meropenem disodium salt). The panel below entitled CLINICAL ISOLATE represents the positive control, whereby a clinical carbapenem-resistant *A. baumannii* strain fully degrades meropenem leading to characteristic peaks in the spectra (m/z 379 decarboxylated sodium salt of meropenem; m/z 401 meropenem with a disrupted amide bond; m/z 423 sodium salt of meropenem with a disrupted amide bond; m/z 445 disodium salt of meropenem with a disrupted amide bond). The two lower panels (IN4 and EF1) show the degradation of meropenem in presence two respective wastewater isolates, recovered in this study. While the IN4 strain induces full meropenem degradation, in the same way than the clinical strain, the degradation by EF1 is incomplete.

Aerobically grown total heterotrophic bacteria were determined on Nutrient agar (Biolife) after incubation at 22 °C for 72h [21].

Carbapenem-resistant bacteria were determined on CHROMagar Acinetobacter supplemented with CR102 (CHROMagar), which allows the growth of carbapenem-resistant isolates after incubation at 42 °C for 48h.

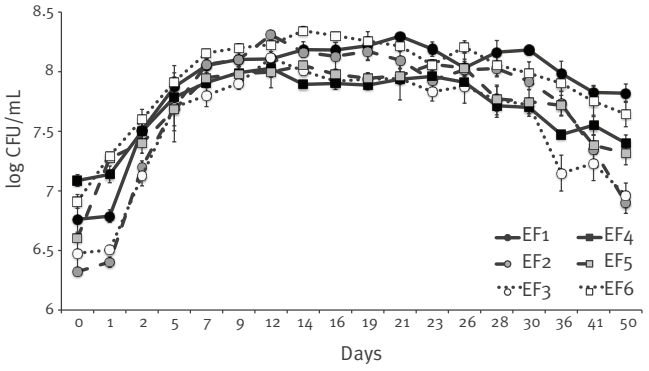
The numbers of intestinal enterococci, aerobically grown total heterotrophic bacteria, and carbapenem-resistant bacteria were determined as colony forming units (CFU), logarithmically transformed, and expressed as log CFU per 1 mL of water. The prevalence of carbapenem-resistant bacteria among total heterotrophic bacteria was calculated.

Isolation and characterisation of *Acinetobacter baumannii* isolates

There is no simple protocol for the isolation of viable *A. baumannii* from environmental samples. *A. baumannii* is usually overgrown with accompanying flora even on selective and differential media. For the isolation of carbapenem-resistant *A. baumannii* from wastewater selective CHROMagar Acinetobacter supplemented with commercial supplement CR102 was used. Cefsulodin sodium salt hydrate (Sigma-Aldrich) was added at 15 mg/L to suppress the growth of *Pseudomonas* and *Aeromonas* spp. The isolation of *A. baumannii* was performed at 42 °C for 48h to suppress the growth of most other species of *Acinetobacter* and *Stenotrophomonas* with intrinsic resistance to carbapenems, as well as species of *Pseudomonas*. Single

Figure 3

Survival of six *Acinetobacter baumannii* isolates (EF1–6) recovered from effluent wastewater in the autoclaved effluent wastewater during 50 days, Croatia, 2014



CFU: colony forming units.

colonies of *A. baumannii* were isolated from plates inoculated with 0.01–0.1mL of influent water and 0.1–1.0mL of effluent water. Presumptive colonies of *A. baumannii* were recultivated (42 °C for 24h) on the same selective plates and then on Nutrient agar.

Pure cultures of presumptive *A. baumannii* grown at 42 °C on Nutrient agar were firstly characterised by routine bacteriological techniques to assess the following characteristics: Gram negative coccobacilli, negative oxidase, positive catalase reaction, no reaction on the Kligler Iron Agar (Biolife). Further identification of presumptive *A. baumannii* was carried out by ATB 32GN and Vitek 2 systems (BioMerieux) [23,24].

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) which was shown to be a reliable method for rapid identification of *A. baumannii* [25,26] was used for confirmation on cell extracts according to Sousa et al. [27]. In brief, overnight cultures pre-grown on Nutrient agar were suspended in 70% ethanol. After centrifugation and removal of the supernatant, cultures were extracted with 70% formic acid and an equal volume of acetonitrile. The suspension was centrifuged and cell extracts were spotted onto a MALDI target plate and air dried at room temperature. Subsequently, the sample was overlaid with a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid as a matrix, dried, and subjected to analysis. Spectra were obtained by using Microflex LT (Bruker Daltonics) in the linear positive mode in the range 2–20 kDa. Each recorded spectrum is the result of six series of 40 single laser shots in different locations. Identification of isolates was performed in technical triplicate and biological duplicate. Recorded mass spectra were processed with the MALDI Biotyper 3.0 software package (Bruker Daltonics) using standard settings.

The genetic relatedness of *A. baumannii* isolates from wastewater to clinical isolate was assessed by pulsed-field gel electrophoresis (PFGE). Representatives of *A. baumannii* belonging to the international clonal lineage I (RUH 2037) and II (RUH 134), which have been confirmed as two dominant clones causing outbreaks in Croatia since 2002 and which are still present in Croatian hospitals [8,9,12-14], were used as PFGE reference strains. PFGE analysis was performed using CHEF-DRII/III system (Bio-Rad) with *Apa*I (New England BioLabs) as restriction enzyme. The images of gel-electrophoresed restriction products were processed using Gel-Doc 1000 system (Bio-Rad) and Compar software. PFGE profiles were analysed and compared using Molecular Analyst Software for Fingerprinting (Bio-Rad). Matching and dendogram were performed with the unweighted pair group method with arithmetic averages (UPGMA) analysis [28,29] using Dice similarity coefficient with optimisation and a position tolerance of 1.0%. The isolates were classified into clusters based on their genetic similarity (cut-off of ≥ 90%).

Antibiotic resistance of *Acinetobacter baumannii* isolates

Susceptibility to β-lactams (imipenem, meropenem), fluoroquinolones (ciprofloxacin, levofloxacin), aminoglycosides (amikacin, gentamicin, tobramycin), and trimethoprim/sulfamethoxazole were determined by disc-diffusion tests. The minimum inhibitory concentration (MIC) values were confirmed using AST-XN05 and AST-N233 testing card for Vitek2 system or using E-tests (AB Biodisk) for colistin (which was also included for susceptibility testing), and interpreted according to the European Committee on Antimicrobial Susceptibility Testing criteria [30].

The carbapenemase-induced carbapenem degradation was confirmed by MALDI-TOF MS as described by Burckhardt and Zimmermann [31] and Hrabak et al. [32], with slight modifications. In brief, a loopful of overnight bacterial culture was added to 0.5mL of 0.2mM meropenem trihydrate (Tokyo Chemical Industry Co., Ltd.) in 0.05M NaCl. The solutions were incubated at 37 °C for 2.5h with stirring at 150rpm. After centrifugation, the supernatants were spotted onto a MALDI-TOF MS targets by a sandwich method using 2,5-dihydroxybenzoic acid (DHB) as a matrix, and subjected to analysis. A carbapenem-resistant clinical isolate of *A. baumannii* carrying the OXA-72 carbapenemase [13] was used as a positive control, while a meropenem solution without addition of bacteria served as the negative control.

The presence of the genes of bla_{OXA} lineage which encode OXA-type carbapenemases was checked in 14 isolates of *A. baumannii*, selected on the basis of PFGE pattern and antibiotic susceptibility profiles. Multiplex polymerase chain reaction (PCR) with specific respective primers was used to amplify $bla_{OXA\text{-}51\text{-}like}$, $bla_{OXA\text{-}40\text{-}like}$, $bla_{OXA\text{-}23\text{-}like}$ and $bla_{OXA\text{-}58\text{-}like}$ genes, according to Woodford et al. [33].

Table 1

Physical, chemical and bacteriological characteristics of influent and effluent wastewater from a secondary water treatment plant, by sampling date, Zagreb, Croatia, 2014

Parameter	Influent		Effluent	
	11 June	28 October	16 April	28 October
Water flow (m^3/day)	250,540	509,994	248,038	495,356
Temperature (°C)	18.6	15.7	16.7	16.9
Dissolved O_2 (mg/L)	0.09	6.80	8.59	9.54
pH	7.76	7.92	7.73	7.83
COD (mg O_2/L)	353	177	26	24
BOD_5 (mg O_2/L)	184	88	3	5
Total nitrogen (mg/L)	31.3	30.9	23.9	20.7
Total phosphorus (mg/L)	5.46	2.68	1.94	1.29
Intestinal enterococci (log CFU/mL)	4.5±0.2	4.7±0.2	2.0±0.2	1.8±0.2
Total heterotrophic bacteria (log CFU/mL)	7.5±0.1	7.3±0.1	4.9±0.1	4.8±0.1
Carbapenem-resistant bacteria (log CFU/mL)	4.3±0.2	2.5±0.2	1.1±0.2	1.4±0.2
Prevalence of carbapenem-resistant bacteria (%)[a] ± SD	57±2	34±2	22±4	29±4

CFU: colony forming unit; COD: chemical oxygen demand; BOD_5: biochemical oxygen demand in five days; SD: standard deviation.

[a] Prevalence of carbapenem-resistant bacteria among total heterotrophic bacteria was calculated as (log CFU/mL $_{\text{carbapenem resistant}}$/log CFU/mL $_{\text{heterotrophic}}$) x 100.

Survival of *Acinetobacter baumannii* in effluent wastewater

Survival of six isolates of *A. baumannii* (isolates EF1–6) recovered from effluent wastewater was monitored in the sterilised effluent wastewater collected on 28 October 2014. Effluent wastewater was filtered through filter paper (blue band) and membrane filter of pore size 0.45µm. Wastewater was partitioned per 200mL into Schott bottles and autoclaved (121°C for 20min). The isolates of *A. baumannii* were pre-grown separately on CHROMagar Acinetobacter at 42 °C for 24h. The biomass from each plate was then vortexed in sterile 0.05M NaCl, and 1mL of each suspension was inoculated into bottles containing the effluent wastewater. The bottles were sealed with a sterile gum cap and aeration was insured by a central hole through which filtered air (1L/min) was provided. The bottles were incubated at 16.7±0.2 °C in a thermostat (Memmert IPP400) with stirring (120 rpm) during 50 days. Any loss of the solution volume caused by evaporation was compensated by refilling with sterile 0.05M NaCl. For each measurement of bacterial numbers 1mL was taken from the bottle.

Numbers of *A. baumannii* were counted in triplicate after dilution in 0.05M NaCl on Nutrient agar after incubation at 42 °C for 24h. The numbers of CFU were logarithmically transformed and reported as log CFU per 1 mL of water.

Results

Physical, chemical and bacteriological characteristics of wastewater

Temperatures of pH-neutral wastewater were relatively constant (Table 1). The physico-chemical characteristics of wastewater slightly varied on two sampling occasions of influent or effluent. Most evident however, were variations in inflow and outflow and concentration of dissolved oxygen where the influent in June was anoxic (0.09 mg dissolved O_2/L). Both in June and in October, the ratio of five-day biochemical oxygen demand (BOD_5) to chemical oxygen demand (COD) in the influent was 0.5, which is considered high (0.3–0.6) and suggests nutrient rich biodegradable influent wastewater [34]. Concentrations of nutrients in effluent wastewater were however in compliance with the national emission standards [35].

National emission standards do not require the monitoring of microorganisms at a described type of wastewater treatment plant. High levels (› 1.8 log CFU/mL) of intestinal enterococci (Table 1) that are reliable indicators of faecal pollution [36] were found in the influent and effluent water at each sampling occasion. The prevalence of carbapenem-resistant bacteria among total heterotrophic bacteria ranged from 34 to 57% in influent and 22 to 29% in effluent.

Isolation and characterisation of *Acinetobacter baumannii* isolates

For the whole study, a total of 21 *A. baumannii* were isolated; 15 from influent and six from effluent wastewater. Colonies of *A. baumannii* were circular, convex, smooth, red with a paler central area. All isolates of *A.*

Table 2

Minimum inhibition concentration values of tested antibiotics and presence of acquired genes of *bla*$_{OXA}$ lineage[a] in isolates of *Acinetobacter baumannii*, Zagreb, Croatia, 2014

Isolate	Minimum inhibition concentration values of antibiotics (mg/L)									*bla* $_{OXA\text{-}like}$
	MEM	IPM	LVX	CIP	TOB	GEN	AMK	SXT	CST	
IN4	>16^{R}	>16^{R}	>8^{R}	4^{R}	>16^{R}	8^{R}	4	20	0.50	*bla* $_{OXA\text{-}23\text{-}like}$
IN5	>16^{R}	>16^{R}	>8^{R}	4^{R}	>16^{R}	>16^{R}	4	20	0.25	Not tested
IN6	>16^{R}	>16^{R}	>8^{R}	16^{R}	2	>16^{R}	8	10	0.19	Not tested
IN8	>16^{R}	>16^{R}	>8^{R}	8^{R}	2	>16^{R}	8	10	0.25	–[b]
IN9	>16^{R}	>16^{R}	>8^{R}	16^{R}	8^{R}	2	2	20	0.25	*bla* $_{OXA\text{-}40\text{-}like}$
IN10	>16^{R}	>16^{R}	>8^{R}	8^{R}	>16^{R}	>16^{R}	2	16	0.25	*bla* $_{OXA\text{-}40\text{-}like}$
IN11	>16^{R}	>16^{R}	>8^{R}	8^{R}	>16^{R}	>16^{R}	2	16	0.25	Not tested
IN12	>16^{R}	>16^{R}	>8^{R}	4^{R}	8^{R}	>16^{R}	4	16	0.50	*bla* $_{OXA\text{-}40\text{-}like}$
IN13	>32^{R}	>32^{R}	>8^{R}	8^{R}	4^{R}	>16^{R}	4	20	0.50	*bla* $_{OXA\text{-}23\text{-}like}$
IN14	>16^{R}	>16^{R}	>8^{R}	4^{R}	16^{R}	>16^{R}	8	16	0.75	*bla* $_{OXA\text{-}23\text{-}like}$
IN15	>16^{R}	>16^{R}	>8^{R}	4^{R}	2	>16^{R}	4	20	0.50	Not tested
IN16	>16^{R}	>16^{R}	4^{R}	16^{R}	4^{R}	4^{R}	4	20	0.50	*bla* $_{OXA\text{-}23\text{-}like}$
IN17	32^{R}	>32^{R}	>8^{R}	16^{R}	16^{R}	>16^{R}	8	16	0.50	Not tested
IN18	>16^{R}	>16^{R}	>8^{R}	8^{R}	8^{R}	>16^{R}	4	20	0.50	*bla* $_{OXA\text{-}23\text{-}like}$
IN19	>16^{R}	>16^{R}	4^{R}	4^{R}	8^{R}	8^{R}	16^{R}	16	0.50	Not tested
EF1	>16^{R}	>16^{R}	4^{R}	4^{R}	>16^{R}	>16^{R}	4	20	0.50	Not tested
EF2	>16^{R}	>16^{R}	4^{R}	8^{R}	8^{R}	8^{R}	4	20	0.25	*bla* $_{OXA\text{-}23\text{-}like}$
EF3	>16^{R}	>16^{R}	>8^{R}	4^{R}	4^{R}	>16^{R}	4	20	0.50	*bla* $_{OXA\text{-}23\text{-}like}$
EF4	>32^{R}	>32^{R}	>8^{R}	4^{R}	>16^{R}	>16^{R}	16^{R}	32	0.75	*bla* $_{OXA\text{-}23\text{-}like}$
EF5	>16^{R}	>16^{R}	>8^{R}	4^{R}	>16^{R}	>16^{R}	32^{R}	32	0.50	*bla* $_{OXA\text{-}23\text{-}like}$
EF6	>16^{R}	>16^{R}	>8^{R}	4^{R}	>16^{R}	>16^{R}	64^{R}	32	0.50	*bla* $_{OXA\text{-}23\text{-}like}$

AMK: amikacin; CIP: ciprofloxacin; CST: colistin; GEN: gentamicin; IPM: imipenem; LVX: levofloxacin; MEM: meropenem; SXT: trimethoprim-sulfamethoxazole; TOB: tobramycin; R: resistant according to European Committee on Antimicrobial Susceptibility Testing criteria.

[a] The constitutive gene *bla*$_{OXA\text{-}51\text{-}like}$ was confirmed in all isolates.

[b] No acquired *bla*$_{OXA\text{-}like}$ gene.

baumannii from wastewater gave reliable score values (range: 2.048–2.409) when compared with *A. baumannii* strains in MALDI Biotyper database. All isolates were also determined by Vitek 2 system as *A. calcoaceticus-baumannii* complex.

An influent isolate named IN19 appeared resistant to *Apa*I digestion and was therefore not subjected to subsequent PFGE cluster analysis. For the remaining *A. baumannii* isolates recovered from wastewater, none were similar to isolates belonging to the international clonal lineage I or II based on the PFGE dendrogram (Figure 1). Three effluent wastewater samples collected on 16 April clustered together as well as all but one of the influent samples from 11 June, and all but one of the influent samples from 28 October. The effluent samples from 28 October, however, showed the absence of clusterisation.

Isolates IN4 and IN5, IN13 and IN17, IN14 and IN16 showed pairwise 100% identical PFGE profile. These PFGE identical pairs were respectively collected on the same date, all from influent water.

Antibiotic resistance of *Acinetobacter baumannii* isolates

According to MIC values of tested antibiotics (Table 2) all 21 *A. baumannii* isolates from wastewater were resistant to carbapenems. Although always susceptible to trimethoprim-sulfamethoxazole and colistin, all isolates were resistant to at least five of the nine antibiotics tested, and therefore could be classified as MDR. The isolates identical in PFGE profile (pairs IN4 and IN5, IN13 and IN17, IN14 and IN16, Figure 1) displayed differences in phenotypic antimicrobial sensitivity profiles. Isolate IN4 showed lower MIC value for gentamicin and higher MIC value for colistin as compared with isolate IN5. Differences in MIC values of amikacin, ciprofloxacin, meropenem, trimethoprim-sulfamethoxazole and tobramycin were observed for isolates IN13 and IN17. Isolates IN14 and IN16 showed differences in MIC values of all tested antibiotics, except for imipenem and meropenem (Table 2).

The carbapenemase-induced carbapenem degradation by *A. baumannii* isolates from wastewater was confirmed by MALDI-TOF MS. No spontaneous degradation of meropenem was observed and characteristic peaks (m/z 383, 405, 427) were evident after 2.5h

of incubation (Figure 2). All isolates from wastewater degraded the meropenem within 2.5h.

Selected types of spectra are shown in Figure 2. The positive control carbapenem-resistant clinical isolate of *A. baumannii* yielded a typical spectrum of complete meropenem degradation with characteristic peaks at m/z 379, 401, 423, and 445. Three isolates (IN4, IN5, IN8) of *A. baumannii* from wastewater degraded the meropenem as efficiently as the clinical isolate, as represented by the spectra of isolate IN4 in Figure 2. In the spectra of the remaining *A. baumannii* isolates recovered from wastewater in this study, residues of meropenem sodium and disodium salts (m/z 405 and 427) were visible in addition to the meropenem degradation products. This type of meropenem degradation is represented by the spectra of isolate EF1 in Figure 2 and suggests that such wastewater isolates degraded meropenem slower than the clinical isolate. A contact time of more than 2.5h was needed for these environmental isolates to fully degrade 0.2mM of meropenem. These isolates degraded the meropenem molecule first, followed by the degradation of meropenem sodium and disodium salts.

Monitoring of the meropenem degradation by MALDI-TOF MS does not provide detection of the type of carbapenemase carried nor detection of other carbapenem-resistance mechanisms [31]. The presence of genes of bla_{OXA} lineage which encode OXA-type carbapenemases was further confirmed by PCR. All of 14 tested *A. baumannii* isolates harboured the constitutive $bla_{OXA\text{-}51\text{-}like}$ gene. Among these, 10 isolates had the acquired $bla_{OXA\text{-}23\text{-}like}$, three the acquired $bla_{OXA\text{-}40\text{-}like}$ genes and one isolate did not have any acquired bla_{OXA} gene (Table 2).

For the three pairs of identical isolates by PFGE, the bla_{OXA} genes in two pairs were only determined for one isolate respectively. For the remaining pair comprising IN14 and IN16, the acquired $bla_{OXA\text{-}23\text{-}like}$ gene was confirmed for both isolates in the pair (Table 2 and Figure 1). The $bla_{OXA\text{-}23\text{-}like}$ gene was moreover found in two tested isolates from the three effluent samples of 16 April, which clustered together by PFGE. It was also present in the four of six tested influent water sample isolates from 28 October, which together formed a separate in PFGE. The four remaining isolates with $bla_{OXA\text{-}23\text{-}like}$ gene did not cluster together by PFGE and included three effluent isolates from 28 October and one influent isolate from 11 June. Although influent samples from 11 June formed a larger PFGE group, this group contained a sub-cluster with two isolates harbouring $bla_{OXA\text{-}40\text{-}like}$ genes. The third isolate with $bla_{OXA\text{-}40\text{-}like}$ gene was from an influent water sample collected on 28 October.

Survival of *Acinetobacter baumannii* in effluent wastewater

Six isolates of *A. baumannii* recovered from effluent wastewater showed excellent survival in the autoclaved effluent wastewater from 28 October (Figure 3). The low concentration of nutrients in this effluent wastewater was sufficient to support the multiplication of bacteria. Multiplication of isolates occurred after one day of incubation, and log phase of growth was evident up to nine days of incubation. The stationary phase of growth was continued up to 30 days, after which a slight decrease of log CFU was observed up to 50 days of incubation. After 50 days of monitoring the numbers (log CFU) of *A. baumannii* isolates were 4.4 to 15.7% higher than initial numbers.

Discussion

In this investigation, wastewater collected at a water treatment plant in Zagreb in April, June and October 2014 tested positive for *A. baumannii* at each occasion. A previous study in 2002 [37] reported the presence of eight phosphate-accumulating *A. baumannii* strains in the activated sludge of a municipal wastewater treatment plant in Izmir, Turkey. The strains in the latter study were however only characterised biochemically (API 20NE identification kit) so they could have belonged to other species of the genus *Acinetobacter* considered important for enhanced biological phosphate removal from wastewaters [24,27,38]. In contrast, none of the *A. baumannii* isolates in the current study were able to accumulate intracellular polyphosphate granule as confirmed by Neisser staining (data not shown).

The prevalences of carbapenem-resistant bacteria among total heterotrophic bacteria, which were estimated each time the water was sampled, ranged from 34 to 57% in influent water and 22 to 29% in effluent water. As carbapenems are heavily used in clinical environments, such prevalences suggest hospital wastewater in the influent water and bacteria of clinical origin in the effluent. The finding of OXA-40-like carbapenemase in three of 14 *A. baumannii* isolates recovered from wastewater strengthens this hypothesis. Indeed, OXA-40-like carbapenemases constitute the dominant mechanism of carbapenem resistance in clinical isolates of *A. baumannii* from most Croatian hospitals [9,13,14]. In addition to this, a number of strains isolated from wastewater degraded meropenem in the same way as a control clinical isolate. The dominance (10 of 14) of wastewater isolates harbouring the $bla_{OXA\text{-}23\text{-}like}$ gene was nevertheless unexpected, since the clinical isolates of *A. baumannii* which produce OXA-23-like carbapenemases have only been observed sporadically in the country [14]. It is possible that isolates carrying the $bla_{OXA\text{-}23\text{-}like}$ gene are more resistant to adverse environmental conditions in sewage as well as in the wastewater treatment process, thus surviving longer, but this needs to be further examined.

In spite of this, all wastewater *A. baumannii* isolates recovered in this study were MDR and showed comparable levels of antibiotic resistance to clinical *A. baumannii* isolates in Croatia [12]. As environmental bacterial isolates are generally more susceptible to antibiotics than clinical isolates [39], these results also point to

hospitals as the likely source of MDR *A. baumannii* in the wastewaters considered. In a preliminary screening, we confirmed the presence of MDR *A. baumannii* in the wastewater of one hospital in Zagreb (data not shown). The observation of MDR *A. baumannii* in hospital wastewaters has also been previously reported in Brazil and China [17,18].

High numbers of intestinal enterococci that are reliable indicators of faecal pollution [36] were also found in the wastewater together with *A. baumannii*, suggesting the presence of sanitary water and bacteria of faecal origin. MDR *A. baumannii* has been found in digestive tracts of hospitalised patients [40] as well as in animal faeces and urine [41]. The digestive tracts of colonised patients and animals could be important epidemiological reservoirs of MDR *A. baumannii*, from which bacteria could migrate through wastewater into the natural environment. The wastewater from veterinary clinics [41] and stormwater, which could leach the bacteria from solid waste [20] may also represent a source of *A. baumannii*. Multilocus sequence typing (MLST) of housekeeping genes should be further performed in order to assess the genetic relatedness of *A. baumannii* isolates from municipal wastewater and clinical isolates, as well as isolates from hospital wastewaters in Zagreb.

When allochthonous bacterial species come in the environment, some abiotic and biotic ecological factors have been shown to determine their survival and multiplication. Little is known however about the influence of environmental factors on *A. baumannii* strains which are significant for public health in wastewater. Six isolates of *A. baumannii* recovered from effluent wastewater multiplied and survived in the effluent wastewater for up to 50 days of monitoring. The average annual water flow velocity of the Sava River, which receives the effluent wastewater, is 0.75m/s, and 12 days are needed for the water mass to travel from the location of effluent discharge to the firth of the Sava into the Danube River. Therefore, the viable MDR *A. baumannii* could possibly spread through the natural water bodies or accumulate in river sediments. A separate study finding a MDR *A. baumannii* isolate related to a clinical isolate in the Seine River in France further supports this possibility [19].

Zhang et al. [39] reported that the tertiary wastewater treatment process contributed to the selective increase of MDR *Acinetobacter* spp. in final effluent and natural recipient. Lower numbers of carbapenem-resistant bacteria together with lower prevalence of such bacteria among total heterotrophic bacteria in effluent than in influent wastewater does not support this in the case of our secondary wastewater treatment process. Higher numbers of viable MDR *A. baumannii* isolated per sampling occasion from influent than from effluent wastewater rather suggest a beneficial role of the secondary wastewater treatment process on the distribution of clinically important bacteria in the environment. However, certain numbers of carbapenem-resistant bacteria and *A. baumannii* are released after the secondary treatment through the effluent wastewater into the natural recipient, which is the Sava River. The presence of viable MDR *A. baumannii* in both raw and treated municipal wastewater poses a serious concern about the spread of this emerging pathogen in nature.

Disinfection of effluent water has been suggested as a promising tool for prevention of the accumulation of MDR bacteria in waterbodies [42]. However, currently the standards for discharge of treated municipal wastewater do not prescribe the elimination of MDR bacteria. Furthermore, carbapenem-resistant MDR *A. baumannii* were isolated from hospital wastewaters both prior and after disinfection by chlorination [18]. Genes encoding New Delhi metallo-beta-lactamase-1 (NDM-1) were present in domestic and industrial wastewater and each stage of the wastewater treatment process, including the chlorinated effluent [43]. Although the viable bacteria were not determined, these NDM-1 genes were 99.8% identical to the NDM-1 genes carried by *A. baumannii*. The findings of MDR *A. baumannii* and NDM-1 genes after the process of chlorination suggest that conventional disinfection of effluent may not be the best strategy for mitigating the propagation of *A. baumannii* in the environment. Other disinfection techniques or barrier approaches at the source of contamination may be a more promising approach.

Tracking the source and further dispersion of *A. baumannii* contamination is needed to initiate any response plan for its control in the environment. The isolates of *A. baumannii* recovered from the environment should be compared with hospital, veterinary/livestock and community-acquired strains by using additional types of molecular assays such as whole genome sequencing [16], whereby there may be identifiable differences between these strains. Larger screening and epidemiological studies should also be performed to investigate the impact of human waste on the spread of MDR *A. baumannii* in the natural environment. In the current era when new antimicrobials for MDR Gram-negative microorganisms are scarce, it is most important to understand the epidemiology of this human pathogen. The results of this study represent a step forward in this direction.

Acknowledgements

This research was supported by the University of Zagreb (project no. 202751) and in a part by the Croatian Science Foundation (project no. IP-2014-09-5656). We are grateful to prof. Zlatko Mihaljevic, Faculty of Science, University of Zagreb for providing us information about water flow velocity of the Sava River.

Conflict of interest

None declared

Authors' contributions

J Hrenovic designed the study. J Hrenovic, I Goic-Barisic, S Kazazic, A Kovacic, and M Ganjto, performed the experiments and analysed data. M Tonkic interpreted part of the results. J Hrenovic wrote the first draft. All authors reviewed, provided comments and approved the final version of manuscript.

References

1. Howard A, O'Donoghue M, Feeney A, Sleator RD. Acinetobacter baumannii: an emerging opportunistic pathogen.Virulence. 2012;3(3):243-50. DOI: 10.4161/viru.19700 PMID: 22546906
2. Croatian Academy of Medical Sciences (CAMS). Antibiotic resistance in Croatia, 2012. Zagreb: CAMS; 2013:90-4.
3. Antunes LCS, Imperi F, Carattoli A, Visca P. Deciphering the multifactorial nature of Acinetobacter baumannii pathogenicity.PLoS One. 2011;6(8):e22674. DOI: 10.1371/journal.pone.0022674 PMID: 21829642
4. Espinal P, Martí S, Vila J. Effect of biofilm formation on the survival of Acinetobacter baumannii on dry surfaces.J Hosp Infect. 2012;80(1):56-60. DOI: 10.1016/j.jhin.2011.08.013 PMID: 21975219
5. Towner KJ. Acinetobacter: an old friend, but a new enemy.J Hosp Infect. 2009;73(4):355-63. DOI: 10.1016/j.jhin.2009.03.032 PMID: 19700220
6. Roca I, Espinal P, Vila-Farrés X, Vila J. The Acinetobacter baumannii Oxymoron: commensal hospital dweller turned pan-drug-resistant menace.Front Microbiol. 2012;3(1):148.PMID: 22536199
7. Zavascki AP, Bulitta JB, Landersdorfer CB. Combination therapy for carbapenem-resistant Gram-negative bacteria. Expert Rev Anti Infect Ther. 2013;11(12):1333-53. DOI: 10.1586/14787210.2013.845523 PMID: 24191943
8. Goic-Barisic I, Bedenic B, Tonkic M, Katic S, Kalenic S, Punda-Polic V. First report of molecular characterization of carbapenem-resistant Acinetobacter baumannii in different intensive care units in University Hospital Split, Croatia.J Chemother. 2007;19(4):462-4. DOI: 10.1179/joc.2007.19.4.462 PMID: 17855194
9. Goic-Barisic I. Multidrug-resistant Acinetobacter baumannii (MRAB) - ten years after the onset of these isolates in Croatia. Infectol Glasn.2012;32(2):67-70. Croatian.
10. Opazo A, Domínguez M, Bello H, Amyes SG, González-Rocha G. OXA-type carbapenemases in Acinetobacter baumannii in South America.J Infect Dev Ctries. 2012;6(4):311-6.PMID: 22505439
11. Higgins PG, Poirel L, Lehmann M, Nordmann P, Seifert H. OXA-143, a novel carbapenem-hydrolyzing class D beta-lactamase in Acinetobacter baumannii.Antimicrob Agents Chemother. 2009;53(12):5035-8. DOI: 10.1128/AAC.00856-09 PMID: 19770279
12. Goic-Barisic I, Bedenic B, Tonkic M, Novak A, Katic S, Kalenic S, et al. Occurrence of OXA-107 and ISAba1 in carbapenem-resistant isolates of Acinetobacter baumannii from Croatia. J Clin Microbiol. 2009;47(10):3348-9. DOI: 10.1128/JCM.02394-08 PMID: 19692565
13. Goic-Barisic I, Towner KJ, Kovacic A, Sisko-Kraljevic K, Tonkic M, Novak A, et al. Outbreak in Croatia caused by a new carbapenem-resistant clone of Acinetobacter baumannii producing OXA-72 carbapenemase. J Hosp Infect. 2011;77(4):368-9. DOI: 10.1016/j.jhin.2010.12.003 PMID: 21316806
14. Vranić-Ladavac M, Bedenić B, Minandri F, Ištok M, Bošnjak Z, Frančula-Zaninović S, et al. Carbapenem resistance and acquired class D beta-lactamases in Acinetobacter baumannii from Croatia 2009-2010. Eur J Clin Microbiol Infect Dis. 2014;33(3):471-8. DOI: 10.1007/s10096-013-1991-9 PMID: 24197438
15. Fournier PE, Richet H. The epidemiology and control of Acinetobacter baumannii in health care facilities.Clin Infect Dis. 2006;42(5):692-9. DOI: 10.1086/500202 PMID: 16447117
16. Farrugia DN, Elbourne LD, Hassan KA, Eijkelkamp BA, Tetu SG, Brown MH, et al. The complete genome and phenome of a community-acquired Acinetobacter baumannii. PLoS One. 2013;8(3):e58628.
17. Ferreira AE, Marchetti DP, De Oliveira LM, Gusatti CS, Fuentefria DB, Corção G. Presence of OXA-23-producing isolates of Acinetobacter baumannii in wastewater from hospitals in southern Brazil.Microb Drug Resist. 2011;17(2):221-7. DOI: 10.1089/mdr.2010.0013 PMID: 21254809
18. Zhang C, Qiu S, Wang Y, Qi L, Hao R, Liu X, et al. Higher isolation of NDM-1 producing Acinetobacter baumannii from the sewage of the hospitals in Beijing. PLoS One. 2013;8(6):e64857. DOI: 10.1371/journal.pone.0064857 PMID: 23755152
19. Girlich D, Poirel L, Nordmann P. First isolation of the blaOXA-23 carbapenemase gene from an environmental Acinetobacter baumannii isolate.Antimicrob Agents Chemother. 2010;54(1):578-9. DOI: 10.1128/AAC.00861-09 PMID: 19884362
20. Hrenovic J, Durn G, Goic-Barisic I, Kovacic A. Occurrence of an environmental Acinetobacter baumannii strain similar to a clinical isolate in paleosol from Croatia.Appl Environ Microbiol. 2014;80(9):2860-6. DOI: 10.1128/AEM.00312-14 PMID: 24584245
21. Standard Methods for Examination of Water and Wastewater. 21th edn. New York: American Public Health Association, American Water Works Association, Water Environment Federation; 2005.
22. HRN ISO 7899-2. Water quality – Detection and enumeration of intestinal enterococci, Part 2: membrane filtration method. Geneva: Organization for Standardization; 2000.
23. Clinical and Laboratory Standards Institute (CSLI). Performance standards for antimicrobial susceptibility testing, 17th informational supplement, M100-S17. Wayne, PA: CSLI; 2007.
24. Lee MJ, Jang SJ, Li XM, Park G, Kook JK, Kim MJ, et al. Comparison of rpoB gene sequencing, 16S rRNA gene sequencing, gyrB multiplex PCR, and the VITEK2 system for identification of Acinetobacter clinical isolates. Diagn Microbiol Infect Dis. 2014;78(1):29-34. DOI: 10.1016/j.diagmicrobio.2013.07.013 PMID: 24157058
25. Mencacci A, Monari C, Leli C, Merlini L, De Carolis E, Vella A, et al. Typing of nosocomial outbreaks of Acinetobacter baumannii by use of matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol. 2013;51(2):603-6. DOI: 10.1128/JCM.01811-12 PMID: 23175257
26. Spinali S, van Belkum A, Goering RV, Girard V, Welker M, Van Nuenen M, et al. Microbial typing by matrix-assisted laser desorption ionization-time of flight mass spectrometry: do we need guidance for data interpretation? J Clin Microbiol. 2015;53(3):760-5. DOI: 10.1128/JCM.01635-14 PMID: 25056329
27. Sousa C, Botelho J, Silva L, Grosso F, Nemec A, Lopes J, et al. MALDI-TOF MS and chemometric based identification of the Acinetobacter calcoaceticus-Acinetobacter baumannii complex species. Int J Med Microbiol. 2014;304(5-6):669-77. DOI: 10.1016/j.ijmm.2014.04.014 PMID: 24877727
28. Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH, et al. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol. 1995;33(9):2233-9. PMID: 7494007
29. Seifert H, Dolzani L, Bressan R, van der Reijden T, van Strijen B, Stefanik D, et al. Standardization and interlaboratory reproducibility assessment of pulsed-field gel electrophoresis-generated fingerprints of Acinetobacter baumannii. J Clin Microbiol. 2005;43(9):4328-35. DOI: 10.1128/JCM.43.9.4328-4335.2005 PMID: 16145073
30. European Committee on Antimicrobial Susceptibility Testing (EUCAST). EUCAST Reading guide. Version 4.0. Växjö: EUCAST; 2014.
31. Burckhardt I, Zimmermann S. Using matrix-assisted laser desorption ionization-time of flight mass spectrometry to detect carbapenem resistance within 1 to 2.5 hours.J Clin Microbiol. 2011;49(9):3321-4. DOI: 10.1128/JCM.00287-11 PMID: 21795515
32. Hrabák J, Walková R, Studentová V, Chudáčková E, Bergerová T. Carbapenemase activity detection by matrix-assisted laser desorption ionization-time of flight mass spectrometry.J Clin Microbiol. 2011;49(9):3222-7. DOI: 10.1128/JCM.00984-11 PMID: 21775535
33. Woodford N, Ellington MJ, Coelho JM, Turton JF, Ward ME, Brown S, et al. Multiplex PCR for genes encoding prevalent OXA carbapenemases in Acinetobacter spp. Int J Antimicrob Agents. 2006;27(4):351-3. DOI: 10.1016/j.ijantimicag.2006.01.004 PMID: 16564159
34. Abdallaa KZ, Hammamb G. Correlation between biochemical oxygen demand and chemical oxygen demand for various wastewater treatment plants in Egypt to obtain the biodegradability indices.Int J Sci Basic Appl Res.2014;13(1):42-8.
35. Official Gazette. Ordinance on emission limit values for wastewater discharges. 2013, no. 80.
36. Mote BL, Turner JW, Lipp EK. Persistence and growth of the fecal indicator bacteria enterococci in detritus and natural estuarine plankton communities.Appl Environ Microbiol. 2012;78(8):2569-77. DOI: 10.1128/AEM.06902-11 PMID: 22327586

37. Paşayeva P, Gezgin Y, Pekin G, Eltem R. Phosphate uptake performance of bacteria isolated from a full-scale Izmir municipal wastewater treatment plant.Environ Technol. 2011;32(5-6):543-9. DOI: 10.1080/09593330.2010.505250 PMID: 21877535
38. Hrenovic J, Tibljas D,, Ivankovic T, Kovacevic D, Sekovanic L. Sepiolite as carrier of the phosphate-accumulating bacteria Acinetobacter junii.Appl Clay Sci. 2010;50(4):582-7. DOI: 10.1016/j.clay.2010.10.024
39. Zhang Y, Marrs CF, Simon C, Xi C. Wastewater treatment contributes to selective increase of antibiotic resistance among Acinetobacter spp.Sci Total Environ. 2009;407(12):3702-6. DOI: 10.1016/j.scitotenv.2009.02.013 PMID: 19321192
40. Corbella X, Pujol M, Ayats J, Sendra M, Ardanuy C, Domínguez MA, et al. Relevance of digestive tract colonization in the epidemiology of nosocomial infections due to multiresistant Acinetobacter baumannii. Clin Infect Dis. 1996;23(2):329-34. DOI: 10.1093/clinids/23.2.329 PMID: 8842272
41. Zordan S, Prenger-Berninghoff E, Weiss R, van der Reijden T, van den Broek P, Baljer G, et al. Multidrug-resistant Acinetobacter baumannii in veterinary clinics, Germany. Emerg Infect Dis. 2011;17(9):1751-4. DOI: 10.3201/eid1709.101931 PMID: 21888812
42. Rizzo L, Manaia C, Merlin C, Schwartz T, Dagot C, Ploy MC, et al. Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: a review. Sci Total Environ. 2013;447(1):345-60. DOI: 10.1016/j.scitotenv.2013.01.032 PMID: 23396083
43. Luo Y, Yang F, Mathieu J, Mao D, Wang Q, Alvarez PJJ. Proliferation of multidrug-resistant New Delhi metallo-β-lactamase genes in municipal wastewater treatment plants in northern China.Environ Sci Technol Lett. 2014;1(1):26-30. DOI: 10.1021/ez400152e

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Research article

Testing for viral material in water of public bathing areas of the Danube during summer, Vojvodina, Serbia, 2014

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Citation style for this article:
Jovanović Galović A, Bijelović S, Milošević V, Hrnjaković Cvjetkovic I, Popović M, Kovačević G, Radovanov J, Dragić N, Petrović V. Testing for viral material in water of public bathing areas of the Danube during summer, Vojvodina, Serbia, 2014. Euro Surveill. 2016;21(15):pii=30196. DOI: http://dx.doi.org/10.2807/1560-7917.ES.2016.21.15.30196

Article submitted on 15 April 2015 / accepted on 14 April 2016 / published on 14 April 2016

From August to September 2014 a water quality study was conducted on five popular public Danube beaches in Vojvodina, Serbia. To assess the safety of Danube water for bathing, physical, chemical, bacteriological tests were performed. While many parameters for monitoring the quality of water are regulated by law, there are neither national nor international legislations addressing the presence of viruses in recreational waters. In this study, we performed analyses that surpassed national requirements, and investigated if adenovirus, enterovirus or rotavirus genetic material was present in samples of recreational water collected for quality monitoring. Of 90 water samples obtained during the study, enterovirus material was not found in any sample, but adenovirus and rotavirus genetic materials were respectively detected in 60 and 31 samples. Statistical analyses showed a significant correlation between adenovirus DNA and total coliforms in the water. Even when water samples were adequate for recreational use, adenoviruses were detected in 75% (57/76) of such samples. Our results indicate that implementation of viral indicators in recreational water might be helpful to better assess public health safety. This might be particularly relevant in areas where urban wastewater treatment is insufficient and surface waters affected by wastewater are used for recreation.

Introduction

Water quality is an essential prerequisite for human health. In the Water Quality and Health Strategy of the World Health Organization, (WHO) 2013–2020 [1], which is centred on the primary prevention of waterborne and water-related diseases, the first strategic objective is to 'obtain the most rigorous and relevant evidence regarding water quality and health'.

While WHO has issued guidelines for safe recreational water environments [2], in the European Union (EU), drinking and bathing water directives (Directive 2000/60/EC, Directive 2006/118/EC, and Directive 2006/7/EC) regulate monitoring and management of water for such purposes. Although many parameters are considered for monitoring water quality, there is currently no national or international regulation regarding the presence of viruses in recreational waters.

Epidemics caused by waterborne viral pathogens can occur however, and for persons affected, infection can potentially result in serious complications (hepatitis [3], meningitis [4], myocarditis [5]), even leading to death in some cases. The most important waterborne viruses are enteric viruses, and comprise adenoviruses, enteroviruses (polio, coxsackievirus A and B, echovirus), hepatitis A and E viruses, noroviruses I and II, and rotaviruses [6-8].

With respect to drinking water, outbreaks caused by waterborne viruses related to water that met bacteriological standards have been reported [9,10]. Viruses on the United States Environment Protection Agency (USEPA)'s drinking water Contaminant Candidate List 4 (CCL 4), a list of 'contaminants that are currently not subject to any proposed or promulgated national primary drinking water regulations, but are known or anticipated to occur in public water systems', include viruses of the Calciviridae (norovirus), Picornaviridae (enterovirus), Adenoviridae (adenovirus) families. These groups are internationally considered relevant in terms of public health with a suggestion that they should be part of monitoring [6,11,12] and there is a growing consensus within the scientific community, about the need to implement viral indicators in legislations that define water quality control [12,13].

Figure

Locations of the beaches investigated for recreational water quality on the Danube in the Autonomous Province of Vojvodina, Serbia, 2014

1: 'Strand' Novi Sad; 2: Futog; 3: 'Begecka jama' in Begec; 4: Backa Palanka; 5: Apatin.

The locations where water was sampled are marked in red and numbered from one to five.

Surface waters can become contaminated by viruses through sewage. Individuals with gastroenteritis or hepatitis excrete from 10^5 to 10^{11} viral particles per g of stool [14] and contact or ingestion of water contaminated by faeces might potentially in turn lead to human infections [11]. In surface waters affected by sewage, over 100 viral species may be found [6,7]. Still, only some are epidemiologically proven to be transmitted by water. The WHO guidelines for safe recreational water environments include enteroviruses as a regulatory parameter of importance for faecal pollution, but no guideline values are provided due to the 'insufficient data for the development of guideline values' [2].

In Serbia, urban wastewater treatment, although regulated by law [15], is still inadequate, and the sewerage network not complete due to financial and technical difficulties. In this context, faecal contamination of surface water is inevitable [16,17]. This represents a potential public health risk especially during bathing season. In the province of Vojvodina, several public beaches on the Danube are crowded with swimmers during the summer. According to the national legislation, physical, chemical and bacteriological parameters of the recreational water are monitored, as well as some biological indicators of water quality (macroinvertebrate, phytobenthos, phytoplankton, and macrophyte analyses). Similar to EU directives however, testing for viruses or viral material is not recognised as part of regular monitoring of bathing waters in the country [18,19].

The objective of our study was to perform analyses that surpass the national requirements and investigate the presence of adenovirus, enterovirus and rotavirus genetic material in recreational waters of the Danube during summer.

Methods

Water sampling

The study was conducted from August to September 2014 at five of the most popular beaches on the Danube in the Autonomous Province of Vojvodina, Serbia. The locations investigated were 'Strand' beach in the city of Novi Sad (1,257.57 river kilometre – rkm; measured from the river mouth) as well as beaches near villages and small towns – Futog (1,267.4 rkm); 'Begecka jama' in Begec (1,276.2–1,284 rkm); Backa Palanka (1,298.56 rkm); and Apatin (1,401.90 rkm) (Figure). Eighteen river water samples per location were respectively obtained by collecting three samples per week for a period of six weeks. This corresponded to a total of 90 recreational water samples for all five locations.

Water sampling, transport of samples and analyses of recreational water on site were done according to national standardised methods [20-23]. On site air and water temperatures were measured as recommended by the World Meteorological Organization [24]. Water sampling for virological analyses was done according to the procedure given by Method 1615, USEPA [25]. Briefly, 30 L of water were collected in pre-sterilised plastic containers. Water samples were taken at waist-depth, 30cm below the surface, closed carefully and transported to the laboratory at 4 °C. Upon arrival, the containers were kept in a refrigerated room (at 4 °C) until further processing for analysis, which occurred no longer than within 24h of sample arrival.

Microbiological indicators and physical and chemical parameters

For all samples, compliance with National Guideline Values (NGV) for microbiological indicators and physical and chemical parameters (the so-called 'ecological and chemical status') was determined. Analyses were undertaken in laboratories accredited by National Accreditation Bodies. Microbiological parameters defined by national regulation and relevant for this study were: faecal coliform concentration, total coliform concentration, intestinal enterococci concentration and the heterotrophic plate count [18,19]. These were determined by the membrane filter method defined in the standardised method, and the Kohl method [18,19,26].

Physical and chemical parameters were: pH by potentiometry; suspended particulate (mg/L) and total mineralisation (mg/L) by gravimetry; dissolved oxygen (mg/L) and chemical oxygen demand (COD) (mg O_2/l) by volumetric titrations; nitrate (mg N/l), nitrite (mg N/l), ammonium ion (mg N/l), total phosphorus (mg P/l) and orthophosphates (mg P/l) by spectrophotometry (VARIAN Cary 100); biological oxygen demand after five days (BOD5) (mg O_2/l) by volumetric titration [27]; total nitrogen (mg N/l) by volumetric

Table 1

Values of microbiological indicators, physical and chemical parameters defining classes of coastal recreational water applicable to Serbia, 2014

Parameter	Units	Guideline values				
		Class I	Class II	Class III	Class IV	Class V
Faecal coliform concentration	cfu/100 mL	≤100	≤1,000	≤10,000	≤100,000	›100,000
Total coliform concentration	cfu/100 mL	≤500	≤10,000	≤100,000	≤1,000,000	›1,000,000
Intestinal enterococci concentration	cfu/100 mL	≤200	≤400	≤4,000	≤40,000	›40,000
Heterotrophic plate count concentration	cfu/100 mL	≤500	≤10,000	≤100,000	≤750,000	›750,000
pH	–	6.5–8.5	6.5–8.5	6.5–8.5	6.5–8.5	‹6.5 and›8.5
Suspended particulate	mg/L	≤25	≤25	NG	NG	NG
Total mineralisation	mg/L	‹1,000 (or natural level)	≤1,000	≤1,300	≤1,500	›1,500
Conductivity	µSv/cm	‹1,000 (or natural level)	≤1,000	≤1,500	≤3,000	›3,000
Dissolved oxygen	mg O_2/L	8.5[a] (or natural level)	≥7[a]	≥5	≥4	‹4
Oxygen saturation – epilimnion stratification	%	90–110	70–90	50–70	30–50	‹30
COD	mg O_2/L	5 (or natural level)	≤10	≤20	≤50	›50
BOD5	mg O_2/L	2[a] (or natural level)	≤5[a]	≤7	≤25	›25
Nitrate (NO_3^-N)	mg N/L	1[a] (or natural level)	≤3[a]	≤6	≤15	›15
Nitrite (NO_2^-N)	mg N/L	0.01 (or natural level)	≤0.03	≤0.12	≤0.30	›0.30
Ammonium ion (NH_4^-N)	mg N/L	0.10[a] (or natural level)	≤0.30[a]	≤0.60	≤1.50	›1.50
Total nitrogen (N)	mg N/L	1 (or natural level)	≤2	≤8	≤15	›15
Total phosphorus (P)	mg P/L	0.05 (or natural level)	≤0.20[a]	≤0.40	≤1	›1
Orthophosphates (PO_4^{3-})	mg P/L	0.02 (or natural level)	≤0.10[a]	≤0.20	≤0.50	›0.50

BOD5: biological oxygen demand after five days; cfu: colony forming unit; COD: chemical oxygen demand; NG: no guideline for this class.

For a water sample to belong to a given class, all the parameter values in the corresponding column for this class have to be fulfilled.

[a] Depends on the type and coastal water magnitude. Analysed samples are from the Danube, a river defined by national regulation as river Type I (i.e. large lowland river, with dominance of fine drifts); guideline values provided in Table 1 for Class I and II are valid for a Type I river [18,19].

titrations [28]; conductivity electrochemically (µS/cm) (METTLER TOLEDO SevenMulti); turbidity by turbidimetry (nephelometric turbidity units – NTU) (HACH 2100N Laboratory Turbidimeter) and oxygen saturation by computing.

According to the national regulation five classes of coastal recreational water by decreasing quality are defined (I, II, III, IV and V), whereby the first three classes (I–III) are considered adequate for human recreational needs, fish farming and drinking – after treatment [18,19]. The microbiological indicators, physical and chemical parameters for each class are described in Table 1. There is no national guideline value for turbidimetry in recreational water [18,19].

Study of viral material present in the water

Assessing the presence of viruses in water is not defined by the current national legislation, so it was performed according to internationally recognised methodology with minor modifications [29]. Briefly, 30L of river water was filtered with nanoceramic filters (NanoCeram-VS Virus Sampler Filter Cartridges, Argonide Corp., US) allowing negatively-charged virus particles to be absorbed onto the positively-charged nanoceramic filters. Following elution with 500 mL of elution buffer (1.5% dessicated beef extract (Sigma-Aldrich) containing 0.05M glycine (Sigma-Aldrich) pH 9.0), the eluate was concentrated by organic flocculation whereby viral particles attached to beef extract proteins at pH 3.5 and were pelleted by centrifugation for 15min at 2,500 x g at 4°C. The pellet was resuspended in 15 mL of 0.15M phosphate buffer pH 7.0. The sample was further concentrated by ultrafiltration using

Table 2

Description of key variables[a] relevant to the investigation of Danube water quality at different locations, Vojvodina, Serbia, 2014

Location	Faecal coliform cfu/100 mL AV ± SD	Total coliforms cfu/100 mL AV ± SD	Intestinal enterococci[b] cfu/100 mL AV	Air T on site °C AV ± SD	pH	Nitrate (NO_3^-N) mg N/L AV ± SD	Nitrite (NO_2^-N) mg N/L AV ± SD	Ammonium ion (NH_4^+N) mg N/L AV ± SD	Turbidity NTU AV ± SD
Strand	4,772.22 ± 4,368.17	1,1961.11± 6,876.14	1,000[c]	18.82 ± 2.43	8.01 ± 0.08	0.99 ± 0.12	0.01 ± 0.006	0.39 ± 0.14	33.16 ± 15.51
Futog	4,894.44 ± 4,695.11	12,838.89 ± 5,218.42	9,000[c]	19.95 ± 2.48	7.99 ± 0.08	0.91 ± 0.12	0.02 ± 0.006	0.34 ± 0.12	31.44 ± 14.91
Begecka jama	2,966.67 ± 3,185.63	7,127.78± 4,450.22	1,400[d]	19.51 ± 2.73	8.16 ± 0.21	0.22 ± 0.20	0.01 ± 0.006	0.46 ± 0.29	8.40 ± 3.40
Backa Palanka	1,805.56 ± 1,436.28	7,050.00± 3,189.73	1,000[c]	18.37 ± 3.68	8.06 ± 0.19	0.01 ± 0.006	0.01 ± 0.006	0.26 ± 0.09	24.60 ± 8.69
Apatin	772.22± 505.04	2,394.44 ± 2,084.53	2,000[c]	21.25 ± 3.60	8.19 ± 0.32	0.02 ± 0.006	0.01 ± 0.006	0.20 ± 0.13	5.52 ± 2.08

AV: average (arithmetic mean); CFU: colony forming unit; N: nitrogen; NTU: nephelometric turbidity unit; SD: standard deviation; T: temperature.

[a] Only key variables from Table 1 were statistically analysed for the purpose of this study. Accordingly heterotrophic plate count and other physical and chemical parameters showed in Table 1 were not taken into further statistical consideration.

[b] Because of the small number of water samples where intestinal enterococci were detected, these were not taken into consideration for further statistics.

[c] Value determined in only one of 18 samples.

[d] Average value calculated according to number of intestinal enterococci determined in two of 18 samples.

Amicon Ultra-15, 30K device (Milipore) to obtain 0.5 mL of sample. Viral nucleic acids (DNA, RNA) were isolated by Ribo Virus kit (Sacace Biotechnologies, Italy), and analysed by real-time polymerase chain reaction (PCR) using a real-time thermocycler Applied Biosystems 7500 (Applied Biosystems, US) as well as the following kits for human diagnostics: RIDAGENE Rotavirus/Adenovirus Duplex (R-Biopharm AG, Germany); Enterovirus Real-TM (Sacace Biotechnologies, Italy).

Statistics

For data processing, the standard parametric and non-parametric statistical methods were used. Numerical data are presented through the arithmetic mean and standard deviation, while qualitative data are assessed by frequency of distribution. To check the normality of continuous data we used Shapiro–Wilk normality test, along with the normal-probability plots. Data of faecal coliforms were $\log_{10}$ transformed to meet assumptions of normality, while transformation (squareroot and $\log_{10}$) of data for total coliforms did not improve their normality. In accordance with the objectives of the analyses and nature of the data, Spearman's correlation coefficient, Fisher exact test, Mann–Whitney U test and Student's t-test were used. Data of faecal coliforms were log transformed to meet assumptions of normality for the Student's *t*-test. In all statistical analyses the presence of viruses was examined as a dependent variable, and coded as '1' to indicate absence of viruses and '2' to indicate presence of viruses. Results were considered statistically significant when the p value for all the applied models was <0.05 and the corresponding 95% confidence interval (95%CI) did not include 1.0 (Student's *t*-test). For all statistical analyses the statistical package SPSS (version 17), Microsoft Office Excel 2003 and Microsoft Visual Fox Pro were used.

Results

Chemical and microbiological findings

In terms of microbiological parameters, the average concentration of faecal coliforms ranged from 772.22 ± 505.04 cfu/100 mL on Apatin beach to 4,894.44 ± 4,695.11 cfu/100 mL on Futog beach, while the average total coliform concentration was in the range of 2,394.44 ± 2,084.53 cfu/100 mL to 12,838.89 ± 5,218.42 cfu/100 mL, at the same respective locations (Table 2).

For chemical analyses, the minimum average value of pH (7.99 ± 0.08) and nitrite (0.02 mg nitrogen/mL ± 0.006) was found at Futog, while the minimum average value of nitrate (0.01 mg nitrogen/mL ± 0.006) was at Backa Palanka. The highest average value of ammonium ion (0.46 mg nitrogen/mL ± 0.29) was found at Begecka jama. Average values of turbidity ranged between 5.52 NTU ± 2.08 and 33.16 NTU ± 15.51 at Apatin and Strand, respectively (Table 2).

Table 3

Quality of the water samples at different locations on the Danube in terms of class of recreational water and presence of viral material, Vojvodina, Serbia, 2014

Location	Samples	Suitable for recreation			Unsuitable for recreation		
		Class II N	Class III N	Total N	Class IV N	Class V N	Total N
Strand (18 samples)	Totals[a]	2	14	16	2	0	2
	With adenoviruses	1	11	12	1	0	1
	With rotaviruses	0	9	9	0	0	0
Futog (18 samples)	Totals[a]	1	16	17	1	0	1
	With adenoviruses	1	14	15	1	0	1
	With rotaviruses	0	4	4	1	0	1
Begecka jama (18 samples)	Totals[a]	2	10	12	3	3	6
	With adenoviruses	2	4	6	0	0	0
	With rotaviruses	1	1	2	0	0	0
Backa Palanka (18 samples)	Totals[a]	2	16	18	0	0	0
	With adenoviruses	2	15	17	0	0	0
	With rotaviruses	0	6	6	0	0	0
Apatin (18 samples)	Totals[a]	2	11	13	0	5	5
	With adenoviruses	1	6	7	0	1	1
	With rotaviruses	2	5	7	0	2	2
Total (90 samples)	**Totals[a]**	**9**	**67**	**76**	**6**	**8**	**14**
	With adenoviruses	**7**	**50**	**57**	**2**	**1**	**3**
	With rotaviruses	**3**	**25**	**28**	**1**	**2**	**3**

N: number of samples.

None of the water samples were defined as Class I.

[a] These totals are the number of samples of a certain category of water quality (defined as water class, or as suitable/unsuitable for recreational use) at each location. Adding up the number of samples testing positive for adenoviruses with the number of samples testing positive for rotaviruses in each category, does not necessarily yield the totals presented, because some individual samples could test positive for both adenovirus and rotavirus. Moreover, some samples included in the total could be negative for both types of viruses.

Classification of the coastal recreation water samples

Taking into account parameters defined by national law – the so called 'chemical and ecological status' for recreational waters, 76 of a total of 90 samples analysed at all locations (84%) complied to the NGV for recreational use (Table 3).

Considering water class distribution at each beach, full compliance with NGV for recreational use over the whole study period was only observed for Backa Palanka, with all 18 water samples taken at this location corresponding to class II or III (2 samples of class II and 16 of class III).

Of the 18 water samples collected respectively at the other beaches, 13 were of class II or III at Apatin, 12 at Begecka jama, 16 at Strand and 17 at Futog (Table 3). One class IV sample was found at Futog, two at Strand and three at Begecka jama (Table 3). Three water samples corresponding to class V were detected at Begecka jama and five at Apatin.

Presence of viral material in the water and relationship with water quality parameters

Enteroviruses were not detected in any of the samples of recreational water analysed, while adenoviruses and rotaviruses were respectively found in 60 (67%) and 31 (34%) samples of the total 90 (Table 3).

Adenoviruses were predominantly present in water collected at Backa Palanka beach (17 of 18 samples), where all analysed samples were in accordance with NGV (Table 3). Rotaviruses were mostly found on the beaches of Strand in Novi Sad and Apatin (9 and 7 samples respectively) where 16 and 13 samples complied to the NGV respectively.

An analysis by Spearman's correlation showed that regardless of the species, the presence of viral material (i.e. adenoviruses and rotaviruses, taken together), was negatively correlated with the surface water temperature ($p<0.05$) and positively correlated with nitrite concentration ($p<0.05$) (Table 4). There was no statistically significant relationship to the class distribution of recreational water samples, nor to the concentration of total coliforms and faecal coliforms.

Table 4

Spearman rank correlation of microbiological indicators, physical and chemical parameters, water samples status defined as classes and the presence of adenoviruses, rotaviruses, and adenoviruses and rotaviruses taken together, Vojvodina, Serbia, 2014

Microbiological indicators/ physical and chemical parameters of water samples status of coastal recreational water	Adenovirus	Rotavirus	Adenovirus and rotavirus
Faecal coliforms concentration	0.265[a]	0.024	-0.089
Total coliforms concentration	0.333[b]	0.049	0.002
pH value	-0.214[a]	-0.040	-0.141
Nitrate (NO_3^-N)	0.273[b]	0.205	0.151
Nitrite (NO_2^-N)	-0.004	0.034	0.213[a]
Ammonium ion (NH_4^-N)	-0.110	-0.156	-0.039
Turbidity	0.312[b]	-0.012	-0.007
Temperature of air on site	-0.066	-0.290[b]	-0.173
Temperature of water on site	-0.451[b]	-0.311[b]	-0.210[a]
Class	-0.355[b]	-0.080	-0.077

[a] Spearman's correlation statistically significant at the level $p<0.05$.

[b] Spearman's correlation statistically significant at the level $p<0.01$.

When viral species were analysed separately, the presence of rotavirus material was significantly negatively correlated with surface water and air temperatures ($p<0.01$ for both parameters). No significant relationship to class distribution was observed.

Like for rotavirus, presence of adenovirus material (Table 4) was significantly negatively correlated with temperature of surface water ($p<0.01$). Water pH value was negatively correlated ($p<0.05$). A positive correlation was obtained between presence of adenovirus material and concentrations of faecal coliforms ($p<0.05$) and total coliforms ($p<0.01$), as well as concentration of nitrate ($p<0.01$) and turbidity ($p<0.01$). For class distribution, a negative correlation was found ($r_o = -0.355$, $p<0.001$).

The relationship between adenoviruses and coliforms in the water as well as adenoviruses and water class were further explored with other statistical tests.

Samples testing positive for adenovirus material were more frequently found when concentrations of total coliforms (Mann–Whitney U test; $p=0.002$) and faecal coliforms (*t* test, $p=0.017$) were elevated, i.e. ≥100,000 cfu/100 mL and ≥10,000 cfu/100 mL respectively (Table 5).

In terms of the correlation between adenoviruses and class, assessment using the Fisher exact test indicated that even when the water samples corresponded to class I-III (n=76 samples), adenoviruses were detected in 75% of samples (n=57) in relation to 25% of samples(n=19), where adenoviruses were not detected ($p=0.000$).

Discussion

In this study, we found that 76 of a total of 90 water samples collected at popular beaches on the Danube in Vojvodina during the summer 2014 complied the NGV for recreational use. Further analyses beyond the requirements defined by current legislation however confirm the presence of adenovirus and rotavirus genetic material respectively in 60 and 31 of the 90 obtained water samples. Although data on viruses in environmental water are scarce for Serbia, a recent report also relates detection of adeno- and/or norovirus material in surface waters of Vojvodina province, at 21 of 30 analysed locations [30]. In that report however, only two of the locations investigated were public beaches and sampling was done only once in the bathing season. Our study in contrast was designed to seek viral material in the Danube at five different popular beach locations, throughout the bathing season.

In addition to checking for the presence of viral material in the water, we conducted in parallel the routine law-required analyses for monitoring water quality. Results suggest faecal contamination of recreational waters in the Danube at all investigated locations, with data not differing significantly from those obtained in previous years [31]. A statistically significant positive correlation was moreover obtained between adenovirus material in the Danube and both the nitrate concentration and turbidity of the water, which are parameters related to faecal contamination. Corroborating these results, adenovirus genetic material was also significantly positively correlated with concentrations of total coliforms and faecal coliforms.

The results found here are in agreement with those of a large European study (EU FP6 Project VIROBATHE study) [13], where significant trends were observed

Table 5

Association between the presence of adenovirus, class distribution and the number of faecal coliforms and total coliforms concentration in analysed recreational samples, Vojvodina, Serbia, 2014

Detection of adenoviruses	Fisher's exact test Water samples classes			Independent samples *t* test Log_{10} of faecal coliforms			Mann–Whitney U test Total number of coliforms			
	Class I–III[a] N (%)	Class IV–V N (%)	P value[d]	N	GM[b] ± SD	Significance	N	Sum of Ranks	Z[c]	P value
Neg.	19 (25)	11 (79)	0.000	30	3.08 ± 0.39	$t^e = 2.218$ $d^f = 88$ $p = 0.017^g$	30	999	–	0.002
Pos.	57 (75)	3 (21)		60	3.32 ± 0.472		60	3,096	3.138	

G: geometric mean; N: number of samples; neg.: negative for adenovirus; pos.: positive for adenovirus; SD: standard deviation.

[a] According to national regulations the first three classes (I-III) allows using the coastal water for human recreational needs including for fish farming and, after adequate treatment, for drinking.

[b] 10^x, where X is the mean of log_{10} transformed values.

[c] Z value of Mann–Whitney U test.

[d] *p* significant (2-tailed).

[e] *t* value of independent samples *t* test.

[f] Equal variances not assumed (tested with Levine's test for variance).

[g] Significant with 95% confidence interval: -0.47 to -0.04.

between categories of increasing faecal indicator organism concentration (*Escherichia coli*, intestinal enterococci and somatic coliphage) and increasing proportions of human adenovirus-positive results in freshwater. An explanation for the results, may lie in the fact that human adenovirus has been detected at high concentration in faeces of infected individuals (10^6 per g of stool) [32].

Quantitative analyses of adenovirus genetic material in European recreational waters in 2010 found 3.2 x 10^2 genomic copies of adenovirus per 100 mL water on average [33], while in another study of the EU FP6 Project VIROBATHE, Wyn-Jones et al. [12] detected adenoviruses in 36% of the samples that they collected in diverse freshwater and marine sites in Europe. The finding that 60 of 90 (67%) water samples collected in our study were contaminated with adenovirus material, is likely attributable to the absence of adequate urban wastewater treatment in Serbia. While it is clear that the only long-term and comprehensive solution to this problem would be a coordinated action of all stakeholders to prevent the contamination of surface waters, the elimination of raw sewage entering rivers may require a number of important measures (e.g. management and improvement of sewerage networks and treatment plants, efforts to reduce spills of sewage during rainfall events by storm tanks, separation of surface and foul infrastructure, sustainable urban drainage schemes) and may not be imminent. Therefore a more extensive control of bathing waters may be considered. Indeed, viral infectivity doses for enteric viruses are very low (10-100 virions) and such viruses persist longer (up to 120 days in freshwater) and are more resistant than enterobacteria to some environmental factors (UV radiation, chemicals) [8].

The presence of viral material in freshwater is moreover not limited to Serbia [12,33-36], and in some EU Member States such as Ireland, raw sewage entering environmental water still represents a problem [37]. In this context, an adequate viral indicator for water, together with surveillance of waterborne virus infections, may generally contribute to a more comprehensive hazard assessment for users of this water. In some countries, such as the US, a special surveillance system for waterborne diseases has been put in place [38,39].

Our study additionally suggests that despite a correlation between adenovirus material and coliforms, bacterial analyses alone cannot always predict the presence of viral material in the water. Even when water samples were found to be suitable for recreational use, 75% of these samples tested positive for adenovirus genetic material. An explanation for this may lie in the fact that adenoviruses have a double stranded DNA genome, which is more sturdy and resistant to various environmental factors than e.g. RNA, which forms the base of rotaviruses' genome. Human adenoviruses are also known to persist longer in the environment than bacterial indicators of faecal contamination and are frequently the most abundant type of virus in surface waters [32]. Because infections with adenovirus may produce serious, even lethal diseases especially in immunocompromised persons, children and elderly [32], monitoring such viruses in recreational water may be of importance.

It should be noted, that the presence of viral material in this study was determined by PCR and does not necessarily mean the presence of infective virus, since PCR detects both non-infective and infective viral genetic material. However, according to the literature data on the survival of adenoviral particles in the environment [40,41] a risk for recreational use could possibly exist.

In order to establish the presence of infective viral particles, more research using cell culture is needed.

In contrast to adenovirus, rotavirus material was present in much lower proportions of our samples (31 samples with rotavirus vs 60 with adenovirus), and this corroborates with the findings of other authors who did similar testing and found rotaviruses much less present than adenoviruses in surface water contaminated with sewage [42]. Although it is difficult to compare the results from different parts of the world, it might be speculated that this result is due to the lower occurrence of such viruses in the human population, as well as their structure [11]. Having RNA as genetic material might make rotaviruses more vulnerable to environmental conditions, as shown here by a significant correlation between rotavirus material presence and air and water temperature. Due to their lower occurrence, it may be concluded that, although of importance [43], rotaviruses would likely not be suitable candidates as a water viral indicator.

Taken together, our data support the need for more measures to avoid faecal contamination of the Danube, and provide more evidence for the idea of having a viral indicator for recreational waters in order to fully assess public health safety. Such an indicator would be of particular importance in regions like Vojvodina (and even in the whole of Serbia), where urban wastewater treatment is not adequate.

Acknowledgements

Project was funded by Provincial Secretariat for Science and Technological Development, as a Project of Special Interest for Sustainable Development, Project No. 114-451-445/2014-03.

Conflict of interest

None declared.

Authors' contributions

AJG: wrote the manuscript, coordinated the study, performed virology analyses; SB: co-writer of the manuscript, interpreted the results; VM: coordinated the study, interpreted the results, revised the manuscript; IH: interpreted the results, revised the manuscript; MP: coordinated the study and chemical analyses; GK and JR: performed water samples for virology analyses, performed PCR assays. ND: performed statistical analyses; VP: coordinated the study, revised the manuscript.

References

1. World Health Organization (WHO). Water Quality and Health Strategy 2013-2020. Geneva: WHO; 2013. Available from: http://www.who.int/water_sanitation_health/publications/2013/water_quality_strategy/en/
2. World Health Organization (WHO). Guidelines for safe recreational water environments. Volume 1, Coastal and fresh waters. Geneva: WHO; 2003.
3. Van der Poel WHM. Food and environmental routes of Hepatitis E virus transmission.Curr Opin Virol. 2014;4:91-6. DOI: 10.1016/j.coviro.2014.01.006 PMID: 24513966
4. Rhoades RE, Tabor-Godwin JM, Tsueng G, Feuer R. Enterovirus infections of the central nervous system.Virology. 2011;411(2):288-305. DOI: 10.1016/j.virol.2010.12.014 PMID: 21251690
5. Gaaloul I, Riabi S, Harrath R, Evans M, Salem NH, Mlayeh S, et al. Sudden unexpected death related to enterovirus myocarditis: histopathology, immunohistochemistry and molecular pathology diagnosis at post-mortem. BMC Infect Dis. 2012;12(1):212. DOI: 10.1186/1471-2334-12-212 PMID: 22966951
6. La Rosa G, Fratini M, della Libera S, Iaconelli M, Muscillo M. Emerging and potentially emerging viruses in water environments.Ann Ist Super Sanita. 2012;48(4):397-406. DOI: 10.4415/ANN_12_04_07 PMID: 23247136
7. Bosch A. Human enteric viruses in the water environment: a minireview.Int Microbiol. 1998;1(3):191-6.PMID: 10943359
8. Fong TT, Lipp EK. Enteric viruses of humans and animals in aquatic environments: health risks, detection, and potential water quality assessment tools.Microbiol Mol Biol Rev. 2005;69(2):357-71. DOI: 10.1128/MMBR.69.2.357-371.2005 PMID: 15944460
9. Bosch A, Lucena F, Díez JM, Gajardo R, Blasi M, Jofre J. Waterborne viruses associated with hepatitis outbreak.J Am Water Works Assoc. 1991;83(3):80-3.
10. Hejkal TW, Keswick B, LaBelle RL, Gerba CP, Sanchez Y, Dreesman G, et al. Viruses in a community water supply associated with an outbreak of gastroenteritis and infectious hepatitis. J Am Water Works Assoc. 1982;74(6):318-21.
11. Gibson KE. Viral pathogens in water: occurrence, public health impact, and available control strategies.Curr Opin Virol. 2014;4:50-7. DOI: 10.1016/j.coviro.2013.12.005 PMID: 24440908
12. Wyn-Jones AP, Carducci A, Cook N, D'Agostino M, Divizia M, Fleischer J, et al. Surveillance of adenoviruses and noroviruses in European recreational waters. Water Res. 2011;45(3):1025-38. DOI: 10.1016/j.watres.2010.10.015 PMID: 21093010
13. Wyer MD, Wyn-Jones AP, Kay D, Au-Yeung H-KC, Gironés R, López-Pila J, et al. Relationships between human adenoviruses and faecal indicator organisms in European recreational waters. Water Res. 2012;46(13):4130-41. DOI: 10.1016/j.watres.2012.04.008 PMID: 22633054
14. Farthing MJG. Viruses and the Gut. Welwyn Garden City, Hertfordshire: Smith Kline & French; 1989.
15. Official Bulletin of the Republic of Serbia. The Law on Waters. No 30/10. Serbian. Available from: http://www.rdvode.gov.rs/doc/dokumenta/zakoni/zakon-o-vodama.pdf
16. Vujovic S, Kolakovic S. Intergrated water approach for Serbia's Resurgence. Water World. 2013; 28(4). Available from: http://www.waterworld.com/articles/wwi/print/volume-28/issue-4/regional-spotlight/intergrated-water-approach-for-serbia-s-resurgence.html
17. Wastewater country profile Serbia and Montenegro, United Nations report 2004. Available from: http://www.un.org/esa/agenda21/natlinfo/countr/serbia/serbiawastewater.pdf
18. Regulations on the parameters of the ecological and chemical status of surface waters and the parameters of the chemical and quantitative status of groundwater. Official Gazette of the Republic of Serbia. 2011; 74.
19. Regulation on limit values of pollutants in surface and ground waters and sediments, and deadlines for achieving them. Official Gazette of the Republic of Serbia. 2012; 50.
20. Institute for standardization of Serbia (ISS). Testing of water. Measurement of temperature. SRPS H.Z1.106:1970. Beograd: ISS; 1970.
21. Institute for standardization of Serbia (ISS). Water quality - Sampling. Part 1: Guidance on the design of sampling programmes and sampling techniques. SRPS EN ISO 5667-1:2008 (sr). Identical with EN ISO 5667-1:2006 CEN/TC 230 and EN ISO 5667-1:2006/AC: 2007 CEN/TC 230. Beograd: ISS; 2008.
22. Institute for standardization of Serbia (ISS). Water quality - Sampling. Part 3: Guidance on the preservation and handling of water samples. SRPS EN ISO 5667-3:2007 (sr). Identical with EN ISO 5667-3:2003 CEN/TC 230. Beograd: ISS; 2007.
23. Institute for standardization of Serbia (ISS). Water quality - Sampling. Part 6: Guidance on sampling of rivers and streams. SRPS ISO 5667-6:1997. Identical with ISO 5667-6:1990 ISO/TC 147/SC 6. Beograd: ISS; 1997.
24. World Meteorological Organization (WMO). Guide to Meteorological Instruments and Methods of Observation. WMO, No.8, 2008 edition, updated in 2010. Geneva: WMO; 2012.
25. United States Environmental Protection Agency (EPA). Method 1615, Measurement of Enterovirus and Norovirus Occurrence in Water by Culture and RT-qPCR, EPA 600/R-10/181, December 2010, Version 1.1, Revised January 2012. Washington DC: EPA; 2012. Available from: www.epa.gov/ord

26. Institute for standardization of Serbia (ISS). Water quality - Detection and enumeration of Escherichia coli and coliform bacteria - Part 1: Membrane filtration method. SRPS EN ISO 9308-1:2010. Identical with EN ISO 9308-1:2000 CEN/TC 230 and EN ISO 9308-1:2000/AC:2008 CEN/TC 230. Beograd: ISS;2010.
27. Institute for standardization of Serbia (ISS). Water quality - Determination of biochemical oxygen demand after n days (BODn) - Part 2: Method for undiluted samples (ISO 5815:1989, modified). SRPS EN 1899-2:2009. Identical with EN 1899-2:1998 CEN/TC 230. Beograd: ISS;2009.
28. Institute for standardization of Serbia (ISS). Water quality - Determination of Kjeldahl nitrogen - Method after mineralization with selenium (ISO 5663:1984). SRPS EN 25663:2009. Identical with EN 25663:1993 CEN/TC 230. Beograd: ISS; 2009
29. Cashdollar JL, Brinkman NE, Griffin SM, McMinn BR, Rhodes ER, Varughese EA, et al. Development and evaluation of EPA method 1615 for detection of enterovirus and norovirus in water. Appl Environ Microbiol. 2013;79(1):215-23. DOI: 10.1128/AEM.02270-12 PMID: 23087037
30. Lazić G, Grubač S, Lupulović D, Bugarski D, Lazić S, Knežević P, et al. Presence of Human and Animal Viruses in Surface Waters in Vojvodina Province of Serbia. Food Environ Virol. 2015;7(2):149-58. DOI: 10.1007/s12560-015-9187-3 PMID: 25687987
31. Arsic M, Ac Nikolic E, Balac D, Bijelovic S, Bjelanovic J, Velicki R, et al. Health status of population living in the City of Novi Sad. Institute of Public Health of Vojvodina, 2013. Available from: http://izjzv.org.rs/uploads/download/Zdravstveno_stanje_stanovnistva/Novi%20Sad/NoviSad_2012.pdf
32. Mena KD, Gerba CP. Waterborne adenovirus.Rev Environ Contam Toxicol. 2009;198:133-67. DOI: 10.1007/978-0-387-09647-6_4 PMID: 19253037
33. Bofill-Mas S, Calgua B, Clemente-Casares P, La Rosa G, Iaconelli M, Muscillo M, et al. Quantification of human adenoviruses in European recreational waters. Food Environ Virol. 2010;2(2):101-9. DOI: 10.1007/s12560-010-9035-4
34. Jiang SC, Chu W. PCR detection of pathogenic viruses in southern California urban rivers.J Appl Microbiol. 2004;97(1):17-28. DOI: 10.1111/j.1365-2672.2004.02269.x PMID: 15186438
35. Albinana-Gimenez N, Miagostovich MP, Calgua B, Huguet JM, Matia L, Girones R. Analysis of adenoviruses and polyomaviruses quantified by qPCR as indicators of water quality in source and drinking-water treatment plants.Water Res. 2009;43(7):2011-9. DOI: 10.1016/j.watres.2009.01.025 PMID: 19230949
36. Ye XY, Ming X, Zhang YL, Xiao WQ, Huang XN, Cao YG, et al. Real-time PCR detection of enteric viruses in source water and treated drinking water in Wuhan, China. Curr Microbiol. 2012;65(3):244-53. DOI: 10.1007/s00284-012-0152-1 PMID: 22645016
37. Environmental Protection Agency. Ireland (EPA). EPA calls for the elimination of raw sewage entering the environment and improvement in the management of wastewater plants. EPA; 2015. [Accessed 10 Apr 2015]. Available from: http://www.epa.ie/newsandevents/news/name,53429,en.html#.VR58mmeQl9A
38. Brunkard JM, Ailes E, Roberts VA, Hill V, Hilborn ED, Craun GF, et al. . Surveillance for waterborne disease outbreaks associated with drinking water---United States, 2007--2008. MMWR Surveill Summ. 2011;60(12):38-68.PMID: 21937977
39. Hlavsa MC, Roberts VA, Anderson AR, Hill VR, Kahler AM, Orr M, et al. . Surveillance for waterborne disease outbreaks and other health events associated with recreational water --- United States, 2007--2008.MMWR Surveill Summ. 2011;60(12):1-32.PMID: 21937976
40. Kotwal G, Cannon JL. Environmental persistence and transfer of enteric viruses.Curr Opin Virol. 2014;4:37-43. DOI: 10.1016/j.coviro.2013.12.003 PMID: 24413147
41. Ogorzaly L, Bertrand I, Paris M, Maul A, Gantzer C. Occurrence, survival, and persistence of human adenoviruses and F-specific RNA phages in raw groundwater.Appl Environ Microbiol. 2010;76(24):8019-25. DOI: 10.1128/AEM.00917-10 PMID: 20952644
42. Victoria M, Fumian TM, Rocha MS, Dalmao F, Leite JPG, Girones R, et al. Gastroenteric virus dissemination and influence of rainfall events in urban beaches in Brazil. J Appl Microbiol. 2014;117(4):1210-8. DOI: 10.1111/jam.12592 PMID: 24980661
43. Kiulia NM, Hofstra N, Vermeulen LC, Obara MA, Medema G, Rose JB. Global occurrence and emission of rotaviruses to surface waters.Pathogens. 2015;4(2):229-55. DOI: 10.3390/pathogens4020229 PMID: 25984911

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Research article

Environmental surveillance of viruses by tangential flow filtration and metagenomic reconstruction

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Citation style for this article:
Furtak V, Roivainen M, Mirochnichenko O, Zagorodnyaya T, Laassri M, Zaidi SZ, Rehman L, Alam MM, Chizhikov V, Chumakov K. Environmental surveillance of viruses by tangential flow filtration and metagenomic reconstruction. Euro Surveill. 2016;21(15):pii=30193. DOI: http://dx.doi.org/10.2807/1560-7917.ES.2016.21.15.30193

Article submitted on 14 July 2015 / accepted on 14 April 2016 / published on 14 April 2016

An approach is proposed for environmental surveillance of poliovirus by concentrating sewage samples with tangential flow filtration (TFF) followed by deep sequencing of viral RNA. Subsequent to testing the method with samples from Finland, samples from Pakistan, a country endemic for poliovirus, were investigated. Genomic sequencing was either performed directly, for unbiased identification of viruses regardless of their ability to grow in cell cultures, or after virus enrichment by cell culture or immunoprecipitation. Bioinformatics enabled separation and determination of individual consensus sequences. Overall, deep sequencing of the entire viral population identified polioviruses, non-polio enteroviruses, and other viruses. In Pakistani sewage samples, adeno-associated virus, unable to replicate autonomously in cell cultures, was the most abundant human virus. The presence of recombinants of wild polioviruses of serotype 1 (WPV1) was also inferred, whereby currently circulating WPV1 of south-Asian (SOAS) lineage comprised two sub-lineages depending on their non-capsid region origin. Complete genome analyses additionally identified point mutants and intertypic recombinants between attenuated Sabin strains in the Pakistani samples, and in one Finnish sample. The approach could allow rapid environmental surveillance of viruses causing human infections. It creates a permanent digital repository of the entire virome potentially useful for retrospective screening of future discovered viruses.

Introduction

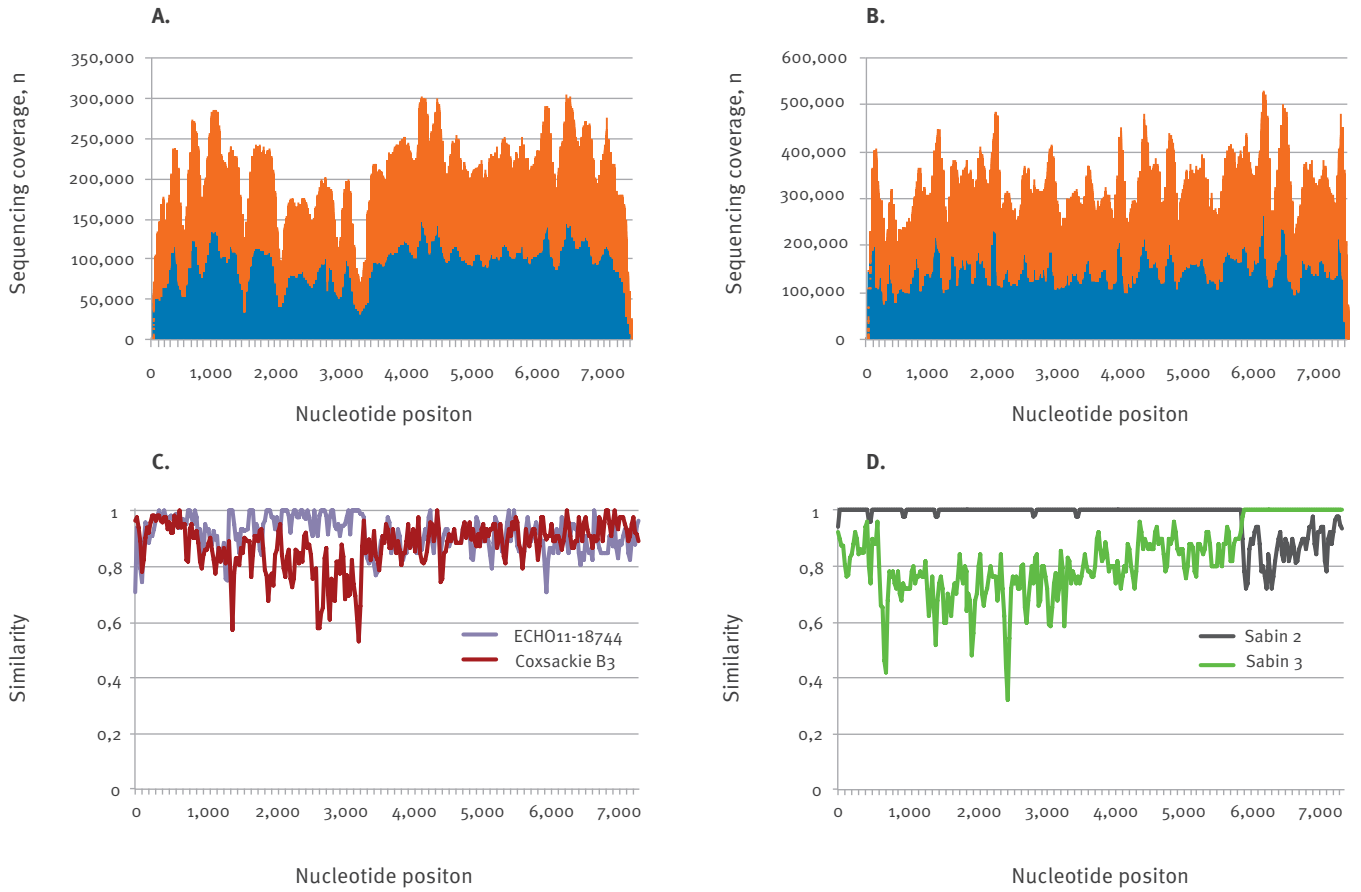
Surveillance for the presence of pathogenic viruses is important for development of rational disease control strategies. It is an integral part of the worldwide campaign to stop the transmission of wild and vaccine-derived polioviruses (VDPV). The campaign uses oral polio vaccine (OPV) made from attenuated strains of three serotypes of poliovirus that can mutate and become virulent VDPVs. The progress of the campaign is monitored by detecting cases of acute flaccid paralysis (AFP) followed by virological confirmation by serological methods, polymerase chain reaction (PCR) tests, and partial nucleotide (nt) sequencing [1-3]. AFP has infectious and non-infectious causes and therefore polio surveillance involves processing of a large number of samples, most of which do not contain poliovirus. An extensive laboratory network is involved in this activity, making it labour-intensive, time-consuming, and expensive.

The sensitivity of AFP surveillance is inherently limited because in a fully susceptible population only a small fraction of infections leads to AFP. In immunised communities, infection to paralysis ratio is even higher, allowing polioviruses causing AFP to transmit silently [4,5]. Confirmation that poliovirus circulation has stopped must be based on detection of poliovirus itself rather than its clinical manifestations. Poliovirus could be consistently isolated from the environment in regions with no recorded cases of paralytic polio [2,4,6]. Highly evolved VDPVs that may have been excreted by unidentified chronically infected individuals have also been found [7-9].

Current sewage concentration methods are based on two-phase separation [10-12], absorption onto membrane filters [13], and other protocols [14]. While some of these allow only a qualitative characterisation of viruses present in a sample, others, such as two-phase separation can also provide quantitative data. The performance of these methods is however affected by sample pH and the presence of different impurities [15,16]. Traditional size-selective ultrafiltration is impractical because filters are rapidly blocked by impurities. In contrast, devices in which liquid moves tangentially

Figure 1

Depth of sequencing coverage and SimPlot analysis of recovered sequences generated by analysis of whole RNA libraries derived from two cell cultures, each infected with a different sewage sample collected in the Helsinki region, Finland, 2013



A. Depth of sequencing coverage generated by analysis of whole RNA library derived from a RD cell culture infected with sewage sample F1. This sewage sample was a priori shown not to contain poliovirus by a standard World Health Organization procedure using strain-specific polymerase chain reaction [21]. Red and blue indicate forward and reverse sequencing reads, respectively.

B. Depth of sequencing coverage generated by analysis of whole RNA library derived from a L20B cell culture infected with sewage sample F2. This sewage sample was a priori shown to contain poliovirus. Red and blue indicate forward and reverse sequencing reads, respectively.

C. SimPlot diagram generated using consensus sequences of the virus present in F1 sewage sample compared with coxsackievirus B3 and echovirus 11, accession numbers AY752944 and AJ276224.

D. SimPlot diagram generated using consensus sequences of the virus present in F2 sewage sample compared with Sabin 2 and Sabin 3, accession numbers AY184220 and AY184221.

to the filter surface (tangential flow filtration, TFF) prevent the significant reduction of flow rates. TFF was successfully used for concentration of viruses, bacteria, and parasites from environmental samples [17-19].

Conventional protocols for the identification of polioviruses in sewage concentrates include cultivation in cell cultures susceptible to poliovirus [12,20], followed by identification using strain-specific PCR and partial sequencing of their genomes [21]. The process is quite laborious and is often complicated by the presence of a mixture of viruses. Massively parallel or deep sequencing generates vast amounts of sequence information and is often used for the analysis of heterogeneous viral populations and metagenomic studies. Metagenomics have many applications in the environmental sciences [22-24] and were also successfully used for the identification of respiratory viruses in clinical samples [25]. Here we explore the utility deep sequencing for the analysis of TFF-concentrated environmental samples.

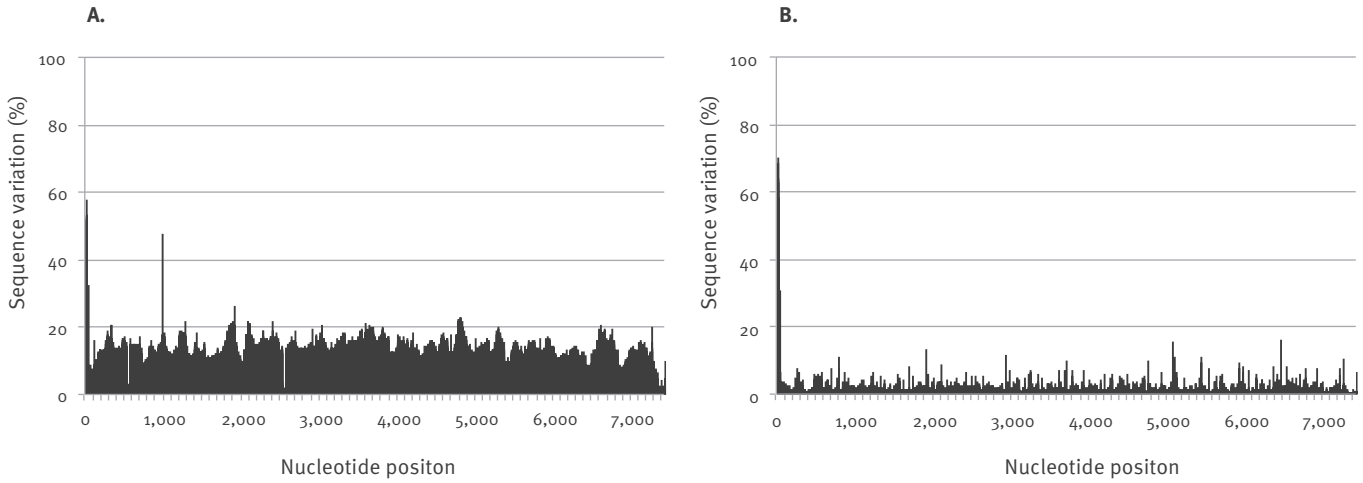
Methods

Tangential flow ultrafiltration of sewage samples

0.5 L sewage samples were collected in 2013 in the Helsinki region of Finland (two samples) and Islamabad, Pakistan (eight samples). They were centrifuged for 10 min at 3,000 *g* resulting in a solid and liquid fraction. The solid (sludge) fraction was

Figure 2

Comparison of sequence diversity profiles in two sewage samples with heterogeneous and homogeneous viral populations, Finland 2013



A. Sequence profiles of the F2 sewage sample containing a virus ca 16% distant from the major viral population.

B. Sequence profile of another sewage sample with a more homogenous viral population

re-suspended and homogenised by Omni homogenizer in 25 mL of 25% beef extract, and the resulting particulate fraction removed by centrifugation at 3,000 *g* for 10 min. The supernatant was combined with the previously obtained liquid fraction and filtered through a 0.2 µm MidiKros TFF filter (Spectrum Laboratories Inc., Rancho Dominguez, CA) followed by rinsing the filter with 10 mL of 25% beef extract. In some cases liquid and solid (i.e. supernatant obtained from elution with beef extract) fractions were processed separately. Next, virus concentration was performed using an automated Spectrum Laboratories KrosFlo Research IIi TFF system and 100 kDa molecular weight cut-off MidiKros filters at 9 psi trans-membrane pressure and 60 mL/min flow rate to the final volume of ca 5 mL (the void volume of the TFF system). The volume was further reduced to 0.5–0.8 mL by manually operating two 5-mL syringes attached to TFF cartridge disconnected from the TFF system.

Isolation of viruses in cell cultures

Sewage concentrates were diluted in Dulbecco minimal essential medium (DMEM) to a final volume of 3 mL and inoculated into cultures of rhabdomyosarcoma (RD) cells and incubated in a 5% CO_2 atmosphere at 37 °C for 1–7 days until cytopathic effect (CPE) developed. The harvest from RD cultures was inoculated into L20B cell cultures (mouse L cells expressing human poliovirus receptor CD155) to selectively amplify polioviruses according to the World Health Organization (WHO) protocol [13]. Culture media from CPE-positive L20B cells and CPE-positive RD cells were used for subsequent real-time PCR analysis and/or deep sequencing.

Detection of polio and enteroviruses in sewage concentrates by real-time PCR

RNA was isolated from samples prepared by different procedures described above using NucleoSpin RNA Virus F kit (Clontech Inc., Mountain View, CA) and used for real-time PCR with strain-specific primers [25]. All PCR reactions were performed in duplicate on a CFX96 Real-Time PCR system (Bio-Rad). The same RNA samples were also used for deep sequencing.

Titration of Sabin 1 poliovirus in spiked sewage samples

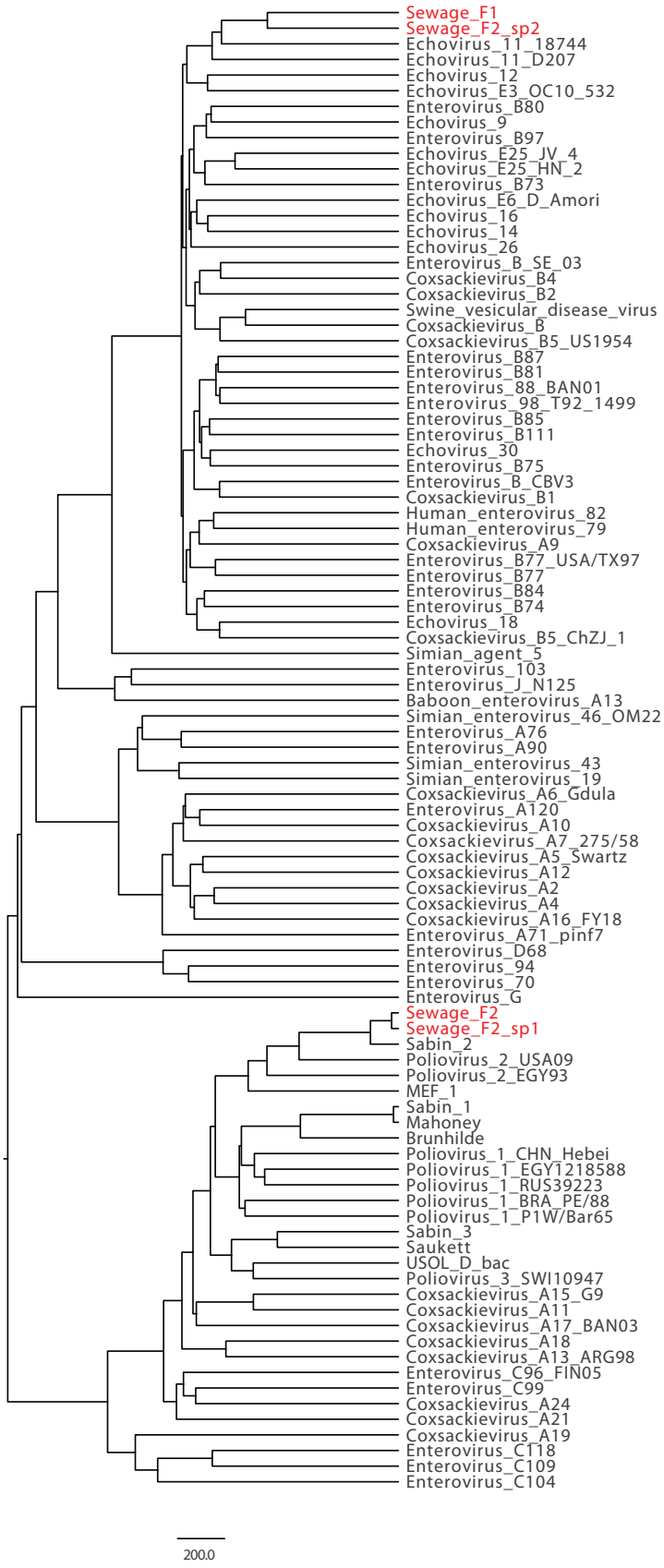
0.5 mL of $2x10^5$ cell culture infectious doses ($CCID_{50}$) of Sabin 1 poliovirus diluted in phosphate buffered saline (PBS) was added to 0.5 L of poliovirus-free sewage sample and incubated for 1 hour. Identical 0.5 mL virus sample served as a control. Virus titres were determined in Hep2C cells by terminal dilutions method according to WHO recommendations [13]. Virus recovery was evaluated as a percentage of infectious virus relative to the control unspiked sample.

Poliovirus enrichment by immunoprecipitation and sequence-independent isothermal amplification

Biotinylated rabbit polyclonal IgG against wild polioviruses of serotypes 1, 2, and 3 were used for immunoprecipitation. Five µL of stock solution containing 0.5 µg rabbit IgG was added to 0.5 mL of TFF-concentrated sewage and incubated for 3 hours on a rocker. Ten µL of streptavidin-coated magnetic beads (Thermo Fisher Scientific Inc., Rockford, IL) washed with PBS were added to the TFF concentrate and incubated at room temperature for 2 hours on a rocker. Magnetic beads were then washed with DMEM and PBS and

Figure 3

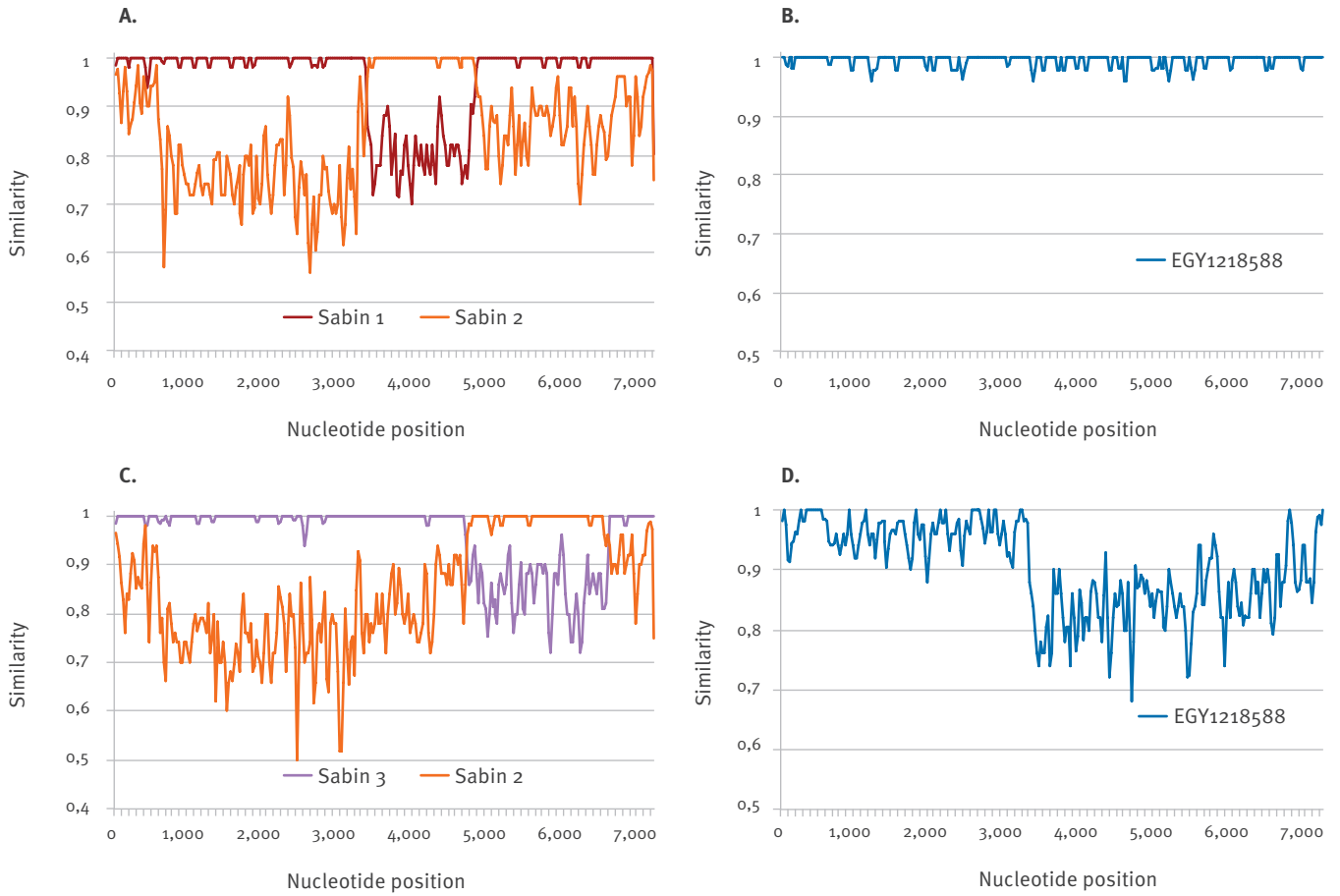
Phylogenetic tree showing the relatedness of viral sequences identified in F1 and F2 sewage samples to reference enterovirus strain sequences, Helsinki region, Finland, 2013



Sequences marked sp1 and sp2 were bioinformatically separated from sewage sample F2, and represent the majority and minority component, respectively.

Figure 4

Simplot analyses showing recombinant structures of some polioviruses present in sewage samples collected in Islamabad, Pakistan, June–July 2013



Reference sequences used for these Simplot charts were Sabin 1 (GenBank accession number: AY184219), Sabin 2 (GenBank accession number: AY184220), Sabin 3 (GenBank accession number: AY184221), and EGY1218588 (GenBank accession number: KJ155496).

A. Recombinant Sabin 1/2 virus.

B. Wild type 1 poliovirus, which, when compared to sequence entries in the entire database of published enterovirus sequences, was most similar to EGY1218588 (which belongs to the south-Asian (SOAS) lineage).

C. Recombinant Sabin 3/2 virus.

D. Wild type 1 poliovirus, which, when compared to sequence entries in the entire database of published enterovirus sequences, was most similar to EGY1218588 (which belongs to the SOAS lineage). The similarity between the virus in panel D and EGY1218588 was less than the similarity between the virus in panel B and EGY1218588. The difference between the two wild type viruses in panels B and D could be explained by a recombination event in the sequence of the virus in panel D at the boundary between the P1 and P2 regions.

stored frozen at -20 °C. The procedure was performed separately for each serotype of poliovirus. Isothermal amplification using REPLI-g WTA single cell kit (Qiagen, Valencia, CA) was performed according to the manufacturer's protocol.

Deep sequencing

RNA isolated from samples prepared by different procedures described above was used for total RNA Illumina library preparation using NEB NEXT Ultra RNA library kit (NEB, Ipswich, MA). The quality of libraries was assessed using the qPCR Library Quantification Kit (Kapa Biosystems, Inc., Woburn, MA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). DNA sequencing was performed on Illumina HiSeq 2000 or MiSeq to generate 100 to 300 nt long paired-end reads. In some experiments, samples were sequenced in multiplex format (up to eight samples per sequencing lane) using unique index primers (barcodes).

Bioinformatic analysis

Bioinformatic analysis was performed using a custom 'swarm' software package developed for UNIX (Darwin) environment on MacPro (Apple Inc., Cupertino, CA) and running in parallel mode using up to 22 computation threads. Low quality sequences, adapters and index

Table 1

A list of viruses identified in eight sewage samples collected in Islamabad, Pakistan, June–July 2013

Sewage sample name	Fraction	Cell culture	Majority virus	Minority virus[a]	
				%[b]	Description
P1	Supernatant	RD	Wt polio type 1	15	Sabin 3
		L20B	Wt polio type 1	5	Sabin 3
	Sludge	RD	Coxsackievirus B5	50	Sabin 1
		L20B	Wt polio type 1	5	Wt Polio type 1
	Combined	RD	Wt polio type 1	40	Sabin 1, Sabin 3
		L20B	Wt polio type 1	2	Sabin 1
P2	Supernatant	RD	Wt polio type 1	20	Sabin 3
		L20B	Wt polio type 1	15	Sabin 3
	Sludge	RD	Wt polio type 1	30	Coxsackievirus B1
		L20B	Wt polio type 1	40	Wt Polio type 1
	Combined	RD	Wt polio type 1	35	Sabin 1
		L20B	Wt polio type 1	15	Sabin 3, Sabin 1
P3	Combined	RD	Coxsackievirus B5	10	Sabin 3, Sabin 1
		L20B	Sabin 2	25	Coxsackievirus B1, B3, B5
P4	Combined	RD	Sabin 2	30	Coxsackievirus B3
		L20B	Sabin 3	20	Coxsackievirus B5
P9	Supernatant	RD	Sabin 3	15	Coxsackievirus B5
		L20B	Sabin 3	–[c]	–[c]
	Sludge	RD	Coxsackievirus B5	20	Sabin 3
		L20B	Sabin 3	40	Wt Polio type 1
	Combined	RD	Coxsackievirus B5	50	Sabin 3, Sabin 3/1 recombinant
		L20B	Sabin 3	10	Coxsackievirus B5, Sabin 3/1 recombinant
P10	Supernatant	RD	Coxsackievirus B5	30	Coxsackievirus B5
		L20B	Wt polio type 1	–[c]	–[c]
	Sludge	RD	Sabin 2	15	Coxsackievirus B3, B5
		L20B	Sabin 2	5	Sabin 1
	Combined	RD	Sabin 2	50	Coxsackievirus B5
		L20B	Sabin 2	35	Coxsackievirus B5
P11	Supernatant	RD	No product	–[c]	–[c]
		L20B	Wt polio type 1	–[c]	–[c]
	Sludge	RD	No product	–[c]	–[c]
		L20B	Wt polio type 1	–[c]	–[c]
	Combined	RD	Wt polio type 1	–[c]	–[c]
		L20B	Wt polio type 1	45	Wt Polio type 1
P12	Supernatant	RD	Wt polio type 1	50	Sabin 2
		L20B	Sabin 2	–[c]	–[c]
	Sludge	RD	No product	–[c]	–[c]
		L20B	Sabin 1	20	Sabin 3
	Combined	RD	Sabin 1	50	Sabin 1
		L20B	Sabin 1	25	Sabin 3

Wt: wild type.

[a] The minority virus is inferred from the finding, next to the majority consensus sequence (of the 'majority' virus), of another consensus sequence which is reconstructed after separation of deep sequence reads using the algorithm described in the text.

[b] Percentage indicates the proportion of deep sequence reads that match to the alternative consensus(es).

[c] Only one virus (i.e. the 'majority' virus) was found in the sewage fraction considered, so there is no 'minority' virus for this fraction.

Table 2

Relative abundance of the most prevalent RNA viruses present in a sewage sample collected in Pakistan, 2013

Virus	Proportions of reads among all reads that match the sequence of a given virus (%)
Tomato mosaic virus	41.20
Cucumber green mottle mosaic virus	26.10
Tobacco mild green mosaic virus	14.20
Pepper mild mottle virus	6.70
Tobacco mosaic virus	6.00
Youcai mosaic virus	2.00
Tomato mottle mosaic virus isolate MX5	1.10
Adeno-associated virus - 2	0.50
Melon necrotic spot virus	0.30
Poliovirus	0.20
Other	1.50

sequences were removed, and the remaining reads were aligned (mapped) on curated databases of full-length sequences of different virus groups (GenBank). The mapping procedure involved k-mer guided Smith–Waterman alignment that will be described elsewhere. Profiles of sequence heterogeneity were computed, and consensus sequences were built by identifying the most frequent nts at each genomic position. In cases where significant sequence heterogeneity was detected, sequence reads were separated into discrete sub-populations using the following algorithm. Sequence reads mapped against aligned reference genomes were segregated into discrete subsets by cluster analysis performed in overlapping 'windows' of 50–100 nt covering the entire genome ('sliding window' method). The identity of each species defined in a particular window was matched with similar information obtained for the adjacent overlapping window, and propagated along the entire genome. Consensus sequences were computed for each subset of sequence reads as described above. Unique sequences of wild type polioviruses determined in this study were deposited in GenBank (accession numbers: KU161395–KU161399).

Results

Concentration of sewage samples by tangential flow filtration and recovery of viruses

The entire concentration procedure of 0.5 L sewage samples took ca 2 hours. Final sewage concentrates in 0.5–0.8 mL volume were used for RNA isolation (see above) or inoculation into cell cultures.

Analysis by the standard WHO procedure using strain-specific PCR [21] had previously shown that a sewage sample hereby named 'F1' and collected in Finland did not contain poliovirus, while another sample 'F2', from the same country, was positive for Sabin 2 poliovirus.

To determine the efficiency of the TFF protocol, the F1 sample was spiked with 10^5 $CCID_{50}$ of Sabin 1 poliovirus. The recovery of spiked poliovirus in the combined sludge/supernatant fraction was 62%, 84%, and 93% in three independent experiments.

Sensitivity determined in spiking experiments may differ from the sensitivity of virus detection in real sewage samples. Therefore to validate the concentration method, another sewage sample F2 from Finland which was demonstrated to contain Sabin 2 poliovirus was processed by the TFF protocol. The concentrates of F1 (poliovirus-free) and F2 were used to infect RD and then L20B cell cultures. RD cultures were virus positive for both samples, while only F2 induced CPE in L20B cells.

Deep sequencing of cell culture-grown viruses

Whole-RNA Illumina libraries prepared from supernatants of RD and L20B cultures infected with F1 and F2 concentrates were deep-sequenced using Illumina Hiseq2000. Individual sequence reads were aligned to genomic sequences of 744 representative enteroviruses. Figures 1A and 1B show the depth of sequencing coverage along the viral genome in forward and reverse orientations. The most prevalent nt at each genomic position was considered to represent the consensus, and all others were assumed to be single nt polymorphisms (SNP). Consensuses contained uninterrupted open reading frames coding for 2,196 and 2,208 amino acids for F1 and F2 samples.

Their comparison with references and SimPlot analysis revealed that the closest relative of the virus present in the F1 sample was echovirus 11 with 82% similarity in the P1 region (Figure 1C). This level of homology does not allow us to unambiguously identify the serotype of this virus.

The virus from the F2 sample was a recombinant of Sabin 2 and Sabin 3 viruses with the crossover at the boundary between regions coding for 3C protease and 3D polymerase (nt 5,959–5,981; Figure 1D). The distal part of the genome was identical to the Sabin 3 virus, while the proximal part differed from the Sabin 2 virus at six nt positions (two in 5'-untranslated region (UTR), two in viral protein VP2, one in VP1, and one in 2A protein). This indicates that this was a minimally evolved vaccine poliovirus, however, it contained mutations at both sites associated with neurovirulence (A_{481}⇢G in 5'-UTR and Thr_{143}⇢His in VP1).

Sequences determined by the above procedure represent consensuses of the majority component. Sequence heterogeneity profiles revealed that sewage sample F2 contained ca 16% of one or more viruses distinct from the predominant Sabin 2/3 recombinant (Figure 2A). For comparison, Figure 2B shows an example of the

Table 3

Relative proportion of sequence reads matching the most abundant viruses detected in a sewage sample before and after enrichment by immunoprecipitation using antibodies against poliovirus

Virus found before enrichment	Per cent[a]	Virus found after enrichment	Per cent[a]
Tomato mosaic virus	41.20	Poliovirus	8.90
Cucumber green mottle mosaic virus	26.10	Tobacco mild green mosaic virus	8.30
Tobacco mild green mosaic virus	14.20	Circovirus-like genome RW-C	6.70
Pepper mild mottle virus	6.70	Cucumber green mottle mosaic virus	4.30
Tobacco mosaic virus	6.00	Tobacco mosaic virus	2.70
Youcai mosaic virus	2.00	Tomato mottle mosaic virus isolate MX5	2.70
Tomato mottle mosaic virus isolate MX5	1.10	Lactococcus phage phiLC3	2.00
Adeno-associated virus - 2	0.50	Sulfolobus islandicus rod-shaped virus 1	2.00
Melon necrotic spot virus	0.30	Zamilon virophage	1.90
Poliovirus	0.20	Enterococcus phage EF62phi	1.70
Cosavirus A strain HCoSV-A1	0.10	Cotesia congregata virus	1.70
Bovine viral diarrhoea virus 1	0.10	Clostridium phage phiMMP04	1.50
Other	0.80	Other	54.50

[a] The percentages represent the proportions of reads among all reads that match the sequence of a given virus.

SNP profile of a relatively homogeneous viral sample. To separate viruses present in the mixture, individual sequence reads mapped on the aligned reference dataset were subjected to cluster analysis in a 'sliding window' format (in a series of overlapping 50–100 nt-long sections). Sequence reads separated by this procedure were assembled into full-genome consensus sequences. The majority species in F2 samples grown in L20B cells was identical to Sabin 2/3 recombinant determined by building the overall consensus. The minority species in F2 sample was close to echovirus 11 virus with ca 10% differences from the virus identified in F1 sample (Figure 3).

Analysis of sewage samples from polio-endemic environment

The above results established the feasibility of using TFF concentration and deep sequencing for characterising multiple viruses present in viral populations. Next, the utility of this approach for surveillance in a country that is still endemic for poliovirus was explored. Eight sewage samples collected in Pakistan were processed as described above, concentrates were inoculated into RD and L20B cultures, and harvested viruses were deep-sequenced. Multiple enteroviruses present in most samples were separated bioinformatically as described above. Table 1 lists discrete viruses found in these samples. We found viruses that were very close to all three Sabin vaccine strains, as well as a number of wild type 1 polioviruses. In some samples, there were also sequences related to non-polio enteroviruses, predominantly of species B (coxsackievirus B1, B3, and B5).

The approach used in this work to reconstruct genomic sequences resulted in complete or near complete genomes (in some cases missing 1–10 nt at the ends, due to low sequencing coverage of the terminal regions). It produced more phylogenetic and molecular-epidemiological information than would be available based on VP1 sequencing alone. For instance, some vaccine derivatives were found to be recombinants of two serotypes of Sabin virus, and the crossover point was easily identified by SimPlot analysis (Figure 4A,4C). Similarly, wild type 1 of south-Asian (SOAS) lineage currently prevalent in Pakistan and Afghanistan contained two sub-lineages that could be distinguished by a recombination event at the boundary between the P1 and P2 regions (Figure 4B,4D).

Metagenomic approach

TFF concentration followed by deep sequencing of viruses isolated in cell culture represents an improvement over the conventional protocol by providing significantly more information in a much shorter time. However, it still depends on the ability of viruses to grow in cell culture, potentially biasing the result. A totally agnostic approach based on the direct sequencing of all RNA present in sewage concentrates could produce a more complete picture. Whole-RNA Illumina libraries were prepared directly from TFF concentrates of sewage collected in Pakistan and deep sequenced. Sequence reads were aligned to reference sequences of 5,301 full length viral genomes belonging to all major groups of viruses (National Center for Biotechnology Information, Bethesda, MD). Approximately 5–8% of all sequence reads could be aligned to sequences in the viral database, and most hits belonged to plant viruses (Table 2). Separation of sequences of plant viruses into discrete subpopulations by the algorithm described above revealed the presence of full length genomes of several different viruses, including tomato mosaic

virus, cucumber green mottle mosaic virus, tobacco mild green mosaic virus, pepper mild mottle virus, tobacco mosaic virus, Youcai mosaic virus, and tomato mottle virus (Table 2).

Surprisingly, the most represented of all animal viruses was adeno-associated virus (AAV, 0.5% of all reads) followed by poliovirus (0.2%). Mapping of sequence reads by alignment to sequences in reference databases of individual virus families that produced a substantial number of hits during the preliminary screening, allowed the assembly of full-genome sequences of viruses present in the sample. Sequence reads aligned to sequences in the database containing 18 reference AAV sequences assembled into a full-length genome of 4,675 nt containing two open reading frames corresponding to the non-structural and the capsid proteins of the virus. Phylogenetic analysis showed that it was 97.3% similar to AAV-2. A similar analysis performed for astroviruses revealed the presence of human astrovirus 4, Aichi virus 1, human parechovirus 1, as well as several other mammalian RNA and DNA viruses (data not shown).

Enrichment of tangential flow filtration-concentrated sewage by immunoprecipitation

The number of hits for most other groups of viruses was insufficient to generate good coverage allowing unambiguous assembly of complete viral genomes or reliable heterogeneity analysis. To enable a more sensitive search for specific viruses, polioviruses present in TFF concentrates were enriched by immunoprecipitation. TFF-concentrated sewage samples were incubated with biotinylated rabbit polyclonal anti-polio IgG and the virus was captured using streptavidin-coated microbeads. Complementary DNA (cDNA) from these samples was isothermally amplified using a uniform sequence-independent REPLI-g system. Table 3 shows the specificity of sequence reads from one sewage sample before and after the enrichment that resulted in poliovirus sequences becoming the predominant species. The consensus sequence assembled from these sequence reads was shown by phylogenetic analysis to be of SOAS WPV1 identical to the virus isolated from the same sewage sample in cell culture.

Discussion

This communication demonstrates that TFF can effectively concentrate viruses present in sewage by reducing the volume from ca 500 mL to ca 1 mL in approximately 2 hours. Unlike the conventional protocols [10,12,21] it does not require large volumes of toxic and noxious organic solvents, removes most impurities including PCR inhibitors, and is more amenable to high-throughput implementation.

Traditionally, identification of viruses in sewage concentrates is performed by using selective cell cultures such as mouse L20B cells that express human poliovirus receptor [20], followed by strain-specific PCR and partial nt sequencing [10,12,21]. The improvements proposed here include (i) the use of deep sequencing for virus identification, and (ii) direct metagenomic analysis of sewage concentrates allowing viruses to be identified without cell cultures. The main advantage of deep sequencing is that it is a universal procedure that detects multiple viruses present in mixtures, and generates complete or near-complete genome sequences that contain more molecular-epidemiological information than partial sequencing.

Thus several vaccine-related and wild type polioviruses identified in this study were found to be intertypic recombinants. Finding the recombinant Sabin 2/3 poliovirus in sewage from Finland was unexpected because this country does not use oral polio vaccine (OPV). Even though this 'young' vaccine-derivative contained only six mutations compared with Sabin 2, two of them were previously linked to neurovirulent reversion. Whole genome analysis of wild polioviruses revealed that currently circulating strains in Pakistan belong to at least two sub-lineages distinguished by different origins of their P2-P3 region (Figure 4B,4D).

Most samples contained more than one virus, including non-polio enteroviruses, predominantly of species B, similar to coxsackievirus B1, B3, B5, and some echoviruses. However, since no sufficiently close match was found in the published database, their serotype identity could not be established unambiguously. Future expansion of the sequence database could enable more accurate identification. Nevertheless, our study demonstrates that this approach could be also used for the surveillance of all non-polio enteroviruses.

Deep-sequencing of the entire RNA content of sewage concentrates also revealed the prevalence of multiple plant viruses, consistent with previous studies of human stool samples [26]. Bioinformatic separation of deep-sequence reads belonging to different viruses allowed their assembly into several full-length genomes that were then identified by phylogenetic analysis. Surprisingly, the most prevalent animal virus in sewage from Pakistan was AAV-2. It has never been associated with any known pathology, and cannot grow in cell cultures without a helper adenovirus [27]. The epidemiology of this virus is unknown, and this observation could help to identify its niche in nature. Additional animal viruses were detected in uncultivated sewage concentrates collected in Pakistan, and their complete genomes sequenced. They included wild and attenuated polioviruses and other enteroviruses described in this paper, as well as cosaviruses, astroviruses, parechoviruses, kobuvirus (Aichi), Saffold virus, bovine viral diarrhoea virus, cricket paralysis virus, and others that were not included in this communication and will be described elsewhere.

The most intriguing aspect of this study is the possibility of a direct metagenomic approach for routine surveillance, bypassing the need for viral cell cultures. The use of this 'agnostic' metagenomic approach

additionally raises an interesting possibility of future retrospective search for viruses unknown at the time of sample collection by aligning sequences determined in the past to reference sequences of newly discovered viruses. Therefore, the deep sequence data could serve as a digital repository of the virome present in sewage samples without the need to preserve their viability or biocontainment.

Depending on the sewage sample, a certain number of viruses were represented by a small number of sequence reads. Although these could have potentially resulted from contamination, such as carryover of a small amount of genetic material from previous sequencing runs in the laboratory, we do not believe that findings described in this report resulted from such carryover. Indeed for the viral sequences that could be reconstructed, we do not work with the corresponding viruses in our laboratory. Moreover, bleaching between sequencing runs, which helps to eliminate this problem, was regularly performed.

Low number of reads of certain viruses, however make the depth of the sequencing coverage insufficient for reliable assembly of their complete genomes. Enrichment of polioviruses from sewage concentrates by immunoprecipitation resulted in poliovirus-specific sequence reads becoming predominant in uncultivated sewage concentrates, preserving the remaining material for further screening for other viruses. As a result, the complete genome of wild type 1 poliovirus was assembled and was found to match the sequence of the virus of the SOAS lineage that was identified in this sample by the cell culture method.

In conclusion, the approach described in this paper can provide an effective alternative to current protocols used for environmental surveillance for wild polioviruses and VDPV, other non-polio enteroviruses, as well as viruses of other families. Most countries in Europe have switched to the exclusive use of Inactivated Polio Vaccines (IPV). Good protection from paralysis that it induces combined with inadequate intestinal immunity [28] allows viruses to spread silently, which makes the traditional AFP surveillance less effective. Therefore environmental surveillance using metagenomic approach in combination with TFF or other improved sewage concentration methods could be particularly useful in Europe and other countries that have switched to the use of IPV. Rapidly declining cost of deep sequencing makes this approach cost-effective compared with the conventional sequencing methods. It could significantly shorten the time needed for the identification of pathogenic viruses in the environment, allowing for a much faster deployment of countermeasures.

Erratum

The name of the author Sohail Z. Zaidi had been misspelled. This was corrected on 12 May 2016.

Acknowledgements

Authors thank Dr. Humayun Asghar of the World Health Organization for suggestions. This work was funded by the intramural research program of the US Food and Drug Administration and a grant to M.R. from the World Health Organization.

Conflict of interest

None declared.

Authors' contributions

Konstantin Chumakov and Merja Roivainen designed the study. Merja Roivainen, Sohail Z. Zaidi, Lubna Rehman, and Muhammad M. Alam collected sewage samples, Vyacheslav Furtak, Merja Roivainen, and Vladimir Chizhikov performed TFF concentration, Olga Mirochnichenko performed virus isolation, Tatiana Zagorodnyaya, Majid Laassri, and Vyacheslav Furtak performed deep sequencing. Konstantin Chumakov drafted the manuscript.

References

1. Centers for Disease Control and Prevention (CDC),. Evaluating surveillance indicators supporting the Global Polio Eradication Initiative, 2011-2012.MMWR Morb Mortal Wkly Rep. 2013;62(14):270-4.PMID: 23575241
2. Hovi T, Shulman LM, van der Avoort H, Deshpande J, Roivainen M, DE Gourville EM. Role of environmental poliovirus surveillance in global polio eradication and beyond.Epidemiol Infect. 2012;140(1):1-13. DOI: 10.1017/S095026881000316X PMID: 21849095
3. Asghar H, Diop OM, Weldegebriel G, Malik F, Shetty S, El Bassioni L, et al. Environmental surveillance for polioviruses in the Global Polio Eradication Initiative. J Infect Dis. 2014;210(Suppl 1):S294-303. DOI: 10.1093/infdis/jiu384 PMID: 25316848
4. Manor Y, Handsher R, Halmut T, Neuman M, Bobrov A, Rudich H, et al. Detection of poliovirus circulation by environmental surveillance in the absence of clinical cases in Israel and the Palestinian authority. J Clin Microbiol. 1999;37(6):1670-5.PMID: 10325305
5. Shulman LM, Gavrilin E, Jorba J, Martin J, Burns CC, Manor Y, et al. Molecular epidemiology of silent introduction and sustained transmission of wild poliovirus type 1, Israel, 2013. Euro Surveill. 2014;19(7):20709. DOI: 10.2807/1560-7917.ES2014.19.7.20709 PMID: 24576471
6. El Bassioni L, Barakat I, Nasr E, de Gourville EM, Hovi T, Blomqvist S, et al. Prolonged detection of indigenous wild polioviruses in sewage from communities in Egypt. Am J Epidemiol. 2003;158(8):807-15. DOI: 10.1093/aje/kwg202 PMID: 14561671
7. Shulman LM, Manor Y, Sofer D, Handsher R, Swartz T, Delpeyroux F, et al. Neurovirulent vaccine-derived polioviruses in sewage from highly immune populations. PLoS One. 2006;1(1):e69. DOI: 10.1371/journal.pone.0000069 PMID: 17183700
8. Al-Hello H, Jorba J, Blomqvist S, Raud R, Kew O, Roivainen M. Highly divergent type 2 and 3 vaccine-derived polioviruses isolated from sewage in Tallinn, Estonia.J Virol. 2013;87(23):13076-80. DOI: 10.1128/JVI.01174-13 PMID: 24049178
9. Hovi T, Paananen A, Blomqvist S, Savolainen-Kopra C, Al-Hello H, Smura T, et al. Characteristics of an environmentally monitored prolonged type 2 vaccine derived poliovirus shedding episode that stopped without intervention. PLoS One. 2013;8(7):e66849. DOI: 10.1371/journal.pone.0066849 PMID: 23935826
10. Albertson P. Two-phase separation of viruses. In Maramorosch K, Koprowski H, eds. Methods of virology. 1967:303-21.
11. Más Lago P, Gary HE, Pérez LS, Cáceres V, Olivera JB, Puentes RP, et al. Poliovirus detection in wastewater and stools following an immunization campaign in Havana, Cuba. Int J Epidemiol. 2003;32(5):772-7. DOI: 10.1093/ije/dyg185 PMID: 14559748

12. World Health Organization (WHO). Guidelines for environmental surveillance of poliovirus circulation. Geneva, Switzerland: WHO; 2003.
13. Tambini G, Andrus JK, Marques E, Boshell J, Pallansch M, de Quadros CA, et al. Direct detection of wild poliovirus circulation by stool surveys of healthy children and analysis of community wastewater. J Infect Dis. 1993;168(6):1510-4. DOI: 10.1093/infdis/168.6.1510 PMID: 8245537
14. Lewis GD, Metcalf TG. Polyethylene glycol precipitation for recovery of pathogenic viruses, including hepatitis A virus and human rotavirus, from oyster, water, and sediment samples. Appl Environ Microbiol. 1988;54(8):1983-8.PMID: 2845860
15. Shields PA, Farrah SR. Concentration of viruses in beef extract by flocculation with ammonium sulfate.Appl Environ Microbiol. 1986;51(1):211-3.PMID: 3006587
16. Guttman-Bass N, Catalano-Sherman J. Humic acid interference with virus recovery by electropositive microporous filters.Appl Environ Microbiol. 1986;52(3):556-61.PMID: 3021058
17. Liu P, Hill VR, Hahn D, Johnson TB, Pan Y, Jothikumar N, et al. Hollow-fiber ultrafiltration for simultaneous recovery of viruses, bacteria and parasites from reclaimed water. J Microbiol Methods. 2012;88(1):155-61. DOI: 10.1016/j.mimet.2011.11.007 PMID: 22108496
18. Smith CM, Hill VR. Dead-end hollow-fiber ultrafiltration for recovery of diverse microbes from water.Appl Environ Microbiol. 2009;75(16):5284-9. DOI: 10.1128/AEM.00456-09 PMID: 19561183
19. Thurber RV, Haynes M, Breitbart M, Wegley L, Rohwer F. Laboratory procedures to generate viral metagenomes.Nat Protoc. 2009;4(4):470-83. DOI: 10.1038/nprot.2009.10 PMID: 19300441
20. Pipkin PA, Wood DJ, Racaniello VR, Minor PD. Characterisation of L cells expressing the human poliovirus receptor for the specific detection of polioviruses in vitro.J Virol Methods. 1993;41(3):333-40. DOI: 10.1016/0166-0934(93)90022-J PMID: 8386181
21. Kilpatrick DR, Yang CF, Ching K, Vincent A, Iber J, Campagnoli R, et al. Rapid group-, serotype-, and vaccine strain-specific identification of poliovirus isolates by real-time reverse transcription-PCR using degenerate primers and probes containing deoxyinosine residues. J Clin Microbiol. 2009;47(6):1939-41. DOI: 10.1128/JCM.00702-09 PMID: 19386844
22. Dinsdale EA, Edwards RA, Hall D, Angly F, Breitbart M, Brulc JM, et al. Functional metagenomic profiling of nine biomes. Nature. 2008;452(7187):629-32. DOI: 10.1038/nature06810 PMID: 18337718
23. Cantalupo PG, Calgua B, Zhao G, Hundesa A, Wier AD, Katz JP, et al. Raw sewage harbors diverse viral populations. MBio. 2011;2(5):e00180-11. DOI: 10.1128/mBio.00180-11 PMID: 21972239
24. Ng TF, Marine R, Wang C, Simmonds P, Kapusinszky B, Bodhidatta L, et al. High variety of known and new RNA and DNA viruses of diverse origins in untreated sewage. J Virol. 2012;86(22):12161-75. DOI: 10.1128/JVI.00869-12 PMID: 22933275
25. Prachayangprecha S, Schapendonk CM, Koopmans MP, Osterhaus AD, Schürch AC, Pas SD, et al. Exploring the potential of next-generation sequencing in detection of respiratory viruses. J Clin Microbiol. 2014;52(10):3722-30. DOI: 10.1128/JCM.01641-14 PMID: 25100822
26. Zhang T, Breitbart M, Lee WH, Run JQ, Wei CL, Soh SW, et al. RNA viral community in human feces: prevalence of plant pathogenic viruses. PLoS Biol. 2006;4(1):e3. DOI: 10.1371/journal.pbio.0040003 PMID: 16336043
27. Mueller C, Flotte TR. Clinical gene therapy using recombinant adeno-associated virus vectors.Gene Ther. 2008;15(11):858-63. DOI: 10.1038/gt.2008.68 PMID: 18418415
28. Plotkin SA, Vidor E. Poliovirus vaccine -- inactivated. In: Plotkin SA, Orenstein WA, Offit PA, editors. Vaccines. 5 ed. Philadelphia: W.B. Saunders; 2008. p. 605-29.

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Vibrio cholerae non-O1 bacteraemia: description of three cases in the Netherlands and a literature review

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Citation style for this article:
Engel MF, Muijsken MA, Mooi-Kokenberg E, Kuijper EJ, van Westerloo DJ. Vibrio cholerae non-O1 bacteraemia: description of three cases in the Netherlands and a literature review. Euro Surveill. 2016;21(15):pii=30197. DOI: http://dx.doi.org/10.2807/1560-7917.ES.2016.21.15.30197

Article submitted on 13 April 2015 / accepted on 22 January 2016 / published on 14 April 2016

Vibrio cholerae **non-O1 serogroup (VCNO) bacteraemia is a severe condition with a high case–fatality rate. We report three cases diagnosed in the Netherlands, identified during a national microbiological congress, and provide a literature review on VCNO bacteraemia. A search strategy including synonyms for 'VCNO' and 'bacteraemia' was applied to PubMed, Medline, Web of Science and Embase databases. The three cases were reported in elderly male patients after fish consumption and/or surface water contact. The literature search yielded 82 case reports on 90 cases and six case series. Thirty case reports were from Asia (30/90; 33%), concerned males (67/90; 74%), and around one third (38/90; 42%) involved a history of alcohol abuse and/or liver cirrhosis The presenting symptom often was gastroenteritis (47/90; 52%) which occurred after seafood consumption in 32% of the cases (15/47). Aside from the most frequent symptom being fever, results of case series concurred with these findings. Published cases also included rare presentations e.g. endophthalmitis and neonatal meningitis. Based on the limited data available, cephalosporins seemed the most effective treatment. Although mainly reported in Asia, VCNO bacteraemia occurs worldwide. While some risk factors for VCNO were identified in this study, the source of infection remains often unclear. Clinical presentation may vary greatly and therefore a quick microbiological diagnosis is indispensable.**

Introduction

The genus *Vibrio* is one of the six members of the Vibrionaceae family and includes ten species pathogenic to humans. Probably the most well-known species is *Vibrio cholerae*. Currently, there are over 130 known serogroups based on the presence of somatic O antigens [1,2]. *S*erogroup O1 and to a lesser extent O139, is notorious for cholera outbreaks of dysenteric diarrhoea due to toxin production i.e. cholera toxin: ctx and toxin co-regulated pilus subunit A: tcpA. Infections are mostly confined to the gastro-intestinal tract [1].

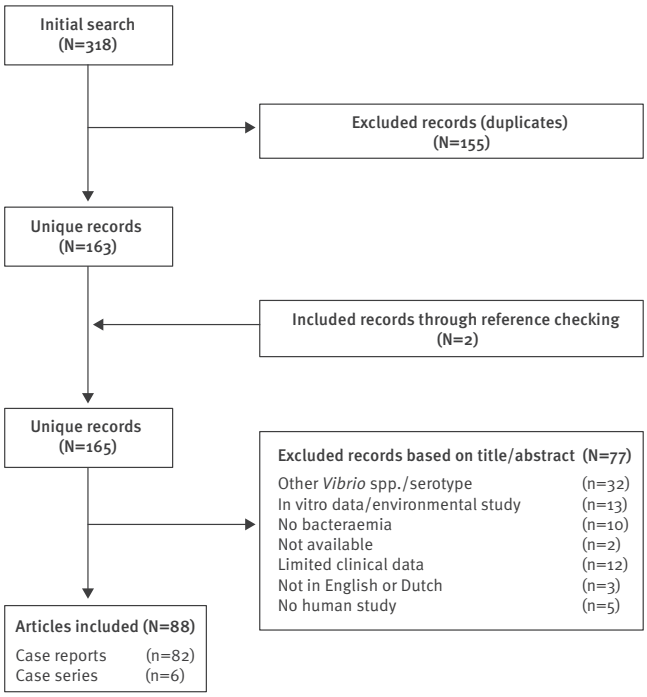
In contrast, *V. cholerae* non-O1 (VCNO) i.e. all serogroups except O1, rarely causes cholera-like outbreaks in form of mild diarrhoea due to toxin-producing VCNO strains but can cause severe extra intestinal infections such as wound infections and bacteraemia [3,4]. Cases of VCNO bacteraemia are reported in various countries and known risk factors are liver disease/cirrhosis and immunosuppression/immunocompromising conditions [1,3,4]. Sources of infection include seafood and contaminated water [5]. In the Netherlands, VCNO has indeed been isolated from recreational surface water (fresh and brackish) and sporadically from livestock [5,6]. In contrast, a Dutch study from 2010 showed that none of the examined shellfish tested positive for *V. cholerae* [7].

V. cholerae is a facultative anaerobic Gram-negative curved or comma-shaped motile bacillus. It can be isolated from blood by using standard culture media such as blood agar [1]. Biochemical properties of this organism include catalase positivity, oxidase positivity, sucrose fermentation and susceptibility for the vibriostatic compound O129. Identification methods are various and include: matrix assisted laser desorption ionization-time-of-flight (MALDI-TOF) analyser (MALDI-TOF, Bruker corporation), VITEK systems (BioMerieux corporation) and polymerase chain reaction (PCR) for 16S and target genes like toxR, ompW and sodB. The non-O1 serogroup can be distinguished from other serotypes by a lack of agglutination with O1-Ogawa and O1-Inaba antigen [1].

In 2013, a patient with a fulminant VCNO sepsis and extensive bullae on the lower extremities was admitted to the Leiden University Medical Centre (LUMC). When searching for literature on VCNO sepsis to help

Figure 1

Flowchart with results from literature search for *Vibrio cholera* non-O1bacteraemia



determine the source of infection and the optimal treatment strategy, we realised that the available literature appeared to be limited to case reports and small case series. In order to provide evidence for clinicians and public health experts about VCNO bacteraemia we report on a series of three cases and summarise the available literature on VCNO bacteraemia.

Methods

Clinical case reports

During the presentation of the LUMC case at the annual Dutch convention for medical microbiology, we inquired if any of the attending medical microbiologists was aware of additional cases of VCNO sepsis detected in the Netherlands. There is no mandatory notification for VCNO isolates in the Netherlands and VCNO sepsis is rare. Thus, retrieving VCNO sepsis cases detected in the Netherlands in another fashion was not feasible.

Literature review

In collaboration with an experienced information specialist of the LUMC library, we formulated a search strategy including synonyms for '*V. cholera* non-O1' and 'bacteraemia' and applied it to PubMed, Medline, Web of Science and Embase databases (Table 1). Articles published before 15 September 2014 were included. Additional articles were identified by checking the references of relevant articles and duplicates were excluded.

Retrieved articles were screened based on title and abstract, and exclusion criteria were: *Vibrio* spp. other than *V. cholerae* non-O1, article not available through the journal's archive/the main author i.e. unanswered email after three months, in vitro data only, environmental samples only, no bacteraemia, no humans, limited clinical data, languages other than English or Dutch. No date limits were applied.

Data of individual case reports were merged and discussed as one patient group. Articles discussing case series were reported separately to prevent overlapping data. Extracted data included: patient demographics, medical history, risk factors i.e. exposure, clinical presentation, laboratory identification method, antimicrobial susceptibility, toxin production, samples cultured aside from blood, treatment and clinical outcome. If antimicrobial resistance was reported for 10 cases or more, they were reported in this article.

Results

Clinical case reports

In addition to the LUMC case, two additional cases were detected in different Dutch medical centres in 2006 and 2007. A relevant selection of the available data per case is presented; none of the isolates were tested for toxin production. All cases were men and above 50 years of age, with infections during the summer season.

Case 1

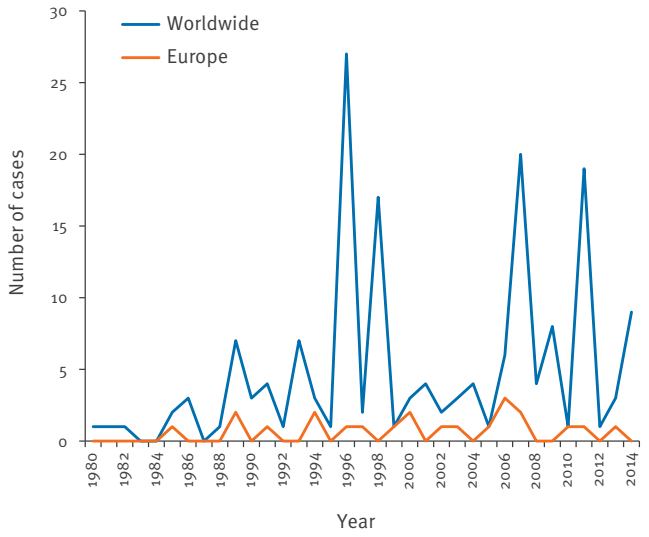
Case 1 was a man in his 50s, with a medical history of tuberculosis, chronic obstructive pulmonary disease, depression, marihuana and excessive alcohol use. One day prior to admission, he felt lethargic and developed a painful discoloration on his right ankle. Three days before hospital admission he had walked barefoot along the Dutch shoreline and ate a ready-made tuna salad. Upon admission at the emergency department (ED) he was hypothermic (34.1°C, norm: 36.5to37.5), blood pressure was 112/70 mmHg (norm: 120/80), heart rate 115 per minute (norm: 60 to 100), O2 saturation was 90% without additional O2 (norm: 93 to 100). While in the ED he developed circulatory failure.

There were no abnormalities on chest auscultation. Inspection of the lower extremities showed oedema, blue discoloration and large bullae on both lower extremities. The chest X-ray showed patchy bilateral abnormalities of which the differential diagnosis comprised acute respiratory distress syndrome, bilateral pneumonia and pre-existing abnormalities after pulmonary tuberculosis.

Analysis of arterial blood at admission showed severe metabolic acidosis with respiratory compensation: pH 7.24 (norm: 7.35 to 7.45), pCO2 4.5 kPa (norm: 4.5 to 6.0), pO2 2.9 kPa (norm: 10.6 to 13.3), base excess -12.1 mmol/L (norm: -2 to 2), O2 saturation 30% (norm: 94 to 99), glucose 1.3 mmol/L (norm: 3.5

Figure 2

Cases of with *Vibrio cholerae* non-O1bacteraemia reported annually in Europe and worldwide, (n=172 including case reports and case series)



to 5.5), lactate 11.4 mmol/L (norm: 0.5 to 2.2). Blood analysis showed a leukopenia, a thrombopenia and elevated liver enzymes. Specifically relevant laboratory results were: haemoglobin (Hb) 8.8 mmol/L (norm: 8.5 to 11.0), leukocytes 2.82 x10^9/L (norm: 4.00 to 10.00), thrombocytes 46 x10^9/L (norm: 150 to 400), INR 1.7(norm 1), C-reactive protein (CRP) 85 mg/L (norm: 0.0 to 0.5), bilirubin 71 µmol/L (norm: 0 to 17), gamma-glutamyltransferase (gamma-GT) 428 U/L (norm: 0 to 55), alkaline phosphatase (AF) 122 U/L (norm: 0-115), aspartate-aminotransferase (ASAT) 179 U/L (norm: 0 to 35), alanine-aminotransferase(ALAT) 68 U/L (norm: 0-45), creatinine was normal.

The putative diagnosis was septic shock due to deep skin infection. Empirical antimicrobial treatment with ciprofloxacin, cefotaxime and selective digestive decontamination with polymyxin E, tobramycin and amphotericin B, was initiated, and the patient was resuscitated, started on vasopressors and admitted to the intensive care unit. Necrotising fasciitis was excluded upon surgical exploration. As VCNO non-O139 was isolated from blood and bullae content on admission day 3, antimicrobial treatment was switched to ciprofloxacin and cefotaxime. On the eighth admission day, the patient developed multi-organ failure (MOF). With the working diagnosis 'hospital acquired infection', gentamicin and flucloxacillin were added. The next day, blood cultures showed *Candida albicans* and before additional treatment was started the patient died from MOF and sepsis. At post-mortem examination *C. albicans* and *Aspergillus fumigatus*, but no *Vibrio* spp. were cultured from several organs including lungs, spleen, liver and intestine.

Microbiology findings

Within 24 hours from presentation at the ED blood cultures and cultures of bullae content grew rod-shaped/curved Gram-negative bacteria which were oxidase-, katalase- and DNase-positive. MALDI-TOF analysis showed *V. albensis* with a score of 2.0 which corresponds with a secure identification on genus level and probable identification on species level (norm: 2.0 to 2.3). Additional biochemical testing (i.e. API 20E, Biomerieux) indicated *V. cholerae*. The microorganism did not agglutinate with O1-Ogawa or O1-Inaba antisera, was sensitive to the vibriostatic compound O/129 and was therefore labelled VCNO. This finding was confirmed and supplemented i.e. non-O 139, by the Dutch National Institute for Public Health and Environment (RIVM). In house susceptibility testing by disk diffusion showed ciprofloxacin and co-trimoxazole sensitivity.

Cultures of the tuna salad packaging did not reveal any *Vibrio* spp.

Case 2

A man in his late 60s with a medical history of heart disease, insulin-dependent diabetes mellitus type II, a cholecystectomy and an aneurysm of the abdominal aorta, presented to the ED with severe diarrhoea. Two weeks earlier, while on one of the Dutch islands, he suffered from severe diarrhoea for two days after having eaten raw herring. There was initial improvement, but the watery diarrhoea recurred and he consulted a general practitioner who referred him to hospital. There was no blood or mucus in his stool. He did not report any surface water contact.

Upon hospital admission, the patient was mildly icteric and had dyspnoea (respiratory rate 29/minute; norm: 12 to 18), lowered O2 saturation of 91% without supplemental oxygen. He was tachycardic (129/minute), blood pressure was slightly elevated (132/92 mmHg) and he had a temperature of 38 °C. Aside from abdominal distention, physical examination of the abdomen, heart, lungs and skin on the extremities showed no abnormalities. Ultrasound scan of the abdomen showed no abnormalities except steatosishepatis and liver cysts (4 cm in diameter). The electrocardiogram and abdominal X-ray were normal. The chest X-ray revealed signs of congestive heart failure.

Parameters of the arterial blood analysis were within normal range. Other laboratory findings at admission showed a mild leucocytosis of 14.0 x10^9/L (norm: 4 to 11) and elevated liver enzymes indicating cholestasis. Laboratory results included: Hb 9.0 mmol/L, thrombocytes 226 x10^9/L, CRP 111mg/L (norm: 0 to 10), glucose 12.0 mmol/L (norm: 3.5 to 7.8), sodium 137 mmol/L (norm: 135 to 145), potassium 3.4 mmol/L (norm: 3.5 to 5.0), creatinine 60 µmol/L (norm: 50 to 110), urea 5.3 mmol/L (norm: 2.5 to 7.5), LDH 269 U/L (norm: 0 to 250), ASAT 194 U/L (norm: 0 to 40), ALAT 292U/L, AF251 U/L (norm: 0 to 120), gamma-GT 631 U/L, bilirubin total 90 µmol/L (norm: 0 to 17), bilirubin direct 66 µmol/L

Table 1

Literature search strategy for *Vibrio cholera* non-O1 bacteraemia

Database	Query	All records	Unique records
PubMed/ Medline[a]	('Vibrio cholerae non-O1'[MeSH] OR (('vibrio cholerae'[MeSH Terms] OR ('vibrio'[All Fields] AND 'cholerae'[All Fields]) OR 'vibrio cholerae'[All Fields]OR vibrio[all fields] OR choler*[all fields]) AND (non-O1[All Fields] OR non-o1[All Fields] OR nonO1[All Fields] OR nono1[All Fields] OR non-O1*[All Fields] OR non-o1*[All Fields] OR nonO1*[All Fields] OR nono1*[All Fields] OR non-O[all fields] OR non-o[all fields] OR nonO[all fields] OR nono[all fields] OR ('non-serogroup'[all fields] AND 'O:1'[all fields]) OR 'Non-OI'[all fields]))) AND ('sepsis'[MeSH Terms] OR 'sepsis'[All Fields] OR 'septic'[all fields] OR 'bacteraemia'[All Fields] OR 'bacteremia'[MeSH Terms] OR 'bacteremia'[All Fields] OR Bacteremi*[all fields] OR Bacteraemi*[all fields] OR Endotoxemia [all fields] OR Endotoxaemia [all fields] OR Endotoxem* [all fields] OR Endotoxaem*[all fields] OR Fungemia[all fields] OR Fungaemia[all fields] OR Fungem*[all fields] OR Fungaem*[all fields] OR Candidemia[all fields] OR Candidaemia [all fields] OR Candidem*[all fields] OR Candidaem*[all fields] OR Parasitemia[all fields] OR Parasitaemia[all fields] OR Parasitem*[all fields] OR Parasitaem*[all fields] OR Viremia[all fields] OR Viraemia[all fields] OR Virem*[all fields] OR Viraem*[all fields]) NOT ('Animals'[MeSH] NOT 'Humans'[MeSH]) AND (english[la] OR dutch[la])	87	87
Web of Science	TS=((((('vibrio' AND 'cholerae') OR 'vibrio cholerae'OR vibrio OR choler*) AND (non-O1 OR non-o1 OR nonO1 OR nono1 OR non-O1* OR non-o1* OR nonO1* OR nono1* OR non-O OR non-o OR nonO OR nono OR ("non-serogroup" AND 'O:1') OR 'Non-OI'OR 'Non-agglutinable' OR 'Non-cholera' OR 'Non-serogroup' OR 'Nonagglutinable' OR 'Noncholera' OR 'Nonserogroup' OR Nonagglutin* OR Noncholer* OR Nonsero* OR 'Non-serogroups' OR 'Nonserogroups' OR 'Non-O1-Vibrio' OR 'Non-O group' OR 'No O1' OR 'No O:1' OR 'Non-O:1' OR 'NOVC' OR 'NVC') OR (('Vibrio O'.mp OR 'Vibrio o'.mp OR 'VibrioO'.mp OR 'Vibrioo'.mp) NOT ('O-139'.mp OR 'O1'.mp OR 'o-139'.mp OR 'o1'.mp OR 'O139'.mp OR 'O-1'.mp OR 'o139'.mp OR 'o-1'.mp))) AND (sepsis OR 'sepsis' OR 'septic' OR 'bacteraemia' OR bacteremia OR 'bacteremia' OR Bacteremi* OR Bacteraemi* OR Endotoxemia OR Endotoxaemia OR Endotoxem* OR Endotoxaem* OR Fungemia OR Fungaemia OR Fungem* OR Fungaem* OR Candidemia OR Candidaemia OR Candidem* OR Candidaem* OR Parasitemia OR Parasitaemia OR Parasitem* OR Parasitaem* OR Viremia OR Viraemia OR Virem* OR Viraem*)) AND la=(English OR Dutch)	62	19
Embase	((vibrio cholerae/ OR ('vibrio'.af AND 'cholerae'.af) OR 'vibrio cholerae'.afOR vibrio.af OR choler*.af) AND (non-O1.af OR non-o1.af OR nonO1.af OR nono1.af OR non-O1*.af OR non-o1*.af OR nonO1*.af OR nono1*.af OR non-O.af OR non-o.af OR nonO.af OR nono.af OR ('non-serogroup'.af AND 'O:1'.af) OR 'Non-OI'.af OR 'Non-agglutinable'.af OR 'Non-cholera'.af OR 'Non-serogroup'.af OR 'Nonagglutinable'.af OR 'Noncholera'.af OR 'Nonserogroup'.af OR Nonagglutin*.af OR Noncholer*.af OR Nonsero*.af OR "Non-serogroups".af OR 'Nonserogroups'.af OR 'Non-O1-Vibrio'.af OR 'Non-O group'.af OR 'No O1'.af OR 'No O:1'.af OR 'Non-O:1'.af OR 'NOVC'.af OR 'NVC'.af) OR (('Vibrio O'.mp OR 'Vibrio o'.mp OR 'VibrioO'.mp OR 'Vibrioo'.mp) NOT ('O-139'.mp OR 'O1'.mp OR 'o-139'.mp OR 'o1'.mp OR 'O139'.mp OR 'O-1'.mp OR 'o139'.mp OR 'o-1'.mp))) AND (exp sepsis/ OR 'sepsis'.af OR 'septic'.af OR 'bacteraemia'.af OR exp bacteremia/ OR 'bacteremia'.af OR Bacteremi*.af OR Bacteraemi*.af OR Endotoxemia .af OR Endotoxaemia .af OR Endotoxem* .af OR Endotoxaem*.af OR Fungemia.af OR Fungaemia.af OR Fungem*.af OR Fungaem*.af OR Candidemia.af OR Candidaemia .af OR Candidem*.af OR Candidaem*.af OR Parasitemia.af OR Parasitaemia.af OR Parasitem*.af OR Parasitaem*.af OR Viremia.af OR Viraemia.af OR Virem*.af OR Viraem*.af) NOT (exp Animals/ NOT exp Humans/) AND (English.la OR Dutch.la)	109	20
Science Direct	TITLE-ABSTR-KEY((vibrio) AND (non-O1 OR non-o1 OR nonO1 OR nono1 OR non-O1* OR non-o1* OR nonO1* OR nono1* OR non-O OR non-o OR nonO OR nono OR ('non-serogroup' AND 'O:1') OR 'Non-OI'OR 'Non-agglutinable' OR 'Non-cholera' OR 'Non-serogroup' OR 'Nonagglutinable' OR 'Noncholera' OR 'Nonserogroup' OR Nonagglutin* OR Noncholer* OR Nonsero* OR 'Non-serogroups' OR 'Nonserogroups' OR 'Non-O1-Vibrio' OR 'Non-O group' OR 'No O1' OR 'No O:1' OR 'Non-O:1' OR 'NOVC' OR 'NVC') AND TITLE-ABSTR-KEY(sepsis OR 'sepsis' OR 'septic' OR 'bacteraemia' OR bacteremia OR 'bacteremia' OR Bacteremi* OR Bacteraemi* OR Endotoxemia OR Endotoxaemia OR Endotoxem* OR Endotoxaem* OR Fungemia OR Fungaemia OR Fungem* OR Fungaem* OR Candidemia OR Candidaemia OR Candidem* OR Candidaem* OR Parasitemia OR Parasitaemia OR Parasitem* OR Parasitaem* OR Viremia OR Viraemia OR Virem* OR Viraem*))	60	37
Total		**318**	**163**

la: language; MeSH: medical subject headings; TS: title/summary.

[a] No additional records were retrieved through searching the Medline database after searching the PubMed database.

(norm: 0 to 5), amylase 12 U/L (norm: 0 to 100), lipase 15 U/L (norm: 0 to 70) and lactate 2.3 mmol/L (norm: 0 to 1.8).

The diagnosis was sepsis and after obtaining blood cultures, treatment with amoxicillin-clavulanic acid and gentamicin was initiated according to the local sepsis treatment protocol. On the second admission day, the Gram-negative rod was isolated and suspected to be *Salmonella* spp. Treatment was switched to co-trimoxazole. Later that day, as the isolate tested oxidase-positive, the working diagnosis was changed to *Pseudomonas* spp. and treatment was switched to ciprofloxacin. Ciprofloxacin was continued after the identification of *V. cholerae*. The patient recovered fully, was discharged after five days with oral ciprofloxacin, and returned to his island holiday.

Microbiology findings

One day after hospital admission, blood cultures became positive with Gram-negative rods, later identified as *V. cholerae* (Phoenix Automated Microbiology System, BD diagnostics and API E, Biomerieux). The isolate was sent to the RIVM and the biochemical profile, fatty acid analysis and 16S rDNA PCR showed *V. cholerae* non-O1 non-O139. Stool cultures remained negative for *Vibrio* spp.. With standard disk diffusion the isolate tested susceptible to co-trimoxazole, cefuroxime, gentamicin, ciprofloxacin, piperacillin, ceftazidime, meropenem, tobramycin and piperacillin/tazobactam. It was intermediately sensitive to amoxicillin, amoxicillin-clavulanic acid, cefazolin and resistant to ceftriaxone.

Case 3

A man in his early 70s presented at the ED with general malaise, dizziness, decreased appetite, coughing and dyspnoea that had been lasting for one week. Relevant medical history comprised heart failure and a hepatojejunostomy for chronic cholangitis more than a decade before presentation. He had not been travelling or swimming, but habitually caught eel in the Ijsselmeer lake that summer and cleaned the eel himself. He did not report having consumed the eel or having had contact with lake water other than taking eel out of fishing nets, or of wounds or lacerations on his hands before his illness.

Upon hospital admission, he had a temperature of 39°C, heart rate of 66/minute, blood pressure of 110/70 mm/Hg and oxygen saturation was normal without oxygen administration. He was disorientated and had trouble concentrating. There were no signs of gastroenteritis or abnormalities on chest examination. No apparent skin lesions were reported.

Arterial blood analysis showed a pH 7.46, pCO2 4.4 kPa (norm: 4.7 to 6.4), bicarbonate 23.1 mmol/L (norm: 22.0 to 29.0), pO2 6.4 kPa (norm: 10.0 to 13.3), base excess 0.1 mmol/L (norm: -3.0 to 3.0), O2 saturation 86%. Analysis of venous blood showed raised inflammatory parameters, impaired renal and liver function tests. CRP 181 mg/L (norm: < 7), leukocytes 10.6 x10^9/L, urea 12,5 mmol/L (norm: 2.9 to 7.5), creatinin 115 umol/L (norm: 01 to 05), AP 336 U/L (norm: 0 to 120), gamma-GT 282 U/L, bilirubin 70 umol/L (norm: 0 to 20), ASAT 132 U/L (norm: 0 to 32), ALAT 104 U/L, glucose 7.9 mmol/L (norm: 0 to 7.8).

The patient was initially empirically treated for sepsis with ceftriaxone and gentamicin, then switched to oral amoxicillin-clavulanic acid. He recovered completely and was discharged after seven days of hospitalisation.

Microbiology findings

Within 24 hours after admission, blood cultures grew Gram-negative rods. On TCBS agar, yellow colonies appeared which tested oxidase negative. Identification through API NE (Biomerieux corporation) showed *V. cholerae* (code 7074745, ID 99.0%), which was confirmed with 16S PCR. The isolate's susceptibility was tested using standard disk diffusion on Muller Hinton agar plates. It was susceptible to amoxicillin, amoxicillin-clavulanic acid, piperacillin, piperacillin-tazobactam, cefoxitin, ceftazidime, meropenem, gentamicin.

Sputum cultures were negative for *Vibrio* spp.. The eel were not examined microbiologically, therefore the source of infection remained unclear.

Review

The initial search yielded 163 unique articles and 155 duplicates (Figure 1). Reference checking resulted in identification of two additional unique articles. Of the 165 retrieved articles, 77 were excluded based on title or abstract, leaving 88 articles including 82 case reports [8-89] and six articles reporting case series [90-95].

Case reports

The 82 articles retrieved covered 90 patients; 23 (26%) of them were female and the mean age was 49 (0–84) years. Cases were reported worldwide; 22 (25%) were identified in Europe, 19 (21%) in the United States of America, 30 (33%) in the Asian continent and 19 (21%) in the remaining continents. The remaining case characteristics are presented in Table 2 (extraction table for data available from authors upon request).

The most frequently reported symptom was gastroenteritis (51/90; 57%) followed by fever without gastroenteritis (26/90; 29%) and bullae (18/90; 20%). However, also rare presentations such as endophthalmitis and neonatal meningitis were reported. Overall known risk factors such as consumption or handling of seafood or consumption or contact with possibly contaminated water were reported in 45 cases (45/90; 50%). Of the 47 patients (47/90; 52%) presenting with gastroenteritis and no bullae, 15 (15/47) reported prior seafood consumption, five (5/47) reported fishing (possibly implying consumption) and for nine cases (9/47) no exposure data were reported; no information

Table 2

Characteristics of clinical cases with *Vibrio cholerae* non-O1 bacteraemia (n=3), and published case reports (n=90) and case series (n=82) identified through literature research

Characteristics		Dutch cases (n=3)	Literature review	
			Case reports (n=90)	Case reports (n=82)
		Number (%) or mean (min-max)		
Demographics	Female[a]	0	23 (26)	12 (15)
	Age	65 (57–72)	49 (0–84)	NR (28–83)
Origin	Europe	3	22 (25)	0
	United States of America	0	19 (21)	0
	Asia	0	30 (33)	74 (90)
	Other[c]	0	19 (21)	6 (7)
Medical history	Uneventful	0	12 (13)	NR
	Liver cirrhosis and/or alcohol abuse	1	38 (42)	27 (33)
	Liver or bile duct disease (including cholecystectomy)	2	6 (7)	7 (9)
	Immunocompromised[d]	0	14 (16)	NR
	Not provided	0	6 (7)	NR
Risk factors	Consumption of seafood	2	21 (23)	12 (15)
	Surface water contact	1	8 (9)	3 (4)
	Surface water contact and seafood handling (e.g. fishing)	1	11 (12)	8 (10)
	Consumption of possibly contaminated water	0	5 (6)	NR
	Other risk factors[e]	0	5 (6)	NR
	Not provided	0	15 (17)	NR
Symptoms at presentation	Gastroenteritis (nausea and abdominal pain/vomiting/mild diarrhoea)	1	47 (52)	32 (39)
	Fever, no gastroenteritis	1	26 (29)	51 (62)
	Bullae	1	14 (17)	8 (10)
	Bullae and gastroenteritis	0	4 (4)	NR
Vibrio cholerae isolated[b]	Faeces	0	5 of 32	0 of 19
	Ascites/peritoneal fluid	0	8 of 20	8 of 33
	Food or water	0	5 of 7	2 of 2
	CSF (meningitis)	0	3 of 5	NR
	Bulla fluid	1 of 1	6 of 6	NR
Clinical outcome	Recovery	2	56 (62)	51 (62)
	Irreversible trauma (e.g. severe neurological impairment)	0	2 (2)	NR
	Death	1	31 (34)	29 (35)
	Unknown	0	1 (2)	NR

CSF: cerebrospinal fluid; NR: Not reported or insufficient data.

[a] The sex of one patient (newborn) was not provided.

[b] Aside from positive blood cultures which were confirmed for all cases.

[c] Australia, Israel, Kuwait, Lebanon, Mauritius, Ottawa (Canada), Puerto Rico, Qatar, Saudi Arabia.

[d] AIDS/HIV, chemotherapy, prednisone, neutropenia, non-Hodgkin lymphoma, myelodysplastic syndrome, idiopathic aplastic anaemia, transplant recipient.

[e] *Vibrio cholerae* vaccine, baby bath/bottle contamination with raw seafood.

on exposure was available for the remaining 18 cases. In five cases (5/90; 6%) authors hypothesised on additional risk factors such as indirect contact with raw seafood e.g. contaminated baby bathwater/bottle (2/5) and administration of the oral *V. cholerae* vaccine (3/5). However, the vaccine contains killed whole *V. cholera* cells with or without recombinant B-sub unit and the nature of the alleged link to VCNO infection was not clarified by the authors. As our case definition of VCNO sepsis included bacteraemia, a positive blood culture was reported for all patients. Additional samples that were positive for VCNO are shown in Table 2. Thirty-one patients died due to their VCNO infection, which results in an overall case–fatality rate of 348 per 1,000 for this selected sample.

For 62 cases (62/90; 69%), susceptibility data of the *V. cholerae* non-O1 isolates were provided (extraction table available from authors upon request). Overall, in vitro resistance was observed to amoxicillin (4/50

tested (intermediately) resistant), trimethoprim-sulfamethoxazole (4/37), ciprofloxacin (2/24), gentamicin (1/37) and doxycycline (3/38). Antimicrobials to which *V. cholerae* non-O1 isolates appeared to be susceptible in vitro, without exception, were cephalotin (n=14 tested), cefuroxime (n=10), cefotaxime (n=14), ceftazidime (n=12), ceftriaxone (n=15), amikacin (n=17) and chloramphenicol (n=33). Toxin production of the isolates was not consistently reported in literature and therefore not included in the analysis.

Case series

Details of the six retrieved case series were extracted and analysed briefly given the selected nature of the sample (Table 2). Altogether, 82 patients with VCNO bacteraemia were retrieved, the majority of reports originating from the Asian continent. As in the three case reports described above, most patients were male. In contrast, fever was the most frequent presenting symptom and risk factors could be identified in only a minority of patients (24/82; 29%).

Overall, the number of cases reported peaked in 1996, 1998, 2007 and 2011 (Figure 2).

Discussion

We presented three cases of VCNO bacteraemia that were identified in the Netherlands between 2006 and 2013as well as 172 cases from literature occurring between 1980 and 2014. Corresponding with current knowledge, both the recently identified cases and the previously reported ones show that patients are typically male, often have a history of liver/bile duct disease and the presenting symptoms often include gastroenteritis, fever and bullae [1]. The suspected sources while seldom confirmed microbiologically, are commonly fish and surface water. In contrast to previous reports, however, we found that a great variety in clinical presentation does occur, ranging from lethargy to meningitis, endophthalmitis, cough and dyspnoea [1]. Severe outcomes include neurological impairment, lower limb amputation and death. All three Dutch cases presented during summer, the season of recreational activities such as fishing, swimming and of flourishing microorganisms in surface waters due to rising temperatures [96-98].

A major strength of our analysis is that it provides a complete overview of what is known about VCNO bacteraemia, whereas other reports merely describe individual cases or a selection of case reports. The search was formulated by an experienced scientist (MFE) and an information specialist (JS) and articles were provided by the LUMC library which has access to over 9,000 leading (bio-)medical journals. Therefore, we consider that this review includes all relevant published articles published in Dutch and English and provides a complete overview of the available literature on VCNO bacteraemia.

The summary of data on antimicrobial susceptibility provided here may assist physicians in choosing an adequate treatment regimen. The data indicate that administration of a cephalosporin is likely to be the best option when dealing with VCNO bacteraemia. However, an important factor that hinders the extrapolation of our data to clinical practice is publication bias, many authors did not report susceptibility data. This is crucial when evaluating resistance data, as many authors solely reported the susceptibility to antimicrobials administered to the patient in question. Additional relevant data on antimicrobial resistance that may very well have been available to the authors was not published.

After searching current literature for the aetiology of VCNO bacteraemia, we could not reveal why males are affected more frequently than females, but found a similar trend in infections with other *Vibrio* spp [99]. The role of immunocompromising conditions in acquiring VCNO bacteraemia seems clear and the influence of liver cirrhosis can probably be attributed to high ferritin levels which are required for the metabolism of *Vibrio* spp [100].

Aside from predisposing conditions and exposure, bacterial virulence may very well play a significant role in the pathogenesis of VCNO bacteraemia. VCNO toxins are being studied and several are known e.g. ctx; large excretion of fluids and electrolytes into the lumen hlyAET; hemolysin, rtxA; actin cross linking, hap; haemagglutinin protease, type 3 and 6 secretion system, nanH; neuraminidase, NAG-ST; heat-stable enterotoxin. The clinical significance of these toxins (e.g. their role in bulla formation, remains yet to be determined [101,102].

In conclusion, VCNO bacteraemia is a disease that can be fatal and poses a threat around the globe especially to patients with a history of alcohol abuse and/or liver cirrhosis. Physicians should be aware of the possibility of VCNO bacteraemia in patients presenting with gastroenteritis, fever or bullae after consumption of or contact with seafood or potentially contaminated water. However, risk factors often remain unidentified, the clinical presentation varies greatly and a quick microbiological diagnosis is indispensable. Cephalosporins are likely the best treatment option for VCNO bacteraemia.

Acknowledgements

We gratefully acknowledge the assistance of Mr. J. Schoones, MA, experienced information specialist at the Walaeus library at the LUMC, with the literature search.

Conflict of interest

None declared.

Authors' contributions

Madelon F. Engel: performing the literature review, gathering information on individual cases, drafting and revising the manuscript. Mariette A. Muijsken: provide information on one of the three cases, revising the manuscript. Esther Mooi-Kokenberg: provide information on one of the three cases, revising the manuscript. Ed J. Kuijper: revising the manuscript. David J. van Westerloo: provide information on one of the three cases, intensively revising the manuscript.

References

1. Versalovic J, Carroll KC, Funke G, Jorgensen JH, Landry ML, Warnock DW. Manual of clinical microbiology. Washington, DC: ASM Press; 2011.
2. Centers for Disease Control and Prevention (CDC). CDC Laboratory Methods for the Diagnosis of Vibrio cholerae. Laboratory Methods for the Diagnosis of Vibrio cholerae. Ch 6. Atlanta: CDC. [Accessed 13 Apr 2016]. Available from: http://www.cdc.gov/cholera/pdf/Laboratory-Methods-for-the-Diagnosis-of-Vibrio-cholerae-chapter-6.pdf
3. Centers for Disease Control and Prevention (CDC),Díaz-Quiñonez A, Hernández-Monroy I, Montes-Colima N, Moreno-Pérez A, Galicia-Nicolás A, Martínez-Rojano H, et al. . Outbreak of Vibrio cholerae serogroup O1, serotype Ogawa, biotype El Tor strain--La Huasteca Region, Mexico, 2013.MMWR Morb Mortal Wkly Rep. 2014;63(25):552-3.PMID: 24964882
4. Haley BJ, Choi SY, Grim CJ, Onifade TJ, Cinar HN, Tall BD, et al. Genomic and phenotypic characterization of Vibrio cholerae non-O1 isolates from a US Gulf Coast cholera outbreak. PLoS One. 2014;9(4):e86264. DOI: 10.1371/journal.pone.0086264 PMID: 24699521
5. Schets FM, van den Berg HH, Marchese A, Garbom S, de Roda Husman AM. Potentially human pathogenic vibrios in marine and fresh bathing waters related to environmental conditions and disease outcome.Int J Hyg Environ Health. 2011;214(5):399-406. DOI: 10.1016/j.ijheh.2011.05.003 PMID: 21664866
6. Visser IJ, ter Laak EA, van Dijk NW, Wouda W. Toxigenic Vibrio cholerae non-O:1 isolated from a goat in The Netherlands. Vet Q. 1991;13(2):114-8. DOI: 10.1080/01652176.1991.9694293 PMID: 1882490
7. Schets FM, van den Berg HH, Rutjes SA, de Roda Husman AM. Pathogenic Vibrio species in dutch shellfish destined for direct human consumption.J Food Prot. 2010;73(4):734-8.PMID: 20377964
8. Albuquerque A, Cardoso H, Pinheiro D, Macedo G. Vibrio cholerae non-O139 and non-139 infection in hepatitis C cirrhosis: A rare event. American Journal of Gastroenterology. 2011 Oct; Conference (var.pagings):S280.
9. Anderson AM, Varkey JB, Petti CA, Liddle RA, Frothingham R, Woods CW. Non-O1 Vibrio cholerae septicemia: case report, discussion of literature, and relevance to bioterrorism. Diagn Microbiol Infect Dis. 2004;49(4):295-7. DOI: 10.1016/j.diagmicrobio.2004.04.016 PMID: 15313536
10. Berghmans T, Crokaert F, Sculier JP. Vibrio cholerae bacteremia in a neutropenic patient with non-small-cell lung carcinoma. Eur J Clin Microbiol Infect Dis. 2002;21(9):676-8. DOI: 10.1007/s10096-002-0794-1 PMID: 12373501
11. Blanche P, Sicard D, Sevali Garcia J, Paul G, Fournier JM. Septicemia due to non-O:1 Vibrio cholerae in a patient with AIDS.Clin Infect Dis. 1994;19(4):813. DOI: 10.1093/clinids/19.4.813 PMID: 7803670
12. Burns KD, Yurack J, McIntyre RW. Non-OI vibrio cholerae septicemia associated with a motor vehicle accident.CMAJ. 1989;140(11):1334-5.PMID: 2720517
13. Chan HL, Ho HC, Kuo TT. Cutaneous manifestations of non-o1 Vibrio cholerae septicemia with gastroenteritis and meningitis.J Am Acad Dermatol. 1994;30(4):626-8. DOI: 10.1016/S0190-9622(94)70071-0 PMID: 8157789
14. Chan MR, Pirsch JD. Nonserogroup O1 Vibrio cholerae in a renal transplant patient.Transplantation. 2007;83(5):668-9. DOI: 10.1097/01.tp.0000255321.86183.47 PMID: 17353792
15. Chang-Chien CH. Bacteraemic necrotizing fasciitis with compartment syndrome caused by non-O1 Vibrio cholerae.J Plast Reconstr Aesthet Surg. 2006;59(12):1381-4. DOI: 10.1016/j.bjps.2006.06.002 PMID: 17113524
16. Chang-Chien CH, Ding HT, Liu C, Yang CS. Vibrio infection associated with finning injury of the hand.Injury. 2007;38(5):614-8. DOI: 10.1016/j.injury.2006.04.134 PMID: 16945371
17. Cheng NC, Tsai JL, Kuo YS, Hsueh PR. Bacteremic necrotizing fasciitis caused by Vibrio cholerae serogroup O56 in a patient with liver cirrhosis.J Formos Med Assoc. 2004;103(12):935-8. PMID: 15624044
18. Choi SM, Lee DG, Kim MS, Park YH, Kim YJ, Lee S, et al. Bacteremic cellulitis caused by non-O1, non-O139 Vibrio cholerae in a patient following hematopoietic stem cell transplantation. Bone Marrow Transplant. 2003;31(12):1181-2. DOI: 10.1038/sj.bmt.1704044 PMID: 12796802
19. Chong Y, Kwon OH, Lee SY, Kim BS, Min JS. Non-O group 1 Vibrio cholerae septicemia and peritonitis--report of two cases. Yonsei Med J. 1985;26(1):82-4. DOI: 10.3349/ymj.1985.26.1.82 PMID: 4072273
20. Christenson B, Soler M, Nieves L, Souchet LM. Septicemia due to a non-o:1, non-o:139 Vibrio cholerae.Bol Asoc Med P R. 1997;89(1-3):31-2.PMID: 9168634
21. Couzigou C, Lacombe K, Girard P-M, Vittecoq D, Meynard J-L. Non-O:1 and non-O:139 Vibrio cholerae septicemia and pyomyositis in an immunodeficient traveler returning from Tunisia.Travel Med Infect Dis. 2007;5(1):44-6. DOI: 10.1016/j.tmaid.2006.06.002 PMID: 17161319
22. Dalsgaard A, Forslund A, Hesselbjerg A, Bruun B. Clinical manifestations and characterization of extra-intestinal Vibrio cholerae non-O1, non-O139 infections in Denmark. Clin Microbiol Infect. 2000;6(11):625-7. DOI: 10.1046/j.1469-0691.2000.00174.x PMID: 11168070
23. de Groot R, van der Horst-Bruinsma IE, Janssens AR, Petit PL. [Sepsis caused by non-O1-Vibrio cholerae: a patient in The Netherlands]. Ned Tijdschr Geneeskd. 1994;138(17):873-5. PMID: 8183400
24. Deris ZZ, Leow VM, Wan Hassan WM, Nik Lah NA, Lee SY, Siti Hawa H, et al. Non-O1, non-O139 Vibrio cholerae bacteraemia in splenectomised thalassaemic patient from Malaysia. Trop Biomed. 2009;26(3):320-5.PMID: 20237446
25. Dhar R, Ghafoor MA, Nasralah AY. Unusual non-serogroup O1 Vibrio cholerae bacteremia associated with liver disease.J Clin Microbiol. 1989;27(12):2853-5.PMID: 2592546
26. Dhar R, Badawi M, Qabazard Z, Albert MJ. Vibrio cholerae (non-O1, non-O139) sepsis in a child with Fanconi anemia. Diagn Microbiol Infect Dis. 2004;50(4):287-9. DOI: 10.1016/j.diagmicrobio.2004.08.004 PMID: 15582302
27. El-Hiday AH, Khan FY, Al Maslamani M, El Shafie S. Bacteremia and spontaneous bacterial peritonitis due to Vibrio cholerae (non-O1 non-O139) in liver cirrhosis.Indian J Gastroenterol. 2006;25(2):107.PMID: 16763354
28. Eltahawy AT, Jiman-Fatani AA, Al-Alawi MM. A fatal non-o1 Vibrio cholerae septicemia in a patient with liver cirrhosis. Saudi Med J. 2004;25(11):1730-1.PMID: 15573214
29. Farina C, Luzzi I, Lorenzi N. Vibrio cholerae O2 sepsis in a patient with AIDS.Eur J Clin Microbiol Infect Dis. 1999;18(3):203-5. DOI: 10.1007/s100960050259 PMID: 10357055
30. Farmachidi JP, Sobesky R, Boussougant Y, Quilici ML, Coffin B. Septicaemia and liver abscesses secondary to non-O1/non-O139 Vibrio cholerae colitis.Eur J Gastroenterol Hepatol. 2003;15(6):699-700. DOI: 10.1097/00042737-200306000-00018 PMID: 12840683
31. Feghali R, Adib SM. Two cases of Vibrio cholerae non-O1/non-O139 septicaemia with favourable outcome in Lebanon. East Mediterr Health J. 2011;17(8):722-4.PMID: 21977578
32. Fernández-Natal I, Alcoba-Leza M. Non-O1 Vibrio cholerae bacteraemia without diarrhoea.Lancet. 1996;348(9019):67. DOI: 10.1016/S0140-6736(05)64401-7 PMID: 8691963
33. Fernández JM, Serrano M, De Arriba JJ, Sánchez MV, Escribano E, Ferreras P. Bacteremic cellulitis caused by Non-o1, Non-o139 Vibrio cholerae: report of a case in a patient with hemochromatosis.Diagn Microbiol Infect Dis. 2000;37(1):77-80. DOI: 10.1016/S0732-8893(99)00153-4 PMID: 10794945
34. George N, Fredrick F, Mohapatra A, Veeraraghavan B, Kakde ST, Valson AT, et al. Non-O1, non-O139 Vibrio cholerae sepsis in a patient with nephrotic syndrome. Indian J Nephrol. 2013;23(5):378-80. DOI: 10.4103/0971-4065.116329 PMID: 24049279
35. Halabi M, Haditsch M, Renner F, Brinninger G, Mittermayer H. Vibrio cholerae non-O1 septicaemia in a patient with liver cirrhosis and Billroth-II-gastrectomy.J Infect. 1997;34(1):83-4. DOI: 10.1016/S0163-4453(97)80016-3 PMID: 9120332
36. Heath CH, Garrow SC, Golledge CL. Non-O1 Vibrio cholerae: a fatal cause of sepsis in northern Australia.Med J Aust. 2001;174(9):480-1.PMID: 11386599
37. Hsieh YH, Chan CC, Wang SS, Chiang JH, Chou DY, Chang FY, et al. Recurrent spontaneous bacterial empyema in cirrhosis: a case report. Zhonghua Yi Xue Za Zhi (Taipei). 1998;61(7):421-6. PMID: 9699395
38. Huhulescu S, Indra A, Feierl G, Stoeger A, Ruppitsch W, Sarkar B, et al. Occurrence of Vibrio cholerae serogroups other than O1 and O139 in Austria. Wien Klin Wochenschr.

2007;119(7-8):235-41. DOI: 10.1007/s00508-006-0747-2 PMID: 17492351

39. Inoue T, Kitai S, Hayaishi S, Kudo M. Septicemia due to Vibrio cholerae serogroup non-O1/non-O139 strain in a cirrhotic patient.Clin J Gastroenterol. 2012;5(6):383-7. DOI: 10.1007/s12328-012-0332-3 PMID: 26181318
40. Ismail EA, Shafik MH, Al-Mutairi G. A case of non-O:1 Vibrio cholerae septicemia with meningitis, cerebral abscess and unilateral hydrocephalus in a preterm baby.Eur J Clin Microbiol Infect Dis. 2001;20(8):598-600. DOI: 10.1007/s100960100553 PMID: 11681447
41. Issack MI, Appiah D, Rassoul A, Unuth MN, Unuth-Lutchun N. Extraintestinal Vibrio infections in Mauritius.J Infect Dev Ctries. 2008;2(5):397-9. DOI: 10.3855/jidc.205 PMID: 19745511
42. Kambal AMM, Ajarim DS, Fawzy E, Babay HH, Chowdhury MNH. Septicemia caused by a non-o1 Vibrio cholerae.Ann Saudi Med. 1996;16(2):200-2.PMID: 17372457
43. Kerketta JA, Paul AC, Balaji V,, Kirubakaran VB, Jesudason MV, Moses PD. Non-o1 Vibrio cholerae septicemia and meningitis in a neonate.Indian J Pediatr. 2002;69(10):909-10. DOI: 10.1007/BF02723720 PMID: 12450305
44. Khan S, Kumar A, Meparambu D, Thomas S, Harichandran D, Karim S. Fatal non-O1, non-O139 Vibrio cholerae septicaemia in a patient with chronic liver disease.J Med Microbiol. 2013;62(Pt 6):917-21. DOI: 10.1099/jmm.0.049296-0 PMID: 23449872
45. Kontoyiannis DP, Calia KE, Basgoz N, Calderwood SB. Primary septicemia caused by Vibrio cholerae non-O1 acquired on Cape Cod, Massachusetts.Clin Infect Dis. 1995;21(5):1330-3. DOI: 10.1093/clinids/21.5.1330 PMID: 8589171
46. Lalitha MK, Dayal U, Cherian AM. Non-agglutinating vibrio (non o-1 V. cholerae) septicemia.Indian J Pathol Microbiol. 1986;29(1):27-30.PMID: 3781609
47. Laosombat V, Pruekprasert P, Wongchanchailert M. Non-o:1 Vibrio cholerae septicemia in thalassemia patients.Southeast Asian J Trop Med Public Health. 1996;27(2):411-3.PMID: 9280014
48. Lee MH, Leu HS, Huang SH. Bacteremic cellulitis caused by non-O1 Vibrio cholerae: report of a case.J Formos Med Assoc. 1993;92(5):472-4.PMID: 8104603
49. Lopez-Brea M, Jimenez ML, de las Cuevas C, Alcala-Zamora J, Alonso P. Non-o1 Vibrio cholerae septicaemia.Trans R Soc Trop Med Hyg. 1985;79(6):878-9. DOI: 10.1016/0035-9203(85)90151-8 PMID: 3832501
50. Lu B, Zhou H, Li D, Li F, Zhu F, Cui Y, et al. The first case of bacteraemia due to non-O1/non-O139 Vibrio cholerae in a type 2 diabetes mellitus patient in mainland China. Int J Infect Dis. 2014;25:116-8. DOI: 10.1016/j.ijid.2014.04.015 PMID: 24905769
51. Lukinmaa S, Mattila K, Lehtinen V, Hakkinen M, Koskela M, Siitonen A. Territorial waters of the Baltic Sea as a source of infections caused by Vibrio cholerae non-O1, non-O139: report of 3 hospitalized cases.Diagn Microbiol Infect Dis. 2006;54(1):1-6. DOI: 10.1016/j.diagmicrobio.2005.06.020 PMID: 16368474
52. Magnusson MR, Pegg SP. Vibrio cholerae non-O1 primary septicaemia following a large thermal burn.Burns. 1996;22(1):44-7. DOI: 10.1016/0305-4179(95)00077-1 PMID: 8719316
53. Mannion PT, Mellor S. Unreviewed reports.Br Med J (Clin Res Ed). 1986;292(6518):450. DOI: 10.1136/bmj.292.6518.450 PMID: 20742605
54. Marek A, Inkster T, Anderson E, Jenkins C, Boyd J, Kerr S, et al. Non-toxigenic Vibrio cholerae bacteraemia: case report and review of the literature. J Med Microbiol. 2013;62(Pt 9):1357-9. DOI: 10.1099/jmm.0.060400-0 PMID: 23722436
55. Mattics C. An inland water source of Vibrio cholerae responsible for sepsis.Clin Microbiol Newsl. 1989;11(7):54-6. DOI: 10.1016/0196-4399(89)90011-1
56. McCleskey FK, Hastings JR, Winn RE, Adams ED. Non-o1 Vibrio cholerae bacteremia--complication of a LeVeen shunt.Am J Clin Pathol. 1986;85(5):644-6. DOI: 10.1093/ajcp/85.5.644 PMID: 3706203
57. Naidu LS, Bakerman PR, Saubolle MA, Lewis K. Vibrio cholerae non-o:1 meningitis in an infant.Pediatr Infect Dis J. 1993;12(10):879-81. DOI: 10.1097/00006454-199310000-00016 PMID: 8284127
58. Namdari H, Klaips CR, Hughes JL. A cytotoxin-producing strain of Vibrio cholerae non-O1, non-O139 as a cause of cholera and bacteremia after consumption of raw clams.J Clin Microbiol. 2000;38(9):3518-9.PMID: 10970422
59. Newman C, Shepherd M, Woodard MD, Chopra AK, Tyring SK. Fatal septicemia and bullae caused by non-o1 Vibrio cholerae.J Am Acad Dermatol. 1993;29(5 Pt 2):909-12. DOI: 10.1016/0190-9622(93)70269-Y PMID: 8408840
60. Patel NM, Wong M, Little E, Ramos AX, Kolli G, Fox KM, et al. Vibrio cholerae non-O1 infection in cirrhotics: case report and literature review. Transpl Infect Dis. 2009;11(1):54-6. DOI: 10.1111/j.1399-3062.2008.00339.x PMID: 18811633
61. Petsaris O, Nousbaum JB, Quilici ML, Le Coadou G, Payan C, Abalain ML. Non-O1, non-O139 Vibrio cholerae bacteraemia in a cirrhotic patient.J Med Microbiol. 2010;59(Pt 10):1260-2. DOI: 10.1099/jmm.0.021014-0 PMID: 20616193
62. Phetsouvanh R, Nakatsu M, Arakawa E, Davong V, Vongsouvath M, Lattana O, et al. Fatal bacteremia due to immotile Vibrio cholerae serogroup O21 in Vientiane, Laos - a case report. Ann Clin Microbiol Antimicrob. 2008;7(1):10. DOI: 10.1186/1476-0711-7-10 PMID: 18439249
63. Phu Huong Lan N, Vu Thieu NT, Thi Thu YN, Dung LT, Thanh TH, Campbell JI, et al. Two cases of non-toxigenic Vibrio cholerae non-O1, non-O139 bacteremia in Ho Chi Minh City. J Clin Microbiol. 2014.
64. Piersimoni C, Morbiducci V, Scalise G. Non-O1 Vibrio cholerae gastroenteritis and bacteraemia.Lancet. 1991;337(8744):791-2. DOI: 10.1016/0140-6736(91)91409-N PMID: 1672411
65. Pitrak DL, Gindorf JD. Bacteremic cellulitis caused by non-serogroup O1 Vibrio cholerae acquired in a freshwater inland lake.J Clin Microbiol. 1989;27(12):2874-6.PMID: 2592550
66. Platia E, Vosti KL. Noncholera vibrio septicemia.West J Med. 1980;132(4):354-7.PMID: 7385839
67. Poulos JE, Cancio M, Conrad P, Nord HJ, Altus P. Non o-1 Vibrio cholerae septicemia and culture negative neutrocytic ascites in a patient with chronic liver disease.J Fla Med Assoc. 1994;81(10):676-8.PMID: 7798872
68. Rabadan PM, Vilalta E. Non-O:1 Vibrio cholerae bacteremia.Rev Infect Dis. 1989;11(4):667. DOI: 10.1093/clinids/11.4.667 PMID: 2638813
69. Raju AZ, Mathai D, Jesudasan M, Suresh M, Kaur A, Abraham OC, et al. Nonagglutinable Vibrio cholerae septicaemia. J Assoc Physicians India. 1990;38(9):665-6.PMID: 2266091
70. Restrepo D, Huprikar SS, VanHorn K, Bottone EJ. O1 and non-O1 Vibrio cholerae bacteremia produced by hemolytic strains. Diagn Microbiol Infect Dis. 2006;54(2):145-8. DOI: 10.1016/j.diagmicrobio.2005.08.008 PMID: 16426794
71. Rubin LG, Altman J, Epple LK, Yolken RH. Vibrio cholerae meningitis in a neonate.J Pediatr. 1981;98(6):940-2. DOI: 10.1016/S0022-3476(81)80596-3 PMID: 7229800
72. Rudensky B, Marcus EL, Isaacson M, Lefler E, Stamler B, Sechter I. Non-O group 1 Vibrio cholerae septicemia in Israel. Isr J Med Sci. 1993;29(1):54-5.PMID: 8454453
73. Safrin S, Morris JG, Adams M, Pons V, Jacobs R, Conte JE. Non-O:1 Vibrio cholerae bacteremia: case report and review.Rev Infect Dis. 1988;10(5):1012-7. DOI: 10.1093/clinids/10.5.1012 PMID: 3055182
74. Shelton CH, Martino RL, Ramsey KM. Recurrent non-o:1 Vibrio cholerae bacteremia in a patient with multiple myeloma.Cancer. 1993;72(1):105-7. DOI: 10.1002/1097-0142(19930701)72:1<105::AID-CNCR2820720120>3.0.CO;2-4 PMID: 8508394
75. Siegel MI, Rogers AI. Fatal non-o1 Vibrio cholerae septicemia in chronic lymphocytic leukemia.Gastroenterology. 1982;83(5):1130-1.PMID: 7117796
76. Strumbelj I, Prelog I, Kotar T, Dovecar D, Petras T, Socan M. A case of Vibrio cholerae non-O1, non-O139 septicaemia in Slovenia, imported from Tunisia, July 2005.Euro Surveill. 2005;10(10):E051020.6.PMID: 16790890
77. Stypulkowska-Misiurewicz H, Pancer K, Roszkowiak A. Two unrelated cases of septicaemia due to Vibrio cholerae non-O1, non-O139 in Poland, July and August 2006.Euro Surveill. 2006;11(11):E061130.2.PMID: 17213560
78. Suankratay C, Phantumchinda K, Tachawiboonsak W, Wilde H. Non-serogroup O:1 Vibrio cholerae bacteremia and cerebritis. Clin Infect Dis. 2001;32(7):E117-9. DOI: 10.1086/319596 PMID: 11264049
79. Tan KK, Sin KS, Ng AJ, Yahya H, Kaur P. Non-O1 Vibrio cholerae septicaemia: a case report.Singapore Med J. 1994;35(6):648-9. PMID: 7761898
80. Thisyakorn U, Reinprayoon S. Non-o1 Vibrio cholerae septicemia: a case report.Southeast Asian J Trop Med Public Health. 1990;21(1):149-50.PMID: 2402675
81. Thomas M, Cherian T, Raghupathy P. Non-O:1 Vibrio cholerae bacteremia and peritonitis in a patient with nephrotic syndrome.Pediatr Infect Dis J. 1996;15(3):276-7. DOI: 10.1097/00006454-199603000-00025 PMID: 8852924
82. Toeg A, Berger SA, Battat A, Hoffman M, Yust I. Vibrio cholerae bacteremia associated with gastrectomy.J Clin Microbiol. 1990;28(3):603-4.PMID: 2324281

83. Wang K, Chao CH, Liu IM, Liu CY. Non-o:1 Vibrio cholerae bacteremia: a case report and literature review.Zhonghua Yi Xue Za Zhi (Taipei). 1991;48(3):232-6.PMID: 1657343
84. West BC, Silberman R, Otterson WN. Acalculous cholecystitis and septicemia caused by non-O1 Vibrio cholerae: first reported case and review of biliary infections with Vibrio cholerae.Diagn Microbiol Infect Dis. 1998;30(3):187-91. DOI: 10.1016/S0732-8893(97)00235-6 PMID: 9572025
85. Wiström J. A case of non-O:1 Vibrio cholerae bacteremia from northern Europe.J Infect Dis. 1989;160(4):732. DOI: 10.1093/infdis/160.4.732 PMID: 2794570
86. Wiwatworapan W, Insiripong S. Non-O1/non-O139 Vibrio cholerae septicemia with peritonitis.Southeast Asian J Trop Med Public Health. 2008;39(6):1098-101.PMID: 19062702
87. Yang CC, Lee BJ, Yang SS, Lin YH, Lee YL. A case of non-O1 and non-O139 Vibrio cholerae septicemia with endophthalmitis in a cirrhotic patient.Jpn J Infect Dis. 2008;61(6):475-6.PMID: 19050358
88. Youmbissi JT, Malik TQ, Ajitkumar S, Rafi A, Al Kursany IA, Karkar A. Non-O1 Vibrio choleraesepticemia and peritonitis complicating peritoneal dialysis.Infect Med. 2003;20(3):160-2.
89. Young CC, Chuang YC, Young CD. Non-O:1 Vibrio cholerae bacteremia: report of two cases.Kansenshogaku Zasshi. 1991;65(11):1479-83. DOI: 10.11150/kansenshogakuzasshi1970.65.1479 PMID: 1791351
90. Ko WC, Chuang YC, Huang GC, Hsu SY. Infections due to non-O1 Vibrio cholerae in southern Taiwan: predominance in cirrhotic patients.Clin Infect Dis. 1998;27(4):774-80. DOI: 10.1086/514947 PMID: 9798033
91. Lee YL, Hung PP, Tsai CA, Lin YH, Liu CE, Shi ZY. Clinical characteristics of non-O1/non-O139 Vibrio cholerae isolates and polymerase chain reaction analysis of their virulence factors.J Microbiol Immunol Infect. 2007;40(6):474-80.PMID: 18087626
92. Lin CJ, Chiu CT, Lin DY, Sheen IS, Lien JM. Non-O1 Vibrio cholerae bacteremia in patients with cirrhosis: 5-yr experience from a single medical center.Am J Gastroenterol. 1996;91(2):336-40.PMID: 8607503
93. Trubiano JA, Lee JY, Valcanis M, Gregory J, Sutton BA, Holmes NE. Non-O1, non-O139 Vibrio cholerae bacteraemia in an Australian population.Intern Med J. 2014;44(5):508-11. DOI: 10.1111/imj.12409 PMID: 24816311
94. Tsai YH, Huang TJ, Hsu RW, Weng YJ, Hsu WH, Huang KC, et al. Necrotizing soft-tissue infections and primary sepsis caused by Vibrio vulnificus and Vibrio cholerae non-O1. J Trauma. 2009;66(3):899-905. DOI: 10.1097/TA.0b013e31816a9ed3 PMID: 19276771
95. Yang CJ, Wang CS, Lu PL, Chen TC, Chen YH, Huang MS, et al. Bullous cellulitis in cirrhotic patients--a rare but life-threatening infection caused by non-O1, non-O139 Vibrio cholerae bacteraemia. J Med Microbiol. 2011;60(Pt 6):861-2. DOI: 10.1099/jmm.0.024497-0 PMID: 21292862
96. Sterk A, Schets FM, de Roda Husman AM, de Nijs T, Schijven JF. Effect of Climate Change on the Concentration and Associated Risks of Vibrio Spp. in Dutch Recreational Waters.Risk Anal. 2015;35(9):1717-29. DOI: 10.1111/risa.12365 PMID: 25809307
97. Pfeffer CS, Hite MF, Oliver JD. Ecology of Vibrio vulnificus in estuarine waters of eastern North Carolina.Appl Environ Microbiol. 2003;69(6):3526-31. DOI: 10.1128/AEM.69.6.3526-3531.2003 PMID: 12788759
98. Frank C, Littman M, Alpers K, Hallauer J. Vibrio vulnificus wound infections after contact with the Baltic Sea, Germany. Euro Surveill. 2006;11(8):E060817.1.PMID: 16966781
99. Oishi H, Ura Y, Mitsumizo S, Nakashima M. [A collective review of Vibrio vulnificus infection in Japan]. Kansenshogaku Zasshi. 2006;80(6):680-9. DOI: 10.11150/kansenshogakuzasshi1970.80.680 PMID: 17176855
100. Hor LI, Chang TT, Wang ST. Survival of Vibrio vulnificus in whole blood from patients with chronic liver diseases: association with phagocytosis by neutrophils and serum ferritin levels.J Infect Dis. 1999;179(1):275-8. DOI: 10.1086/314554 PMID: 9841854
101. Ceccarelli D, Chen A, Hasan NA, Rashed SM, Huq A, Colwell RR. Vibrio cholerae non-O1/non-O139 carrying multiple virulence factors and V. cholerae O1 in the Chesapeake Bay, Maryland. Appl Environ Microbiol. 2015.
102. Cinar HN, Kothary M, Datta AR, Tall BD, Sprando R, Bilecen K, et al. Vibrio cholerae hemolysin is required for lethality, developmental delay, and intestinal vacuolation in Caenorhabditis elegans. PLoS One. 2010;5(7):e11558. DOI: 10.1371/journal.pone.0011558 PMID: 20644623

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Vlaams Infectieziektebulletin
Department of Infectious Diseases Control, Flanders
Quarterly, print and online. In Dutch, summaries in English.
http://www.infectieziektebulletin.be

Bulletin d'information de la section d'Epidémiologie
Institut Scientifique de la Santé Publique, Brussels
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http://www.iph.fgov.be/epidemio/epifr/episcoop/episcoop.htm

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Bulletin of the National Centre of Infectious and Parasitic Diseases, Sofia
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http://www.ncipd.org/

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Newsletter of the Network for Surveillance and Control of Communicable Diseases in Cyprus
Medical and Public Health Services, Ministry of Health, Nicosia
Biannual, print and online. In Greek.
http://www.moh.gov.cy

Czech Republic

Zpravy CEM (Bulletin of the Centre of
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Centrum Epidemiologie a Mikrobiologie Státního
Zdravotního Ústavu, Prague
Monthly, print and online. In Czech, titles in English.
http://www.szu.cz/cema/adefaultt.htm

EPIDAT (Notifications of infectious diseases in the Czech Republic)
http://www.szu.cz/cema/epidat/epidat.htm

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EPI-NEWS
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http://www.ssi.dk

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Department of Infectious Disease Epidemiology, National Public Health Institute, Helsinki
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http://www.ktl.fi/portal/suomi/osastot/infe/tutkimus/tartuntatautien_seuranta/tartuntatautilaakarin_kommentit/

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Bulletin épidémiologique hebdomadaire
Institut de veille sanitaire, Saint-Maurice Cedex
Weekly, print and online. In French.
http://www.invs.sante.fr/beh/default.htm

Germany

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Robert Koch-Institut, Berlin
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http://www.rki.de/DE/Content/Infekt/EpidBull/epid__bull__node.html

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Hellenic Centre for Disease Control and Prevention (HCDCP/KEELPNO), Athens
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http://www2.keelpno.gr/blog/?lang=en

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Epinfo (az Országos Epidemiológiai Központ epidemiológiai információs hetilapja)
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Weekly, online. In Hungarian.
http://www.oek.hu/oek.web?to=839&nid=41&pid=7&lang=hun

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Health Protection Surveillance Centre, Dublin
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Notiziario dell'Istituto Superiore di Sanità
Istituto Superiore di Sanità, Reparto di Malattie Infettive, Rome
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Bolletino Epidemiologico Nazionale (BEN)
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Epidemiologijas Bileteni
Sabiedribas veselibas agentura
Public Health Agency, Riga
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http://www.sva.lv/epidemiologija/bileteni

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Center for Communicable Disease Prevention and Control, Vilnius
Online. In Lithuanian.
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http://revista.isciii.es

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http://www.cdscni.org.uk/publications

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Health-EU Portal

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ISSN 1025 496X