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Rapid communications

Most but not all laboratories can detect the recently emerged Neisseria gonorrhoeae porA mutants – results from the QCMD 2013 N. gonorrhoeae external quality assessment programme 2
by D Luijt, C Di Lorenzo, AM van Loon, M Unemo

Research articles

The 2012 dengue outbreak in Madeira: exploring the origins 6
by A Wilder-Smith, M Quam, O Sessions, J Rocklov, J Liu-Helmersson, L Franco, K Khan

Surveillance and outbreak reports

Large multistate outbreak of norovirus gastroenteritis associated with frozen strawberries, Germany, 2012 13
by H Bernard, M Faber, H Wilking , S Haller, M Höhle, A Schielke, T Ducomble, C Siffczyk, SS Merbecks, G Fricke, O Hamouda, K Stark, D Werber, on behalf of the Outbreak Investigation Team

Perspectives

Reducing the barriers against analytical epidemiological studies in investigations of local foodborne disease outbreaks in Germany – a starter kit for local health authorities 22
by D Werber, H Bernard

Letters

Letter to the editor: Diagnosis of a single imported dengue case who had travelled to Japan – how serious is it for travellers? 28
by R Ueno, H Nishiura

Authors reply: Diagnosis of a single imported dengue case who had travelled to Japan – how serious is it for travellers? 31
by NG Schwarz, B Kreuels, K Stark, C Frank, J Schmidt-Chanasit

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Most but not all laboratories can detect the recently emerged *Neisseria gonorrhoeae porA* mutants – results from the QCMD 2013 *N. gonorrhoeae* external quality assessment programme

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We describe the results of the Quality Control for Molecular Diagnostics 2013 *Neisseria gonorrhoeae* external quality assessment programme that included an *N. gonorrhoeae* strain harbouring an *N. meningitidis porA* gene which causes false-negative results in molecular diagnostic assays targeting the gonococcal *porA* pseudogene. Enhanced awareness of the international transmission of such gonococcal strains is needed to avoid false-negative results in both in-house and commercial molecular diagnostic assays used in laboratories worldwide, but particularly in Europe.

In recent years, false-negative test results using PCRs targeting the *Neisseria gonorrhoeae porA* pseudogene have been reported from Australia, Scotland, Sweden and England [1-4]. Further investigations revealed that the gonococcal strains were not clonal, but all had replaced large segments or their entire *N. gonorrhoeae porA* pseudogene with an *N. meningitidis porA* gene.

This report describes the results of the Quality Control for Molecular Diagnostics (QCMD) 2013 *N. gonorrhoeae* External Quality Assessment (EQA) programme. It included an *N. gonorrhoeae* strain containing an *N. meningitidis porA* gene which gives rise to false-negative results in molecular diagnostic assays targeting the gonococcal *porA* pseudogene. QCMD (www.qcmd.org) is an independent international organisation which provides a wide range of molecular EQA services in the field of infectious diseases to over 2,000 participants in over 100 countries.

Quality assessment

The *N. gonorrhoeae porA* mutant strain included in the QCMD 2013 *N. gonorrhoeae* proficiency testing programme (NgDNA13) was isolated in 2011 in Sweden [3]. The strain was cultured on gonococcal agar media and diluted in an *N. gonorrhoeae*-negative urine sample to a concentration of 1.0×10^4 copies/mL. Following lyophilisation, the sample was distributed on 17 June 2013 to participating laboratories as part of the QCMD NgDNA13 proficiency testing panel, along with instructions on how the samples were to be processed. Laboratories tested the panel samples using their routine molecular diagnostic method for the detection of *N. gonorrhoeae*. Test results, together with details of the assays used, were returned to QCMD via a dedicated online data collection system. The deadline for submitting results was 19 July 2013.

Results

The NgDNA13 panel was sent to 286 laboratories in 35 countries, 23 of which are located in the World Health Organization (WHO) European Region. A total of 304 datasets with unambiguous test results (i.e. positive or negative) for the *N. gonorrhoeae porA* mutant were returned (Table 1). Some laboratories used several molecular diagnostic methods and delivered more than one dataset. Participants were also requested to specify the target gene of their assay, and 281 datasets (92%) contained this information (Table 2).

In total, 27 datasets (9%) reported the *N. gonorrhoeae porA* mutant sample as negative, and there was a highly significant association between reporting use of the *porA* pseudogene as target gene and reporting negative results in the *N. gonorrhoeae* molecular diagnostics (chi-square test, $p<0.001$). In total, 29 datasets reported the *porA* pseudogene as their sole assay target. Of these 29 datasets, 18 reported a negative result and 11 reported a positive result. The additional nine datasets that could not detect the *N. gonorrhoeae porA* mutant reported as target the *piv*NG gene (n=2), *porA* and 16S rRNA gene (n=2), *porA* and *opa* genes (n=1), *opa* genes (n=1), 16S rRNA gene (n=1), cryptic plasmid

and amino acetyltransferase gene (n=1), or did not report the gene (n=1) (Table 2).

Results in 268 datasets (88%) were from a commercially available molecular technology. In 36 datasets (12%), an in-house PCR assay had been used. Of the most frequently used commercially available *N. gonorrhoeae* molecular assays, only one manufacturer (Seegene) was reported to use the *N. gonorrhoeae porA* pseudogene as the only target gene. Fourteen datasets (5%) were generated by Seegene assays (Table 2) used in six different countries (data not shown). In addition, 14 of the 36 in-house molecular assays also used the *porA* pseudogene as the sole target gene.

The 27 datasets (9%) reporting the *N. gonorrhoeae porA* mutant sample as negative, had been created with different in-house PCR assays (n=13), Seegene assays (n=6), Sacace assays (n=2), Geneproof assay (n=2), Abbott assay (n=1), Bioneer assay (n=1), BD ProbeTec assay (n=1) and Siemens assay (n=1) (Table 2).

Table 1

Participating laboratories in the QCMD 2013 *Neisseria gonorrhoeae* external quality assessment programme, 2013 (n=286)

Country	Number of participants	Number of datasets returned with positive or negative result[a]
Austria	3	3
Azerbaijan	1	1
Belgium	57	53
Czech Republic	5	5
Denmark	4	3
Estonia	4	6
Finland	1	1
France	18	19
Germany	1	1
Hong Kong	2	3
Hungary	1	2
Iceland	1	1
Indonesia	1	1
Ireland	4	4
Israel	6	5
Italy	5	4
Jamaica	1	1
Kenya	1	1
Luxembourg	2	7
Namibia	1	1
The Netherlands	53	63
Netherlands Antilles	1	1
New Zealand	1	1
Norway	7	5
Portugal	2	2
Slovenia	2	2
South Africa	4	4
South Korea	2	5
Spain	2	1
Sweden	11	11
Switzerland	33	34
Tanzania	1	0
Thailand	2	2
United Kingdom	43	47
United States	3	4
Total	**286**	**304**

[a] Participants may submit more than one dataset, e.g. when they have several *N. gonorrhoeae* assays available.

Discussion

Gonorrhoea remains a major public health problem globally [5], and *N. gonorrhoeae* has developed resistance to all antimicrobials used for treatment of gonorrhoea, which is of grave concern worldwide [6]. In settings with sufficient resources, molecular diagnostic methods have to a large extent replaced conventional culture diagnosis. However, a considerable number of molecular diagnostic assays for *N. gonorrhoeae* (both commercial and in-house) have shown cross-reactivity with other *Neisseria* spp. [7-9]. This suboptimal specificity has led in Europe and Australia to the recommendation that positive tests should be confirmed with another molecular detection assay targeting a different gene sequence [10,11]. The gonococcal *porA* pseudogene is often used as a target sequence in in-house PCRs and in some new commercial assays for confirmatory testing, and in some settings also for primary diagnostic examination.

In the present quality assessment for the detection of *N. gonorrhoeae*, the *opa* genes were the most commonly used individual targets (25%), followed by the DR-9 repeat sequence gene (19%), 16S rRNA (13%) and the *piv*NG gene (11%). Forty-six datasets (15%) were generated by a method targeting the *N. gonorrhoeae porA* pseudogene, either alone (n=29) or in combination with additional genes (n=17). Surprisingly, 11 laboratories using assays reported to target the *N. gonorrhoeae porA* pseudogene alone (Seegene assays and some in-house real-time PCRs), were able to detect the *N. gonorrhoeae porA* mutant sample. These results require further investigation, and it is likely that additional molecular tests and/or targets were used but were not reported. For example, although the *porA* pseudogene was reported as the sole target by all participants, the Seegene AnyplexII STI-7 Detection and Seegene Seeplex STI Master ACE Detection assays are dual-target assays (*porA* pseudogene and one additional gene) according to the manufacturer.

Most molecular diagnostic assays targeted other genetic sequences than the *N. gonorrhoeae porA* pseudogene, and accordingly detected the *porA* mutant sample correctly. However, an enhanced awareness of the potential emergence of gonococcal mutants resulting in false-negative results in the molecular diagnostic tests is essential because of the ongoing evolution

Table 2

Results for the *Neisseria gonorrhoeae porA* mutant sample NgDNA quality assessment programme, 2013 (n=304)

Kit/Assay typea	Assay target[a]	Results on *porA* mutant sample	
		Positive	Negative
Bioneer AccuPower CT and NG Real-Time PCR Kit	*porA* pseudogene	0	1
Seegene Anyplex CT/NG Real time detection	*porA* pseudogene	1	0
Seegene AnyplexII STI-7 Detection	*porA* pseudogene	4	0
Seegene Seeplex STD6 ACE Detection	*porA* pseudogene	0	4
Seegene Seeplex STD6B ACE Detection	*porA* pseudogene	1	1
Seegene Seeplex STI Master ACE Detection	*porA* pseudogene	2	1
Real time in-house PCR	*porA* pseudogene	3	10
Conventional in-house PCR	*porA* pseudogene	0	1
Geneproof Neisseria gonorrhoeae PCR kit	*porA* pseudogene and 16S rRNA gene	0	2
Diagenode C. trachomatis and N. gonorrhoeae Real-Time PCR	*porA* pseudogene and *opa* genes	3	0
Diagenode Neisseria gonorrhoeae Real-Time PCR	*porA* pseudogene and *opa* genes	11	0
Real time in-house PCR	*porA* pseudogene and *opa* genes	0	1
Abbott RealTime CT/NG	*opa* genes	58	1
Goffin Presto CT-NG assay	*opa* genes	3	0
TestLine Clinical Diagnostics Real-time Neisseria gonorrhoeae	*opa* genes	1	0
Real time in-house PCR	*opa* genes	13	0
Roche COBAS 4800 System CT/NG assay	DR-9 repeat sequence	59	0
Roche COBAS Amplicor CT/NG	M-Ngo PII	3	0
Gen-Probe APTIMA Combo 2 Assay	16S rRNA gene	33	0
Gen-Probe APTIMA GC Assay	16S rRNA gene	4	0
Institute of Applied Biotechnologies as Neisseria gonorrhoeae RG detect	16S rRNA gene	1	0
Real time in-house PCR	16S rRNA gene	1	1
BD Probetec ET CT/GC Amplified DNA Assay	pivNG gene	8	0
BD Probetec ET CT/GC/AC Amplified DNA Assay	pivNG gene	13	0
BD ProbeTec GC Qx Amplified DNA Assay	pivNG gene	6	1
Bio-Rad Dx CT/NG/MG assay	pivNG gene	1	0
Siemens VERSANT CT/GC DNA 1.0 Assay (kPCR)	pivNG gene	3	1
Fast-track Diagnostics FTD Gonorrhoea confirmation	*opa* genes and pivNG gene	1	0
Fast-track Diagnostics FTD STD9	*opa* genes and pivNG gene	1	0
Fast-track Diagnostics FTD Urethritis	*opa* genes and pivNG gene	1	0
Fast-track Diagnostics FTD Urethritis basic	*opa* genes and pivNG gene	2	0
Fast-track Diagnostics FTD Urethritis plus	*opa* genes and pivNG gene	1	0
Fast-track Diagnostics FTD Vaginal swab	*opa* genes and pivNG gene	1	0
TIB MOLBIOL NG TaqMan 48	*opaD* gene	1	0
TIB MOLBIOL NG TaqMan 48	90 bp fragment	1	0
TIB MOLBIOL LightMix Kit 480HT CT and NG	*opaD* gene	3	0
TIB MOLBIOL LightMix Kit 480HT CT and NG	90 bp fragment	2	0
TIB MOLBIOL LightMix Kit 480HT CT and NG	gyrA gene	4	0
TIB MOLBIOL LightMix Kit 480HT CT and NG	nvt gene	1	0
Sacace Neisseria gonorrhoeae Real-TM	CCp + Methyltransferase gene	1	0
Sacace Neisseria gonorrhoeae 370/660 IC	Cryptic plasmid and amino acyltransferase gene	0	1
EuroClone Duplica Real Time Neisseria gonorrhoeae	Cryptic plasmid pJD1 and 16S rRNA gene	1	0
Conventional in-house PCR	cppB gene and 16S rRNA gene	1	0
Real-time in-house PCR	cppB gene	1	0
Cepheid GeneXpert CT/NG assay	not reported	16	0
Ecoli s.r.o. Amplisens Neisseria gonorrhoeae-test	not reported	1	0
Sacace C. trachomatis/N. gonorrhoeae/M. genitalium Real-TM	not reported	1	0
Sacace N. gonorrhoeae/C. trachomatis/T. vaginalis/ M. genitalium Real-TM	not reported	0	1
Real-time in-house PCR	not reported	3	0
Conventional in-house PCR	not reported	1	0
Total		**277**	**27**

[a] As reported by 2013 external quality assessment participants

and genetic diversity of *N. gonorrhoeae* and other bacteria. For example, the new variant of *Chlamydia trachomatis* reported from Sweden had a 377 bp deletion in the cryptic plasmid, which contained the target sequences for the *C. trachomatis* molecular assays from two main manufacturers and resulted in many thousands of false-negative tests in Sweden [12-14]. With the replacement of conventional culture methods, laboratories need to be aware of the risk of emergence of these mutant strains that cannot be detected by molecular assays.

In conclusion, enhanced awareness of the international transmission of *N. gonorrhoeae porA* mutant strains is needed to avoid false-negative results in several molecular diagnostic assays, both in-house and commercial. The opportunities to use combinations of different diagnostic methods such as several molecular methods, molecular methods and culture, and multi-target methods, remain exceedingly valuable.

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Conflict of interest

None declared.

Authors' contributions

DL wrote the initial draft. All authors were involved in the analysis of the results and preparations of the final draft of the paper and tables.

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The 2012 dengue outbreak in Madeira: exploring the origins

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In 2012, Madeira reported its first major outbreak of dengue. To identify the origin of the imported dengue virus, we investigated the interconnectivity via air travel between dengue-endemic countries and Madeira, and compared available sequences against GenBank. There were 22,948 air travellers to Madeira in 2012, originating from twenty-nine dengue-endemic countries; 89.6% of these international travellers originated from Venezuela and Brazil. We developed an importation index that takes into account both travel volume and the extent of dengue incidence in the country of origin. Venezuela and Brazil had by far the highest importation indices compared with all other dengue-endemic countries. The importation index for Venezuela was twice as high as that for Brazil. When taking into account seasonality in the months preceding the onset of the Madeira outbreak, this index was even seven times higher for Venezuela than for Brazil during this time. Dengue sequencing shows that the virus responsible for the Madeira outbreak was most closely related to viruses circulating in Venezuela, Brazil and Columbia. Applying the importation index, Venezuela was identified as the most likely origin of importation of dengue virus via travellers to Madeira. We propose that the importation index is a new additional tool that can help to identify and anticipate the most probable country of origin for importation of dengue into currently non-endemic countries.

Introduction

Dengue is an important arboviral disease, endemic mainly in the tropics and sub-tropics. Over 50% of the world's population lives in areas where there is a risk of contracting the disease [1]. Dengue is also the most rapidly spreading mosquito-borne viral disease in the world. In the past 50 years, the incidence of dengue has increased 30-fold with geographic expansion to new countries [2]. An estimated 50 million to 100 million dengue infections occur annually according to the World Health Organization (WHO) [2]. Using consensus and modelling approaches, recent estimates of the burden of dengue are as high as 390 million infections per year [3].

The reasons for the resurgence and geographic expansion of dengue are complex, and include factors such as climate change, virus evolution, deteriorating vector control, and societal changes [4]. Population growth associated with rapid uncontrolled urbanisation is likely to be the main factor that has driven the rapid amplification of dengue in many endemic countries in recent decades [5]. One of the primary factors behind the geographic spread to non-endemic areas is the introduction of infected *Aedes* mosquitoes by shipping [6] and importation of dengue virus via viraemic travellers using through air travel [1,7].

Imported dengue via travellers to currently non-endemic countries has increased steadily in recent decades, as reported by GeoSentinel, a worldwide network of travel medicine providers [8,9]. Dengue is the top cause of febrile illnesses in international travellers returning from south-east Asia [10]. Australia has seen a dramatic rise in the number of dengue cases caused by returning travellers, particularly from south-east Asia, with an increase of approximately 350% between 2004–2007 and 2008–2011 [11,12]. In Europe, various countries have also reported increased numbers of dengue in returning travellers to Europe [13-16].

While imported dengue cases to the United States have resulted in small but contained dengue clusters for many years [17-21], no autochthonous cases were reported in Europe until 2010, when two cases were reported in southern France, followed later that year by cases in Croatia [22,23]. In 2012 came the report of the first major outbreak of dengue in Europe [24] since the 1926–1928 outbreak in Greece [25]. This outbreak occurred in the autonomous region of Madeira, Portugal. Madeira is an archipelago in the Atlantic on the same latitude as the north coast of Africa [26]. The main vector for dengue, *Aedes aegypti*, is reported to

have been introduced to Madeira in 2005 [27] and has been able to establish itself thanks to Madeira's sub-tropical climate.

The outbreak of dengue in Madeira evolved rapidly from its onset on 3 October 2012 and had resulted in over 2,100 cases by March 2013, with 78 cases introduced into 13 other European countries via travellers departing Madeira [24], and was due to dengue virus serotype 1 (DENV-1) [27].

Information on air traffic can be used to predict the risks of vector-borne disease importation [28]. A project known as BioDiaspora was developed to evaluate the probable pathways of international dissemination of infectious diseases via the global airline transportation network [29]. BioDiaspora assesses the ways in which countries around the globe are connected through international travel and consequently how they share risks associated with infectious diseases [30].

In this study, we attempted to determine the most likely country of origin of the dengue virus responsible for the 2012 Madeira epidemic. If we assume that dengue was introduced through viraemic travellers, the risk of importation of dengue is presumably a function of dengue virus activity in the country of origin and the volume of travellers from there to Madeira. To identify the origin of the imported dengue virus, we investigated the interconnectivity via air travel between dengue-endemic countries and Madeira. Properly-annotated sequence data for dengue can help to track the spread of dengue, and, in particular, help to identify the probable origin of virus importation. We therefore also compared the available sequence information from the Madeira epidemic to other sequences available in GenBank.

Methods

To describe global air travel patterns to Madeira from dengue-endemic countries, we analysed worldwide full-route flight itinerary data, taking into consideration all traveller flight connections, from the International Air Transport Association (IATA) between 1 January and 31 December 2012, as collated by BioDiaspora. We identified dengue-endemic countries from the global map created by Brady et al. and selected countries where dengue endemicity has been well documented (complete, good and moderate consensus evidence) [31]. Countries such as the US, Argentina, Australia and China, where dengue activity is limited to very small areas within the country, were excluded. We examined the cities and countries where individuals initiated travel and had a final destination in Madeira. This included direct and indirect flights to Madeira. We quantified the total number of travellers arriving from individual dengue-endemic countries in Madeira in 2012 and created a map depicting the volume of travellers from individual cities within those countries. We also quantified the volume of arriving travellers in monthly intervals for the countries with the highest travel volume to Madeira.

We obtained the 2012 annual incidence rate (IR) for dengue per 100,000 population from WHO regional websites or as specified in the Table [32-36].

The risk of importation of dengue depends on (i) dengue activity in the country of origin and (ii) the volume of travellers from the country of origin to Madeira.

Table

Annual number of air travellers to Madeira, annual incidence rate of dengue infection in the country of embarkation and annual importation index into Madeira in 2012

Country of embarkation	Traveller volume (TV)[a]	Annual incidence rate (IR)[b]	Annual importation index (ID)[c]
Venezuela	15,884	174.86	27.77
Brazil	4,676	295.33	13.81
Thailand[d]	214	116.35	0.25
Aruba	11	653.92	0.07
Philippines[d]	19	172.15	0.03
Costa Rica	20	149.11	0.03
Colombia	4	487.46	0.02
Panama	9	215.18	0.02
Singapore[d]	3	585.39	0.017
Mexico	4	345.6	0.013
Ecuador	37	37.36	0.01
Paraguay	17	86.04	0.01
Puerto Rico	6	123.39	0.007
Sri Lanka[d]	2	206.95	0.004
Vietnam[d]	4	84.81	0.003
Guatemala	3	81.69	0.002
India[d]	9	4.17	0.0004
Bahamas	4	1.46	0.00006
Angola	1,281	NA	NA
Cape Verde	352	NA	NA
Senegal	122	NA	NA
Mozambique	111	NA	NA
Reunion	53	NA	NA
Nigeria	33	NA	NA
Guinea-Bissau	26	NA	NA
Cameroon	15	NA	NA
Mauritius	13	NA	NA
Ghana	12	NA	NA
Madagascar	4	NA	NA

NA: not available.

[a] Annual number of travellers to Madeira.

[b] Annual incidence per 100,000 inhabitants using World Health Organization (WHO) and Regional Office websites [32–35]. WHO Regional Office for Africa dengue incidence by country was not available at the regional level.

[c] Annual importation index units in traveller incident cases per year. The formula used is ID = IR × TV.

[d] Where only incidence was available, 2012 population was taken from [36].

Based on these two parameters, we calculated country-specific risk indices referred to as importation indices. A higher importation index indicates a higher product of dengue activity and air travel volume to Madeira from a particular dengue-endemic country.

We used the following formula for the importation index for a given country:

ID = IR × TV

where ID is the importation index, IR is the incidence rate of dengue in the country of origin and TV is traveller volume (the total number of annual air travellers from a given country to Madeira).

We then selected the two countries with the highest importation index to assess seasonal trends in travel. To observe the seasonality of this importation index, we obtained the monthly and weekly incidences of reported dengue cases for 2012 from online governmental surveillance portals for these two countries [37,38]. Using 2012 traveller volume by month into Madeira, we generated and compared the importation index scaled by month to observe the likelihood that one or both countries may have been the source of the dengue-viraemic traveller(s) during the months that preceded the onset of the outbreak.

Multiple sequence alignment of the Madeira dengue virus serotype 1 sequence (GenBank: KC248375.1) to other DENV-1 sequences deposited in GenBank was carried out using a fast Fourier transform in MAFFT, a multiple sequence alignment programme [39]. The maximum-likelihood phylogenetic tree was inferred from the sequence alignment using RAxML [40]. The robustness of the maximum-likelihood tree was assessed by 1000 maximum-likelihood bootstrap replications. The maximum-likelihood tree was visualised and produced using FigTree v1.4.0 [41].

Results

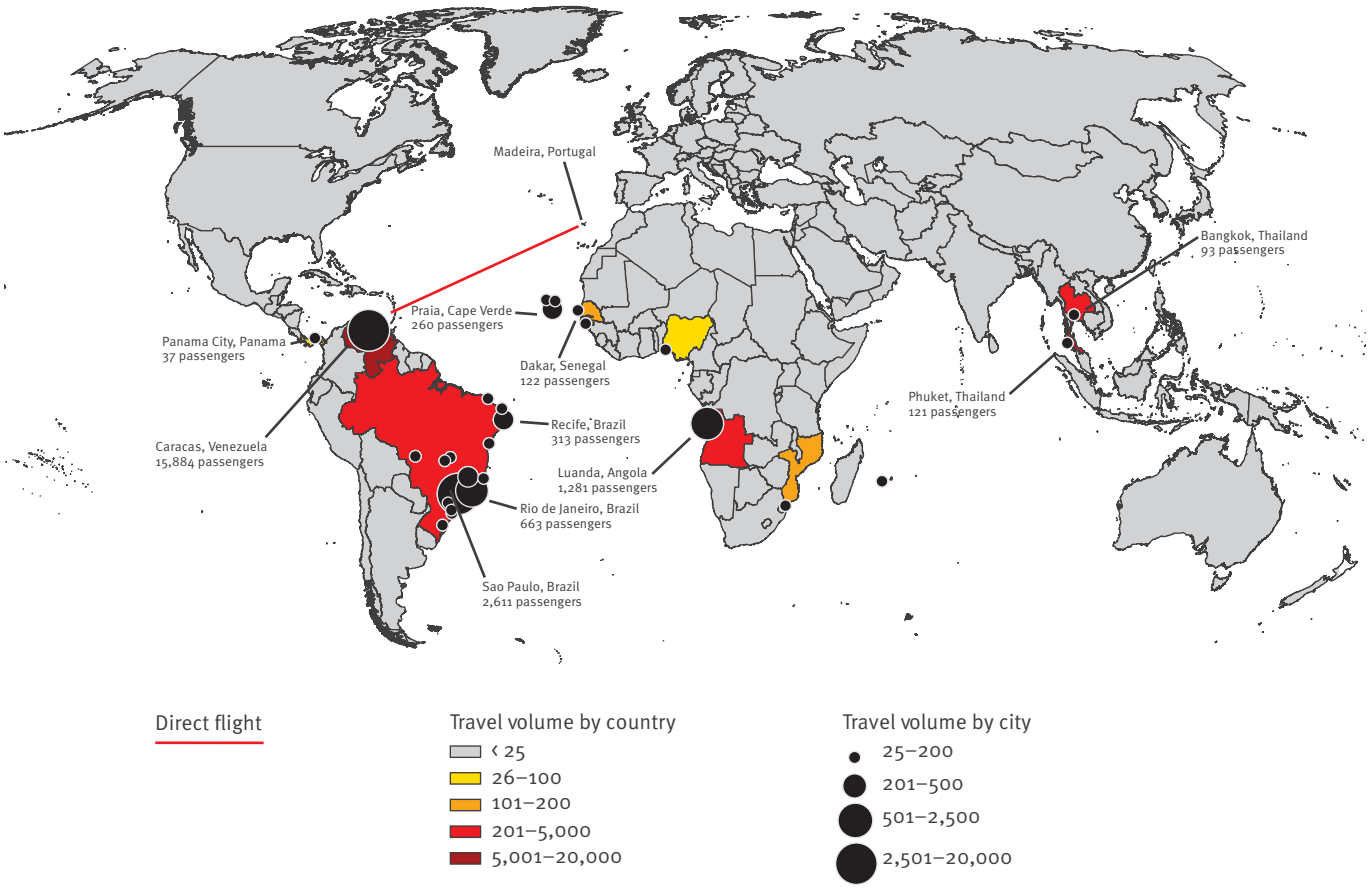
Connectivity to Madeira via air travel

There were 22,948 air travellers to Madeira in 2012, originating from twenty-nine dengue-endemic countries; 89.6% of these international travellers originated from Venezuela and Brazil (Table). The number of air travellers from Venezuela to Madeira was 15,884, almost four times higher than the 4,676 travellers from Brazil. Most of the air travellers from Venezuela boarded in Caracas, whereas in Brazil most travellers boarded in Sao Paulo and Rio de Janeiro.

Figure 1 shows a map of the world with air traffic intensity between dengue-endemic countries and Madeira

Figure 1

Map of air travel volume by country and city from dengue-endemic countries to Madeira, 2012



in 2012. Only Venezuela has direct flights to Madeira, originating in Caracas.

Using the formula above, we calculated the annual importation index for 2012 to be 27.77 ((174.86/100,000) × 15,884) and 13.81 ((295.33/100,000) × 4,676) for Venezuela and Brazil respectively. The ratio of the IDs suggests a dengue importation risk from Venezuela that was 2.01 times higher than from Brazil. We calculated 2012 IDs for all other countries as summarised in the Table.

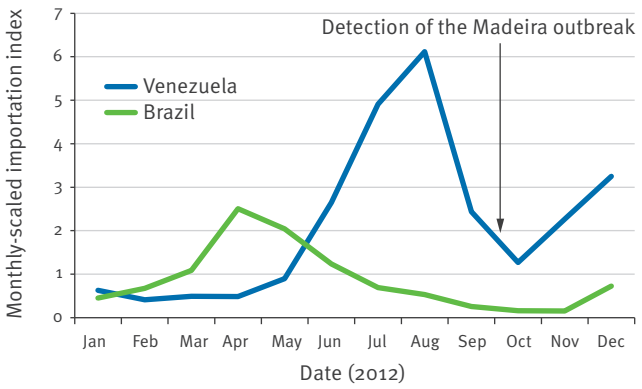
Figure 2 shows the importation indices generated by month for Venezuela and Brazil, using reported cases in 2012 as well as 2012 IATA travel volume by month. Both Brazil and Venezuela have a seasonal peak of interconnectivity with Madeira during the month of July, but have distinctly different seasonal peaks of dengue activity. The monthly-scaled importation index for Brazil, which combined both factors, reaches its annual peak of 3.29 in April, whereas Venezuela reaches a much higher peak, 6.58, in August. Using the monthly index for the months of July, August and September, the index reflects an importation index averaging up to seven times greater for Venezuela than for Brazil.

Dengue sequencing

The 494 base pair sequence used for this analysis indicates that the Madeira virus is most closely related to a DENV-1 strain within Genotype V from South America (Figure 3), particularly those previously sequenced from Colombia (GQ868570; 452/454 identities), Venezuela (JN819415; 450/454 identities) and Brazil (JN713897; 450/454 identities).

Figure 2

Monthly importation index for dengue virus by travellers to Madeira from Venezuela and Brazil, January–December 2012



The dengue outbreak in Madeira was detected in October and first cases were determined to have happened end September [27].

Y axis shows importation index units in traveller incident cases per month. Based on 2012 International Air Transport Association (IATA) travel volume [29], and monthly country incidence obtained from national surveillance systems reports for Venezuela and Brazil [37,38].

Discussion

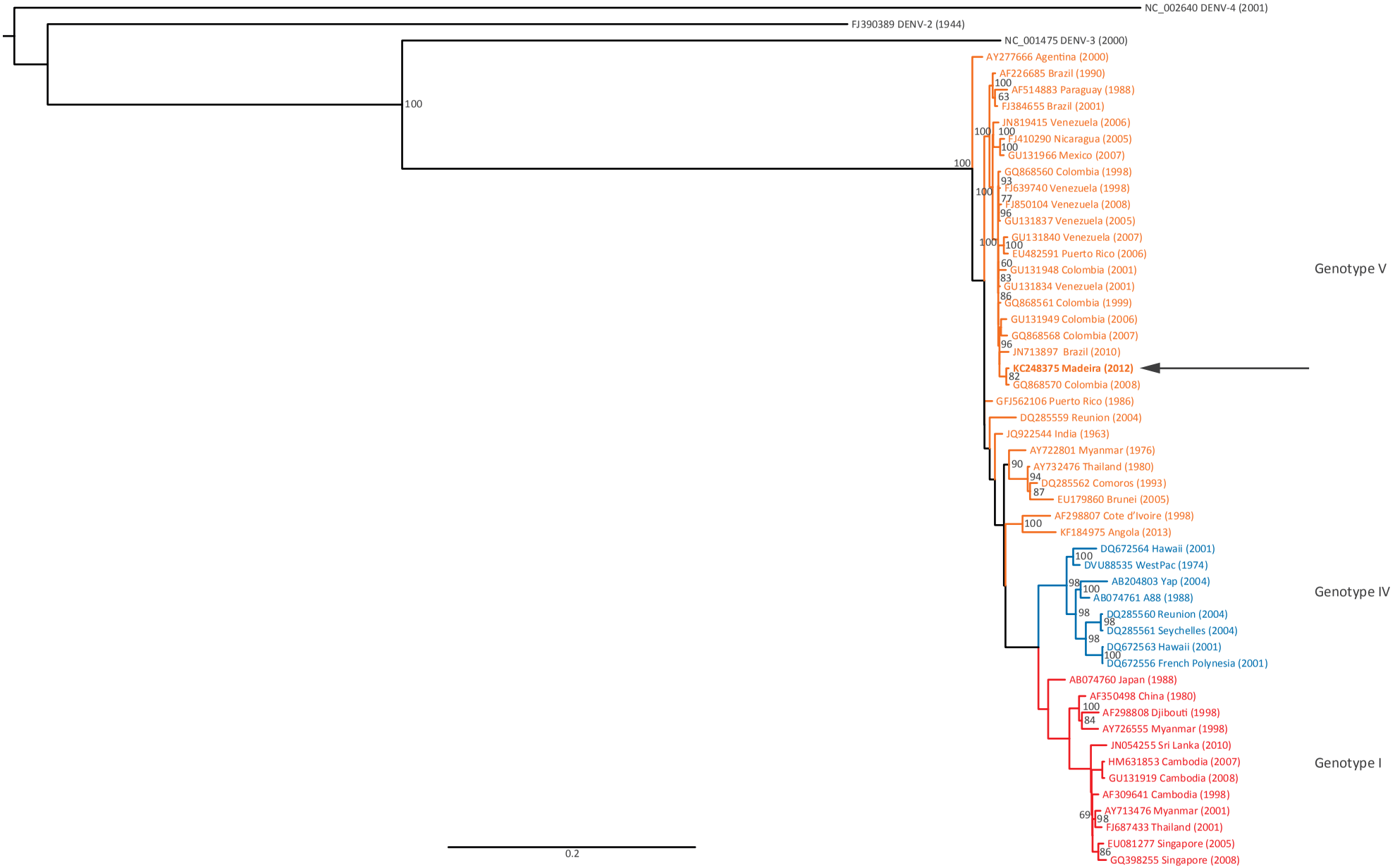
We attempted to explore the origin of importation of dengue into Madeira that resulted in the massive outbreak in 2012, taking into account air travel patterns and dengue sequencing.

In principle, importation of dengue can occur via infected mosquitoes or via viraemic travellers. Importation of *Aedes* mosquitoes via the shipping and cargo industry is well established [42], and this was the most likely route of the introduction of *Aedes* mosquitoes to Madeira in 2005. *Aedes* populations remained established in Madeira due to the favourable climate, and so the conditions exist for a dengue outbreak to occur if the virus is imported [43,44]. It is also possible that dengue virus in infected mosquitoes may survive the long journey due to transovarial transmission. However, data on mosquito populations on cargo and passenger ships are difficult to obtain, as are data on the extent of connectivity to Madeira. Furthermore, the probability of importation of dengue via viraemic travellers is thought to be much higher than through dengue virus importation in mosquitoes [1,6,45,46]. The duration of viraemia is 5 to 7 days [47], and too short to last the journey on cruise ships from most well-documented endemic countries in Asia or the Americas to Madeira. Therefore, we conclude, modern rapid transportation via aeroplane is the most likely source of importation of dengue virus between countries.

Although south-east Asia carries the main burden of dengue worldwide [47], Asia is unlikely to be the origin of the importation of dengue to Madeira, because of much lower interconnectivity to this island. Indeed, even high-risk countries such as Thailand had a very low importation index. Based on the importation index, our findings show that Venezuela is by far the most likely origin of the Madeira outbreak. The importation index was almost two times higher than for Brazil, the country with the second highest interconnectivity with Madeira. Although the extent of dengue activity in Brazil is higher than that of Venezuela, the risk of introducing dengue from Brazil to Madeira is lower because of the lower overall interconnectivity via air travel between Brazil and Madeira. As the outbreak in Madeira was first reported in October 2012 [27], taking the extrinsic and intrinsic incubation time of the *Aedes* mosquitoes into account, the most likely time of importation into Madeira would have been between July to September 2012. Using the monthly index for July, August and September 2012, the importation index for Venezuela is seven times higher during this period than that for Brazil. This is because at this time of the year Venezuela has higher dengue activity than Brazil; dengue is seasonal in most dengue-endemic countries. Our findings, based on the importation index, suggest that Venezuela is the most likely source of importation of dengue to Madeira in late summer 2012, just before the outbreak's first reported case.

Figure 3

Phylogenetic tree of the dengue virus serotype 1 (DENV-1) from the 2012 Madeira outbreak



DENV-1 nucleotide sequence from the Madeira outbreak (KC248375; bold, indicated by the arrow) was aligned with representative DENV-1 sequences from around the world representing multiple genotypes. Orange-coloured isolates are from genotype V, blue-coloured isolates are from genotype IV, and red-coloured isolates are from genotype I.

Annotating existing dengue sequences with GenBank further strengthens this conclusion. The 494 base pair sequence used for this analysis is highly related to South American dengue isolates from Colombia, Brazil and Venezuela, all neighbouring countries in South America. Analysis of NS5 and CprM partial sequences derived from one of the first cases by a Portuguese group and their preliminary phylogenetic analysis based on CprM nucleotide sequences also identified the virus to be related to viruses circulating in Latin America, more specifically from Venezuela and Colombia and the Roraima region in northern Brazil [27]. This finding is similar to that reached by sequencing done by Huhtamo's group based on a Finnish traveller from Madeira [48]. It should be noted that a different region of the DENV-1 genome was used for this analysis than was used by Huhtamo (C-prM instead of E).

This study has the following limitations. The first is that the importation index depends on the dengue activity in the country of origin; however underreporting and high variation between the notification rates of countries to WHO is likely, hence the true probability of importation is likely to be higher than reported in this analysis. Recent estimates of dengue incidence are several-fold higher than those reported by WHO [3]. The second limitation is that incidence data from Africa are lacking, and so we were not able to calculate reliable importation indices for most dengue-endemic countries from Africa. Furthermore, we do not know about undocumented population movements, for example via illegal migration. Interconnectivity between Africa and Madeira by air travel is, however, not high. The strongest interconnectivity via air from an African country to Madeira was Angola. Angola saw a major dengue outbreak due to dengue virus serotype 1 in 2013, shortly after the 2012 Madeira outbreak. Sequencing from the 2013 Angola outbreak performed by our group showed that this outbreak was not closely related to the Madeira outbreak, and was more closely related to a long-term circulating strain from west Africa [49]. The third limitation is that we focused on introduction of dengue virus via air travel and did not account for cargo or cruise ships. However, due to the short incubation time, introduction via air travel is far more likely, and hence we feel justified in our approach. The fourth limitation is that we only used a simple formula. Seyler et al. have previously developed a more complex Monte Carlo model based on the number of viraemic person-days among air travellers arriving in the European Union (EU), taking into account the probability distributions based on quarterly incidences in endemic countries, passenger flow from endemic to EU countries, duration of viraemia, probability of being viraemic upon arrival, and distribution and period of vector activity in the EU [50]. Our primary objective was to explore the most likely origin of importation, not to quantify the risk of establishment of dengue or the extent of the resulting outbreak, and so a simple importation index was deemed sufficient. Because of these limitations, our importation index is only a crude estimate. The importation index is therefore best used to relatively rank the most probable country of origin rather than to quantify the probability and size of dengue outbreaks after the introduction.

In conclusion, dengue sequencing of the Madeira dengue virus points to a Colombian, Venezuelan or Brazilian origin for the Madeira outbreak. The importation index suggests that Venezuela is the most likely country of origin for the dengue outbreak in Madeira in 2012. We propose that the importation index is a simple new additional tool that may aid in identifying and anticipating the most probable country of origin for importation of dengue into currently non-endemic countries.

Authors' contributions

AWS and JR had the study idea; AWS coordinated the analyses and wrote the manuscript. JR, AWS and KK developed the index; MQ and JL-H analysed the data on seasonality; MQ and KK obtained the data for the calculations of the importation index; MQ created the Table and Figure 2; OS created Figure 3; OS and LF analysed existing GenBanks; KK obtained and analysed all the air travel data. All co-authors contributed to the final manuscript.

Conflict of interest

None declared.

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Large multistate outbreak of norovirus gastroenteritis associated with frozen strawberries, Germany, 2012

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From 20 September through 5 October 2012, the largest recorded food-borne outbreak in Germany occurred. Norovirus was identified as the causative agent. We conducted four analytical epidemiological studies, two case–control studies and two surveys (in total 150 cases) in secondary schools in three different federal states. Overall, 390 institutions in five federal states reported nearly 11,000 cases of gastroenteritis. They were predominantly schools and childcare facilities and were supplied almost exclusively by one large catering company. The analytical epidemiological studies consistently identified dishes containing strawberries as the most likely vehicle, with estimated odds ratios ranging from 2.6 to 45.4. The dishes had been prepared in different regional kitchens of the catering company and were served in the schools two days before the peaks of the respective outbreaks. All affected institutions had received strawberries of one lot, imported frozen from China. The outbreak vehicle was identified within a week, which led to a timely recall and prevented more than half of the lot from reaching the consumer. This outbreak exemplifies the risk of large outbreaks in the era of global food trade. It underlines the importance of timely surveillance and epidemiological outbreak investigations for food safety.

Introduction

Infection with norovirus is the most common cause of acute infectious gastroenteritis in European countries [1,2], usually manifesting with self-limiting symptoms of vomiting and diarrhoea, with sudden onset and short duration [3]. Large protracted outbreaks of norovirus gastroenteritis are often recognised in institutions such as hospitals and homes for the elderly [4], with person-to-person transmission predominating. Food-borne norovirus outbreaks are common, but still under-recognised [5,6].

In Germany, outbreaks of acute infectious gastroenteritis are notifiable to the local public health departments according to the Protection Against Infection Act of 2001. The health departments conduct epidemiological investigations and take control measures. They also transmit outbreak information electronically to the public health authority of the respective federal state and, subsequently, to the Robert Koch Institute (RKI) on the national level [7]. On request of the state health authorities, the RKI assists in outbreak investigations, including analytical epidemiological studies.

On mid-day of 27 September 2012, the public health authority of the federal state of Brandenburg informed the RKI about several outbreaks of gastroenteritis in schools and childcare facilities in Brandenburg amassing to at least 500 cases. Diarrhoea or vomiting in affected individuals had started on the evening before. All affected institutions offered lunch provided by Caterer X, a company operating across Germany, and a food-borne outbreak was suspected. According to the public health department of one of the affected counties, Caterer X was already aware of gastroenteritis cases in four other German federal states (Berlin, Saxony-Anhalt, Saxony and Thuringia). The RKI informed the two national food safety authorities, the Federal Institute for Risk Assessment (BfR) and the Federal Office of Consumer Protection and Food Safety (BVL), and the public health authorities of the other 15 federal states about the situation and requested information on similar outbreaks. By the evening of the same day, the RKI had knowledge of more than 4,000 cases relating to outbreaks in schools and childcare

facilities supplied by Caterer X in four neighbouring federal states in the east of Germany.

This report focusses on the epidemiological investigations to identify the outbreak vehicle and to prevent further cases. Details of the laboratory investigations are presented elsewhere [8].

Methods

Descriptive analysis

In daily teleconferences, the public health authorities of the affected federal states and the RKI exchanged information on the number of affected institutions (including aggregated case numbers) and on laboratory results from human samples taken in the context of the outbreak. For the descriptive analysis, we defined a case as a person with diarrhoea or vomiting from 19 September through 7 October 2012, who did not test positive for any pathogen other than norovirus and who attended an affected institution. An institution was considered to be affected if it offered meals by any external caterer and if at least 10 cases had occurred in that institution (or, in small institutions, if 10% of persons were cases). We did not restrict affected institutions to those supplied by Caterer X to remain sensitive to the potential involvement of other caterers in this outbreak.

Analytical studies

On the individual level we conducted two case–control studies (CCS) and two surveys in affected secondary schools in three federal states. For the CCS, we interviewed pupils directly at their schools, for the surveys, a web-based (Survey 1) and an email (Survey 2) questionnaire were used. Exposure histories were recorded for menu items offered in these schools for lunch (as listed on weekly menu plans) and other food items available in the school, e.g. in the cafeteria. The canteens of all four schools had been supplied by different regional kitchens of Caterer X.

The causative agent was unknown at the start of all four studies, but was suspected to be norovirus or bacterial toxins, based on reported symptoms and the sudden and almost simultaneous occurrence of disease within the institutions. Thus, the relevant period of exposure was considered to be the three days before the start of the outbreaks in these institutions (the dates were not identical at the four study sites). In all four studies, we investigated whether eating at the school canteen was associated with illness. We restricted the calculation of food-specific associations to individuals who reported having had lunch at the school canteen on any of the days of the exposure period. Pupils who reported gastroenteric illness in the family in the week before the outbreak period were excluded from the analysis because they could have been secondary cases of illness in their household. We compared cases and controls regarding their food exposures, calculated odds ratios (ORs) and 95% confidence intervals (CI), and assessed statistical significance using Fisher's exact or other appropriate tests. If several food items were associated with disease in univariable analyses with an OR>1, a p value <0.2 and an exposure reported by at least 25% of cases, multivariable logistic regression analysis (exact method for the surveys) was performed with a manual forward selection of variables (cut-off: p<0.2). Statistical analyses were conducted in R [9] for the CCS and Stata [10] for the surveys.

Case–control study 1

CCS1 was conducted on 1 and 2 October in School A in a city in Saxony. The school had experienced a sudden surge of gastroenteritis cases during calendar week 39 (24–30 September) with a peak on Wednesday, 26 September, and had been closed on 28 September due to the outbreak.

We restricted the study to pupils from grades 5 to 7 (10–13 year-olds) because these age groups were predominantly affected in this school. We defined a case as a pupil with onset of vomiting or diarrhoea from 24 to 30 September (outbreak period for CCS1). Of the approximately 70 cases, we selected two thirds for the study using systematic random sampling. Eligible controls were all pupils from three school classes who did not report vomiting or diarrhoea during the outbreak period. The classes were arbitrarily chosen by the deputy head of the school. Assuming an exposure prevalence of 70% among cases, a case-to-control ratio of 1 would have allowed us to detect an OR of 4 with a power of 86% at a significance level of 5%.

We collected information on the participants' age and sex, symptoms, date of symptom onset and on food exposures at the school's canteen during calendar week 39, as indicated by the canteen's menu plan, which listed four meal choices daily. For data entry and immediate univariable analysis on site, we used the Linelist tool, a spreadsheet file developed at the RKI, to assist in the epidemiologic investigation of local outbreaks [11].

On 2 October, we conducted a sub-study restricting the study population to those who had reported eating at the school canteen on Monday 24 September. Cases with symptom onset after 28 September were excluded from the analysis. In this sub-study, participants were explicitly asked for the consumption of strawberry compote because it had not only been part of one main meal but also been offered as a dessert with two of the other three meal choices. The information was collected in an aggregate fashion during a congregation of cases and controls in the school auditorium, by sending each pupil to one of four corners depending on the pupils' outcome and exposure status (a live 2x2 table). The children were first split into two groups based on presence of symptoms, and these groups were divided further based on their recollection of having eaten strawberries. Pupils were explicitly told not

to walk with their friends, but according to their recollection of symptoms and food consumption.

Case–control study 2

CCS2 was conducted on 4 October in School B in the state of Thuringia. The school had not been closed in response to the outbreak. The aim of the study was to investigate whether the results of CCS1 were reproducible in a different geographical region. The methodology was identical to CCS1 with the following exceptions: Pupils from grade 8 (14 years-old) were additionally included, the outbreak period was from 24 through 27 September, and eligible controls came from five arbitrarily chosen classes. Because strawberry compote was offered as dessert with several meal choices, even on the same day, we asked for this food item in an additional question.

Survey 1 (web-based questionnaire)

This study was conducted at School C located in Saxony using an online questionnaire. The school had experienced a sudden surge of gastroenteritis cases during the last two weeks of September and, in response to the outbreak, had been closed on Friday 28 September. Lunches consisted of a main component (e.g. chicken wings), pre-ordered by the pupils and dispensed by canteen staff, and side dishes, salads and desserts for a self-service buffet cart.

Study participants were recruited through a letter, distributed by teachers and addressed to the parents of all pupils of grades 5 to 8 (n=451) present at the school on 5 October. It informed about the aims of the study and invited participation in an online survey. The questionnaire was accessible (password-protected) from 5 October through 8 October. It contained questions on demography (age, sex, grade), potential disease history (symptoms, time course) and food exposure history from 20 through 27 September (choice of three main components and around 10 sides and desserts daily). We defined a case as a pupil with onset of vomiting or diarrhoea from 20 through 29 September. Cases with an onset date 20–23, 24–26 and 27–29 September were defined as first-, second- and third-wave cases, respectively.

Survey 2 (email questionnaire)

This study was conducted at School D in Berlin. On 5 October, 38 cases of gastroenteritis had been notified to the local health department by the head of the school. Because school holidays had just begun at the start of the study, face-to-face interviews were not feasible. We therefore developed a questionnaire covering, in addition to demographic and symptom information, meals served between 24 and 28 September (choice of four dishes daily plus salad buffet). This questionnaire was emailed to the parents of all pupils under the age of 18 years (approximately n=900). Questionnaires could be returned to the RKI via electronic or regular mail between 1 and 5 October. Cases were defined as pupils with onset of diarrhoea and/or vomiting from 24 through 28 September.

Food trace-back investigations

The German Task Force on Food and Feed safety, consisting of food safety authorities of affected states and at the national level, convened on 29 September. The task force coordinated food safety investigations, which also included epidemiological product tracing investigations.

Results

Descriptive analysis

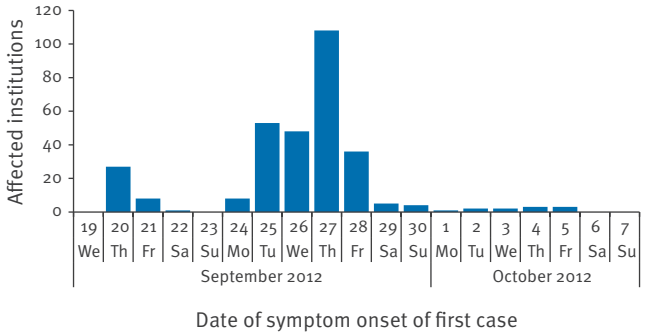
A total of 390 institutions in five federal states in East Germany were reported as affected during the outbreak period. The earliest outbreak in an institution started on 20 September, the latest on 5 October, and most started between 25 and 28 September with a peak on 27 September (n=108 institutions, 28%) (Figure 1). A median of 21 children were affected per institution (inter-quartile range (IQR): 12–37).

The majority of affected institutions were schools (244/390, 63%) and childcare facilities (140/390, 36%), three were facilities for disability care, two were homes for the elderly and one was a rehabilitation clinic.

A total of 10,950 persons, mostly children and teenagers but also staff members, were reported ill in the affected institutions. The median proportion of cases among regular attendees was 14% (IQR: 10–22) across all affected institutions, and 18% (IQR: 12–27) in childcare facilities. At least 38 (0.3%) people required hospitalisation; the majority of illnesses were of short duration and self-limiting. Figure 2 shows the incidence of illnesses among persons under the age of 18 years by district. The federal states of Saxony, Brandenburg and Berlin were predominantly affected, which also reflects the distribution of affected institutions (n=130, 129 and 88, respectively).

Figure 1

Number of affected institutions by date of onset of first case in the respective institution, multistate outbreak of norovirus gastroenteritis, Germany, 2012 (n=309[a])



[a] Date of onset of first case was available for 309 institutions.

As of 8 October 2012, 555 human specimens (339 from ill persons, and a convenience sample of 216 staff members of Caterer X with unknown disease status) were reported by the health authorities of four of the five affected federal states. Of those, 32% were positive for norovirus (40% of ill persons, 20% of staff members). No other viral or bacterial pathogens or bacterial toxins were reported from the respective health departments in connection with the outbreak.

Analytical studies

All four analytical studies, comprising 150 cases and 274 controls, identified dishes containing strawberries as vehicles of infection (either strawberry compote or strawberry fruit quark) (Table).

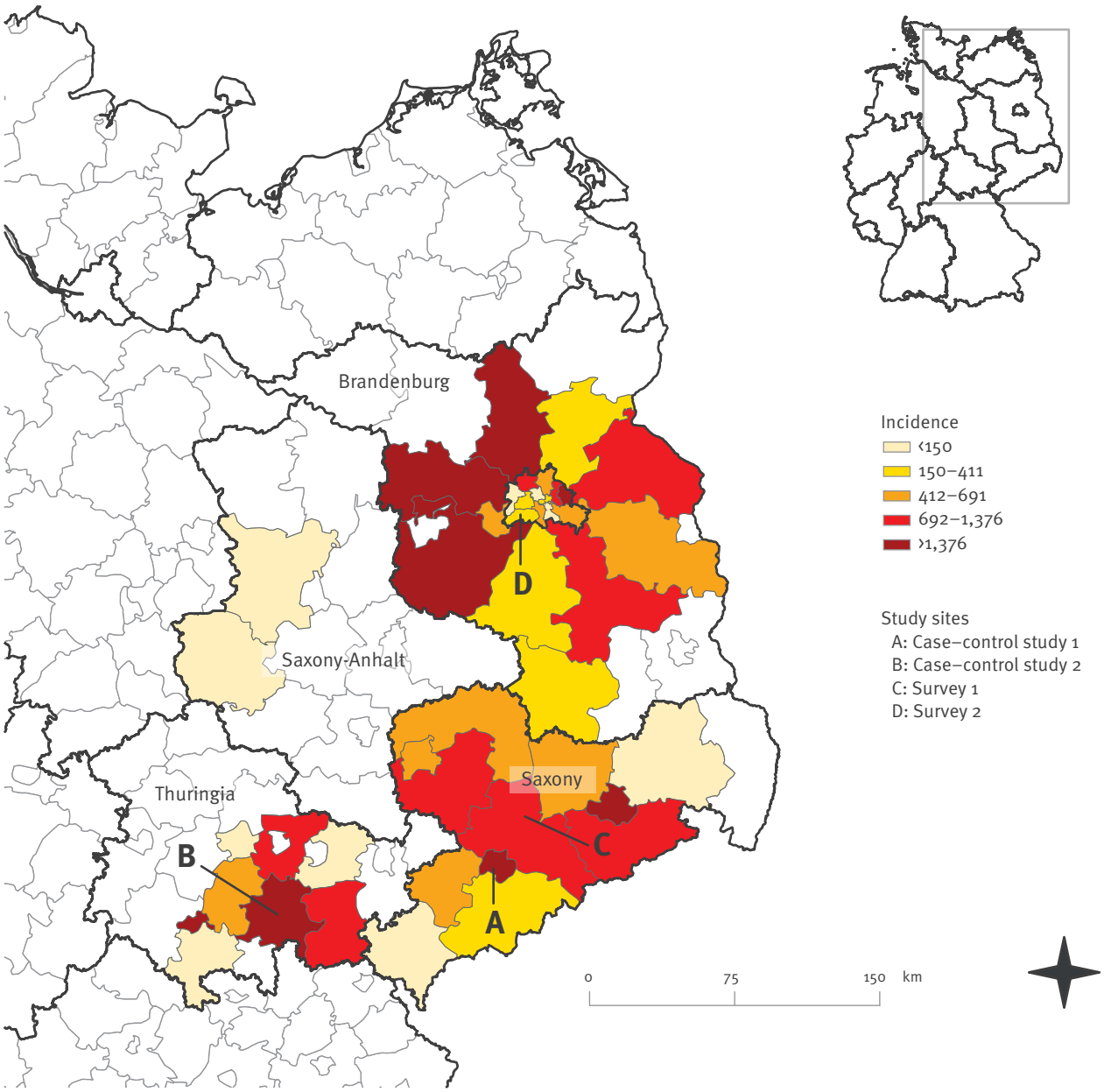
Case–control study 1

We included 43 cases and 54 controls (median age for each: 11 years), three potential secondary household cases were excluded. Symptom onset was from 24 through 30 September, with a steep increase and a peak of the epidemic curve on Wednesday 26 September (n=16 cases) suggesting a point source (Figure 3A).

Most cases, and a higher proportion of cases than controls, had eaten at the school's canteen on Monday 24 and Tuesday 25 September (see Table), but not on the following two days (when a substantial proportion of cases were already sick). The only dish offered on one of these two days that was positively associated

Figure 2

Cases per 100,000 population under the age of 18 years by districts, and locations of analytical study sites, multistate outbreak of norovirus gastroenteritis, Germany, 2012 (n=10,950)



Table

Results of univariable and multivariable risk factor analyses, multistate outbreak of norovirus gastroenteritis, Germany, 2012 (n=424)

School lunch exposure	Cases		Controls		Univariable analysis			Multivariable analysis		
	Total	Exposed (%)	Total	Exposed (%)	OR	95% CI	p value	OR	95% CI	p value
Case–control study 1										
Mon 24 Sep	43	36 (84)	54	41 (76)	1.63	0.59–4.53	0.45	Not performed		
Semolina pudding and strawberry compote	36	26 (72)	40	20 (50)	2.60	1.00–6.77	0.06			
Any strawberry containing dish	36	32 (89)	40	27 (68)	3.85	1.12–13.21	0.03			
Strawberry compote[c]	37	28 (76)	40	11 (28)	8.20	2.66–26.03	<0.0001			
Tue 25 Sep	42	34 (81)	53	37 (70)	1.84	0.70–4.84	0.24			
Wed 26 Sep	43	24 (56)	53	38 (72)	0.50	0.21–1.16	0.13			
Thu 27 Sep	43	17 (40)	53	37 (70)	0.28	0.12–0.66	<0.01			
Case–control study 2										
Mon 24 Sep	39	37 (95)	73	40 (55)	15.26	3.42–68.11	<0.01	[a]		
Semolina pudding and strawberry compote	37	30 (81)	40	17 (43)	5.80	2.06–16.30	<0.01	[a]		
Strawberry compote	37	32 (86)	40	11 (28)	16.87	5.23–54.40	<0.01	16.87	5.23–54.40	<0.01
Tue 25 Sep	39	33 (85)	73	42 (58)	4.06	1.52–10.88	0.01	[a]		
Pasta Bolognese	33	27 (82)	42	25 (60)	3.06	1.04–9.00	0.05	[b]		
Wed 26 Sep	39	3 (8)	73	30 (41)	0.12	0.03–0.42	<0.01	[a]		
Thu 27 Sep	39	9 (23)	73	27 (37)	0.51	0.21–1.24	0.14	[a]		
Survey 1										
First wave										
Thu 20 Sep: Strawberry fruit quark	25	22 (88)	60	15 (25)	22.00	5.28–125.13	<0.001	16.87	5.23–54.40	<0.01
Thu 20 Sep: Fresh plums	20	7 (35)	53	10 (19)	2.32	0.61–8.34	0.15	[b]		
Second wave										
Mon 24 Sep: Strawberry compote	16	12 (75)	27	3 (11)	24.00	3.76–177.14	<0.001	33.80	3.41–∞	<0.01
Tue 25 Sep: Peas and carrots	15	9 (60)	27	2 (7)	18.75	2.63–202.28	<0.001	23.66	2.22–∞	<0.01
Tue 25 Sep: Red cabbage	15	7 (47)	30	5 (17)	4.38	0.88–22.35	0.03	[b]		
Tue 25 Sep: Sauce	18	14 (78)	26	13 (50)	3.50	0.78–18.12	0.06	[b]		
Mon 24 Sep: Semolina pudding	18	13 (72)	32	17 (53)	2.29	0.58–10.08	0.19	[b]		
Tue 25 Sep: Boiled potatoes	14	8 (57)	28	10 (36)	2.40	0.54–10.96	0.19	6.10	0.55–∞	0.14
Third wave										
Wed 26 Sep: Strawberry quark dessert	7	5 (71)	25	1 (4)	60.00	3.31–2,944.34	<0.001	45.42	3.31–2,944.92	<0.01
Mon 24 Sep: Fruit yoghurt	7	5 (71)	26	4 (15)	13.75	1.43–172.24	<0.01	[b]		
Tue 25 Sep: Plain yoghurt	8	4 (50)	24	4 (17)	5.00	0.61–39.84	0.06	[b]		
Survey 2										
Any school lunch 24–28 Sep	14	14 (100)	72	25 (35)	35.00	5.51–∞	<0.0001	Not performed		
Wed 26 Sep: Semolina pudding and strawberry compote	14	10 (71)	25	3 (12)	16.45	2.77–139.30	<0.001			
Wed 26 Sep: Strawberry compote (with any dish)	12	11 (92)	21	5 (24)	30.61	3.19–1,605.80	<0.001			

CI: confidence interval; OR: odds ratio.

Only exposures positively associated with disease occurrence with p<0.2 and explaining at least 25% of cases are shown.

[a] Not considered for multivariable analysis.

[b] Not part of the final multivariable model, i.e. p≥0.2.

[c] Results from a sub-study ('live 2x2 table').

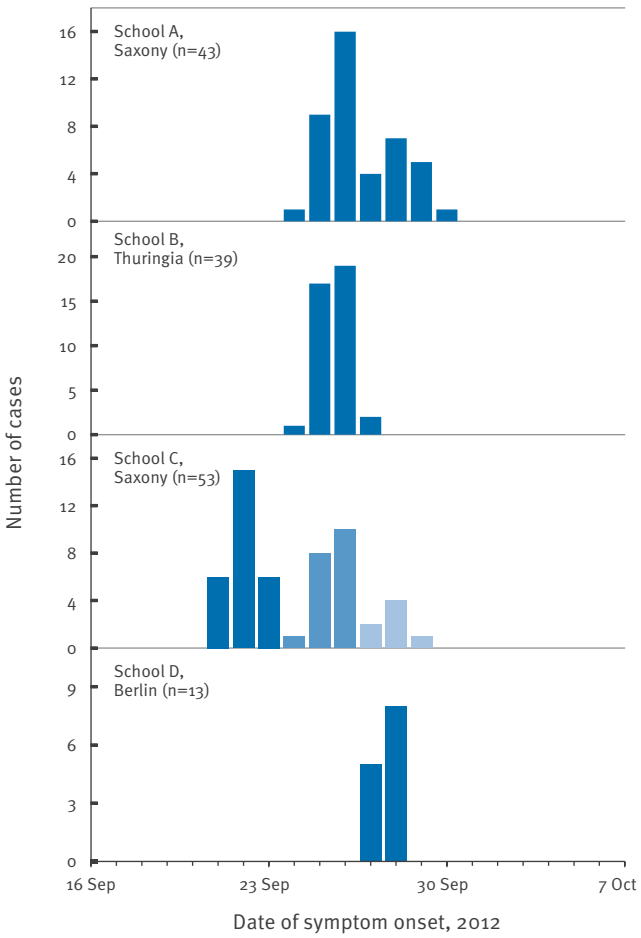
with illness with a p value ‹0.1, was semolina pudding, which had been served on Monday with a choice of cherries, sugar and cinnamon, or cold strawberry compote (Table).

Of the 36 cases, 26 reported to have chosen the semolina pudding. However, on that day strawberry compote had been offered with three of the four meal choices. We therefore compared cases and controls regarding the choice of any meal containing strawberry compote. Cases had chosen significantly (p‹0.05) more often a strawberry compote-containing dish than controls. In the sub-study including those who had eaten at the canteen on Monday 24 September, 28 of 37 cases but only 11 of 40 controls reported to have eaten strawberry compote (OR=8.20; 95% CI: 2.66–26.03; p‹0.01).

Having identified a dish served on Monday 24 September as the likely vehicle of infection, we estimated a median incubation period of two days (IQR: 2–6 days; onset data only available by full days).

Figure 3

Time course of the norovirus outbreak: number of cases included in the four analytical studies, by date of symptom onset, Germany, 2012 (n=148)



The columns for School C are filled with different colours for the three waves of disease (see case definition).

Case–control study 2

We included 39 cases and 73 controls in the analysis (median age: 11 and 12 years, respectively), excluding again three potential household secondary cases. The epidemic curve showed an even steeper increase in case numbers with 36 of 39 of cases with symptom onset on Tuesday 25 (n=17) or Wednesday 26 (n=19) September (Figure 3B).

Most cases, and a higher proportion of cases than controls, had eaten at the school's canteen on Monday 24 and Tuesday 25 September (see Table), but not on the following two days (when a substantial proportion of cases were already sick). On both days, the consumption of one dish was significantly associated with illness: semolina pudding with strawberry compote, sugar and cinnamon on Monday, and pasta with Bolognese sauce on Tuesday (Table). Again, the strawberry compote had been offered with two of the four dishes on Monday, and the association between consumption and illness was even stronger when analysing compote as a separate variable. In multivariable analyses including the exposure variables strawberry compote on Monday and pasta on Tuesday, only the consumption of strawberry compote remained significantly associated with illness (OR=16.87; 95% CI: 5.23–54.4; p‹0.01).

Survey 1 (web-based questionnaire)

We included 54 cases and 75 controls (median age of each: 12 years) in the analysis (participation rate: 29%). The epidemic curve showed three peaks of dates of symptom onset (Figure 3C). Overall, the proportion of persons having had lunch at the school canteen from 20 through 27 September was significantly higher in cases than in controls (98% vs 76%, OR=16.7; 95% CI: 2.4–710.1; p‹0.01).

In univariable analyses (Table) we found an association between being a case in the first wave and the consumption of strawberry quark and fresh plums both served on 20 September. In the multivariable analysis, only the former remained statistically significant (OR=27.13; 95% CI: 5.24–276.40; p‹0.01).

For the cases in the second wave, we found a significant association with having eaten at the school canteen on 24 September (multivariable analysis: OR=11.1; 95% CI: 1.38–88.4; p‹0.05). Of the 35 items served on that and the following day, six were associated with disease and included in the multivariable analysis, in which strawberry compote (OR=33.80; 95% CI: 3.41–∞; p‹0.01) and carrots and peas (OR=23.66; 95% CI: 2.22–∞; p‹0.01) remained significant.

For cases in the third wave, the univariable analysis showed three different food items to be associated with occurrence of disease. In the multivariable analysis only strawberry quark remained statistically significant (OR=45.42; 95% CI: 3.31–2,944.92; p‹0.01).

Survey 2 (email questionnaire)

We received 86 completed questionnaires (response 10%). 14 participants were classified as cases, 72 as controls. Median age of cases and controls was 12 years (range: 9–16 and 9–17, respectively). Onset times peaked on the afternoon of 27 September and the following morning (Figure 3D).

The proportion of persons having eaten at the school's canteen from 24 through 28 September was significantly higher in cases than in controls. Of all dishes on offer during that period, only semolina pudding with strawberry compote resulted in a statistically significant positive association with disease in the univariable analysis (Table). Strawberry compote was also offered as a dessert alongside China vegetables, but only one pupil reported to have chosen this dish. The strongest association was found when asking specifically for the consumption of strawberry compote (independent of main course). The median incubation period, calculated from the most likely time of exposure to strawberries (13:00 on 26 September) and the individual times of symptom onset, was 35 hours (range: 12–40 hours).

Food trace-back investigations

Frozen strawberries had been used in regional kitchens of Caterer X. They were part of a lot of 22 tonnes imported by Company Y in Saxony from a company in China, packaged in 2,201 boxes of 10 kg each. Of the institutions with available information, 98% (368/377) were supplied by regional kitchens of Caterer X, the remainder were supplied by two smaller catering companies. All three caterers were supplied by the same company (Company Y). All affected institutions had received products containing the implicated frozen strawberries. Starting on 5 October, the date of a joint press release by RKI, BfR and BVL, Company Y began withdrawing the lot of frozen strawberries from their customers (the company had already stopped further delivery of the strawberries before that date). Overall, delivery stop and recall ensured that at least 1,136 boxes (more than 11 tons) of strawberries from the incriminated lot did not reach the consumer. The remaining 1,065 boxes (ca 10.7 tons) had either already been used or were destroyed under the supervision of the local food safety authorities after the recall. On Oct 8, Saxony's State Health Laboratory detected norovirus in a sample obtained from an unopened box of the incriminated lot of frozen strawberries [12].

Discussion

We report here the largest recorded food-borne outbreak in Germany. It affected several hundreds of institutions supplied almost exclusively by one large caterer and was associated with strawberries imported frozen in a large lot from China. Norovirus was identified as the causative agent. Although the individual clinical courses of disease were mild, the overall disease burden was considerable. The high number of cases caused substantial distress and impairment of the daily routine in affected institutions and families, considerable concerns about food safety in canteens for children, and nationwide media interest.

The epidemiological studies provided strong evidence for strawberries as the vehicle of infection. Conducted in different geographical regions and using various designs, they consistently and exclusively showed statistically significant associations between illness and the consumption of strawberry dishes. Furthermore, in all studies, affected institutions offered strawberry dishes two calendar days before the peak of illnesses, and in one instance, several strawberry-containing dishes, served on different days, caused several waves of illnesses.

Epidemiological evidence guided food safety investigations on the local, state and national level [12]. Early identification of the vehicle of infection led to a timely withdrawal of more than half of the lot. Assuming a similar level of contamination in the part of the lot that was withdrawn, at least 11,000 cases were averted by the withdrawal, probably even more, seeing as only a fraction of the delivered strawberries had been prepared for consumption. According to the report of the German Task Force on Food and Feed Safety, some of the involved regional kitchens of Caterer X reported not to have heated the strawberries during preparation of the implicated dishes whereas others stated that they had, which may in part explain that not all institutions supplied by the involved regional kitchens reported cases of gastroenteritis [12].In response to this outbreak, recommendations in Germany for institutions catering for vulnerable populations (including schools and child care facilities) have been amended and now specifically include the advice to heat frozen berries [13]. Furthermore, from 1 January 2013, a European Union (EU) regulation requires 5% of consignments of frozen strawberries imported from China into the EU to be tested for norovirus [14].

Infectious disease outbreaks due to contaminated produce have gained importance in the recent past [15], including norovirus outbreaks in Europe linked to frozen raspberries [16-22] or blackberries [23] and the multistate outbreak of hepatitis A due to mixed frozen berries [24]. Also strawberries have repeatedly been incriminated in large hepatitis A outbreaks in the United States [25,26] and in Europe [27]. Germany has recently faced a number of outbreaks caused by contaminated vegetables or fruits including sprouts and watermelons [28-30]. The original contamination of the food vehicles or relevant ingredients occurred in countries that were not known to be affected by outbreaks, which complicated or even prevented thorough source investigations. In none of these outbreaks, including this one, was the mode of contamination elucidated. Undoubtedly, transnational source investigations pose particular challenges [31]; political and economic issues may sometimes hamper effective collaboration. A better understanding of how the berries

became contaminated is crucial for developing long-term prevention measures upstream of the retailer. Several different norovirus genotypes of genogroups I and II were detected in the strawberries [8] (and also in human samples) [32]. Together with the large scale of the outbreak, this lends support to the hypothesis that the use of contaminated water in the production of the strawberries was responsible for the outbreak.

This report exemplifies the risk of large outbreaks in the era of global food trade. Today, unprecedented volumes of produce (here 22 tonnes) are distributed to a large number of markets throughout the world [33], thereby increasing the risk for food safety. Public health surveillance needs to adapt to these challenges, e.g. be able to detect outbreaks caused by widely disseminated foods. Surveillance using molecular subtyping information allows establishing links between disease occurrences in different regions, usually seemingly sporadic cases or small clusters [34]. The outbreak described here appeared as an accumulation of concurrent local outbreaks in several adjacent states. Thus, in addition to molecular surveillance, rapid communication of local outbreaks to the state level, as it happened in the outbreak-detecting state of Brandenburg, enables rapid recognition and investigation of supra-regional events even before the aetiology is known, and should be implemented in routine infectious disease surveillance.

Outbreak Investigation Team

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Conflict of interest

None declared.

Authors' contributions

HB, MF, HW and DW drafted the manuscript. HB and DW were the principle investigators of CCS 1, DW of CCS 2, MF of Survey 1, and SH of Survey 2 (all substantially contributed to conception and design, data collection, analysis and interpretation). HB designed the tool used for data collection and analysis in CCS 1 and 2 and represented the RKI at the Task Force Gastroenteritis collaborating with the food safety authorities. HW was responsible for the collection, analysis and interpretation of descriptive data. MH performed the data analysis of CCS 1 and 2. AS substantially contributed to data interpretation and represented the RKI at the Task Force Gastroenteritis. TD participated in data collection of CCS 1 and was a principle investigator of CCS 2 (data collection and interpretation). CS was a principle investigator for the State Health Authority Brandenburg (collection, analysis and interpretation of descriptive data). SSM was a principle investigator for the State Health Authority Saxony (collection, analysis and interpretation of descriptive data) and contributed to the conception of CCS 1. GF coordinated the German Task Force on Food and Feed Safety. OH and KS substantially contributed to the interpretation of descriptive and study data.
All authors critically revised the manuscript and approved of the final version.

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Reducing the barriers against analytical epidemiological studies in investigations of local foodborne disease outbreaks in Germany – a starter kit for local health authorities

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Thousands of infectious food-borne disease outbreaks (FBDO) are reported annually to the European Food Safety Authority within the framework of the zoonoses Directive (2003/99/EC). Most recognised FBDO occur locally following point source exposure, but only few are investigated using analytical epidemiological studies. In Germany, and probably also in other countries of the European Union, this seems to be particularly true for those investigated by local health authorities. Analytical studies, usually cohort studies or case–control studies, are a powerful tool to identify suspect food vehicles. Therefore, from a public health and food safety perspective, their more frequent usage is highly desirable. We have developed a small toolbox consisting of a strategic concept and a simple software tool for data entry and analysis, with the objective to increase the use of analytical studies in the investigation of local point source FBDO in Germany.

Introduction

Outbreak identification, investigation, and control are primary objectives for public health. The Directive 2003/99/EC on the monitoring of zoonoses and zoonotic agents of the European Parliament and of the European Council defines food-borne disease outbreaks (FBDO) as *two or more human cases of the same disease and/or infection [...] and where the cases are linked, or are probably linked, to the same food source*. FBDO, usually caused by infectious agents, occur frequently in Europe [1]. In 2010, for example, a total of 4,858 FBDO affecting 58,083 persons (data excluding those of Spain) were reported to the European Food Safety Authority (EFSA) [2]. Their effective control depends on the rapid identification of the suspected food vehicle from all the available epidemiological, microbiological, environmental and other evidence and on preventing its further consumption, e.g. by removing it from the market. From a food safety perspective, it is paramount to learn what went wrong during production and preparation of the food so that measures to prevent further outbreaks or sporadic cases can be implemented [3]. Most FBDO are recognised and handled locally [4], emphasising the necessity for local health authorities to be able to conduct timely epidemiological investigations, which can (but do not always have to) include analytical epidemiological studies, usually cohort studies or case–control studies. Importantly, these studies increase the likelihood of successfully identifying the suspected food vehicle. [4]

However, analytical epidemiological studies are seldom employed in the investigation of FBDO, particularly in local outbreaks, where control measures are often prompted by descriptive epidemiology and prior knowledge, i.e. biological plausibility, of common food vehicles. In 528 of 4,858 (11%) FBDO reported to EFSA for 2010, the evidence for a suspected food vehicle was reported as being 'strong', i.e. usually based on better evidence than a suspected vehicle's biological plausibility and on the fact that most or all of the cases had been exposed. Only in 148 of them (27%; 3% of all reported FBDO) had an analytical epidemiological study been conducted. The use of analytical epidemiology varies widely across the Member States of the European Union (EU), and some countries seemingly do not use them at all [2]. In 2010 in Germany, reports on 439 FBDO with an identified causative agent were electronically submitted to the public health institute at national level, the Robert Koch Institute (RKI). In 40 of these 439 (9%), the evidence implicating a suspect food vehicle or meal was considered to be strong, but only in two of the 40 (i.e. 5% of those with strong evidence, and less than 1% of the total) had an analytical epidemiological study been reported, one by the state health department and one by a field epidemiologist trainee [5], both assisting local health authorities.

Obviously, barriers against employing analytical epidemiological studies in FBDO investigations exist. The reasons why analytical epidemiological studies in Germany are seldom employed are many and include lack of human or technical resources, conflicting public

health priorities, late outbreak detection (resulting in no new cases at the start of a possible investigation), and lack of experience in conducting such studies. In our experience, the greatest barriers are associated with three core activities in epidemiological outbreak investigations:

1. Use of long hypothesis-generating (trawling) questionnaires,
2. Use of specific, sometimes complex software for data entry and/or statistical analysis,
3. Identifying and interviewing healthy persons serving as reference population (in cohort studies or case–control studies).

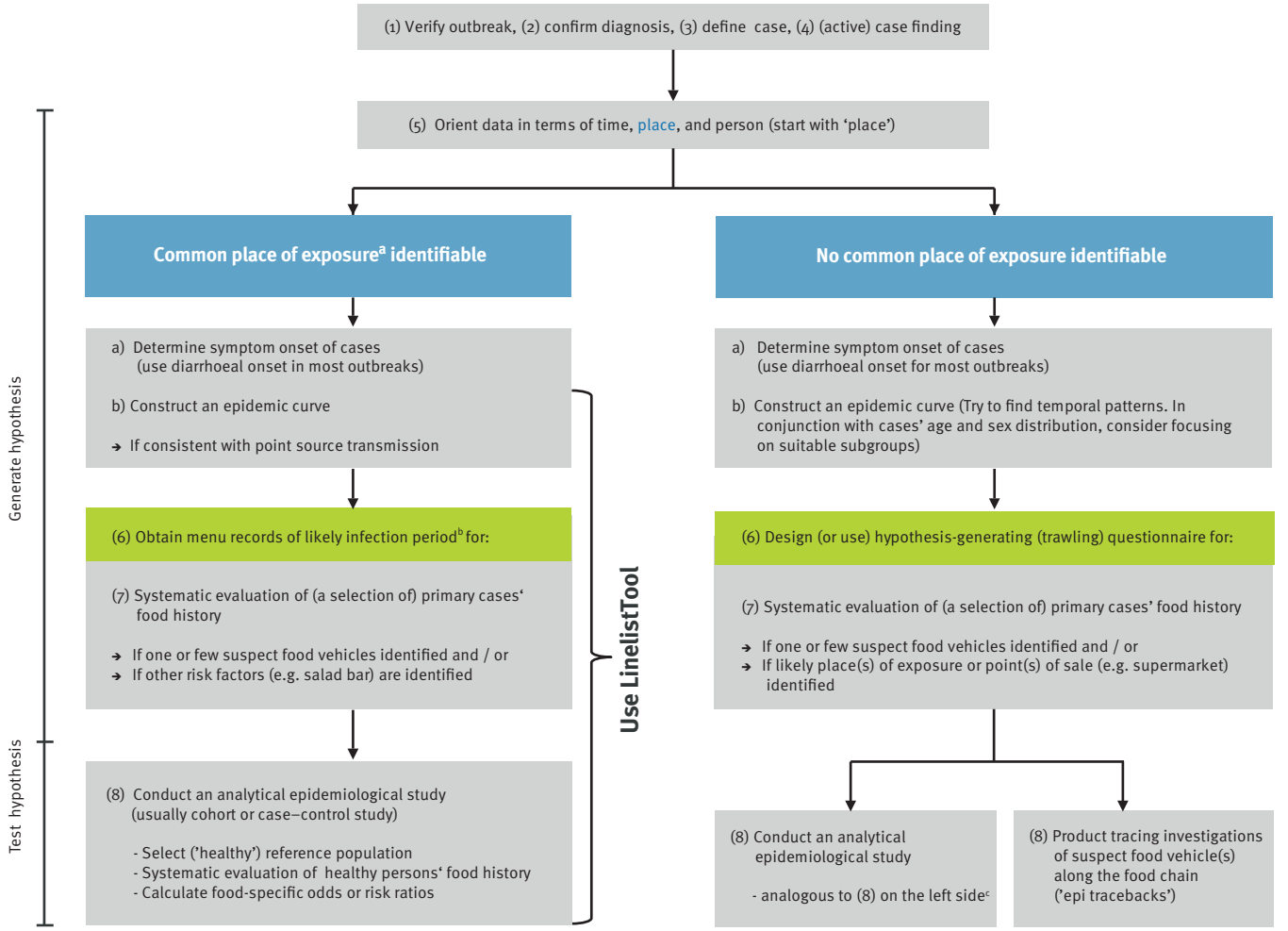
All these activities are usually not used outside of outbreak investigations by local health authorities in Germany. Consequently, they require special resources, and some also require specific skills (points 2 and 3). To support local public health authorities in overcoming some of these barriers (namely points 1 and 2), we have developed a toolbox that includes a strategic concept and an electronic file (Linelist Tool) that allows for data entry and automatic analysis. We thereby hope to increase the use of analytical epidemiological comparisons in investigations of FBDO in Germany.

The concept

The concept of how to investigate epidemiologically a FBDO is based on the steps of an outbreak investigation [6] as taught in many field epidemiology training programmes around the world (e.g. the European Programme for Intervention Epidemiology Training (EPIET) or the Epidemic Intelligence Service (EIS) of

Figure 1

Simplified schematic for epidemiological investigation of foodborne disease outbreaks in Germany



FBDO: foodborne disease outbreaks.

[a] Usually an institution, a restaurant or a social gathering.

[b] Infection period can be estimated for group of primary cases using the incubation period of the causal agent (if known), or those of typical FBDO agents.

[c] Additionally or even alternatively, the association of specific food items with illness may be rapidly assessed using binomial probability, provided that estimates on the prevalence of consumption of these food items in the general population are available or that their range can be reasonably guessed [9].

the United States). The concept has been adapted primarily to distinguish outbreaks where a common local place of exposure can be identified, e.g. a social gathering or an institution, and outbreaks where this is not the case. The concept is displayed in Figure 1. FBDO come in various shapes and sizes, and thus the concept is oversimplifying (e.g. continuing common source outbreaks do occur in institutions, and the concept does not accommodate for them). By experience and for didactic purposes, it is assumed that most local outbreaks are recognised by a sudden increase in case numbers, and so the epidemic curve is suggestive of a point source transmission, indicating a single exposure event.

The salient point is that in these outbreaks, menu records can often be used instead of long trawling questionnaires as the basis for hypothesis-generating interviews with cases. We advise local health authorities to focus primarily on investigating local outbreaks with a point source exposure. The Linelist Tool should offer them assistance in their analytical epidemiological investigation. In the rarer outbreaks where cases are geographically scattered, we advise them to seek assistance from public health authorities at the federal state or the national level, as these investigations are usually more time-consuming, complex and often not restricted to one jurisdiction.

The Linelist Tool

The Linelist Tool is a Microsoft Excel (Microsoft Corporation, Redmond, Washington) file consisting of several spreadsheets. The first spreadsheet (Linelist) allows for the entry of data collected from cases and healthy control subjects (Figure 2).

The other spreadsheets are completed automatically, based on the data entered. In one spreadsheet, an epidemic curve of the outbreak is generated. This should help local investigators to determine the time period when exposure is likely to have occurred, and to identify cases that are likely to be primary cases, i.e. infected by a common source, to seek food histories from (a subset of) them. Other spreadsheets display univariable measures of association for each dichotomous exposure, i.e. food vehicle-specific associations, expressed as odds ratios or risk ratios, depending on whether a cohort or case–control design is used (only responses coded as 'yes' or 'no' are included in the analysis). In addition to these estimates, corresponding approximate 95% confidence intervals [7] indicate the precision of the estimation and can be used to assess statistical significance (Figure 3).

We offer a short training course for local health authorities which we recommend users to complete before applying the tool in an outbreak investigation. The Linelist Tool is currently available only in German

Figure 2

Linelist spreadsheet for entry of data collected on case-patients and control subjects in investigations of foodborne disease outbreaks in Germany

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	AA	AB	AC	AD	AE
1	Date of data entry (complete cell G1 for automatic calculation of age):						19.12.2012																								
2	ID	Name	First name	Ill (y/n)	Date of birth (dd.mm.yyyy)	Age (calculated)	Sex	Diarrhoea (y/n)	Vomiting (y/n)	Fever (y/n)	Abdominal cramps (y/n)	Date of symptom onset (dd.mm.yyyy)	Hospitalised (y/n)	Cinnamon rolls	Spicy sprout salad	Oysters	Raw minced pork	Watermelon	Food item 6	Food item 7	Food item 8	Food item 9	Food item 10	Food item 11	Food item 12	Food item 13	Food item 14	Food item 15	Food item 16	Food item 17	Food item 18
3				y	01.01.2000	13						18.12.2012		y	y	y	y	y													
4				n	01.01.1999	14								n	n	n	n	n													
5				y	01.01.1998	15						15.12.2012		y	n	n	y	n													
6				y	01.01.1997	16						17.12.2012		y	y	n	n	y													
7				y	02.01.1996	17						18.12.2012		y	n	n	y	y													
8				y	02.01.1995	18						18.12.2012		n	y	n	y	y													
9				y	02.01.1994	19						16.12.2012		y	n	n	y	y													
10				n	02.01.1993	20								n	y	n	y	y													
11				n	03.01.1992	21								n	n	n	y	y													
12				n	03.01.1991	22								n	y	n	y	y													
13				y	03.01.1990	23						17.12.2012		y	n	n	y	y													
14				y	03.01.1989	24						03.12.2012		n	y	y	y	y													
15				n	04.01.1988	25								n	n	y	y	n													
16				n	04.01.1987	26								n	y	y	n	n													
17				n	04.01.1986	27								y	n	y	n	n													
18				n	04.01.1985	28								y	y	n	y	n													
19				n	05.01.1984	29								n	n	n	y	n													
20				n	05.01.1983	30								n	y	n	y	n													
21				y	05.01.1982	31						19.12.2012		y	n	n	y	y													
22				y	05.01.1981	32						16.12.2012		y	n	n	n	n													
23				n	06.01.1980	33								n	y	y	y	y													
24				n	06.01.1979	34								n	y	y	y	y													
25				n	06.01.1978	35								n	y	y	y	y													
26				y	06.01.1977	36						18.12.2012		y	n	y	n	n													
27				y	07.01.1976	37						17.12.2012		y	y	n	n	n													
28				n	07.01.1975	38								n	n	y	y	n													
29				y	07.01.1974	39						18.12.2012		n	n	n	n	n													
30				y	07.01.1973	40						17.12.2012		y	n	n	n	y													
31				y	08.01.1972	41						18.12.2012		y	n	n	n	y													
32				n	08.01.1971	42								n	n	y	y	y													
33				n	08.01.1970	43								n	n	n	y	y													
34				n	08.01.1969	44								n	n	n	y	y													
35				n	09.01.1968	45								n	n	n	y	y													
36				n	09.01.1967	46								y	n	n	y	y													
37				n	09.01.1966	47								n	n	n	y	y													
38				n	09.01.1965	48								n	y	n	y	n													
39				n	10.01.1964	49								n	n	n	y	y													
40				y	10.01.1963	50						18.12.2012		y	n	y	y	y													
41				y	10.01.1962	51						18.12.2012		y	n	n	y	n													
42				y	10.01.1961	52						17.12.2012		y	n	n	y	n													
43				n	11.01.1960	53								n	y	n	y	y													
44				n	11.01.1959	54								n	y	y	y	n													

Information | Linelist | Epidemic curve | Cohort study | Case–control study | 2x2 table RR | 2x2 table OR

language and can be downloaded at http://www.rki.de/linelisttool. Planned future developments of the tool include a power table for unmatched case–control studies (based on case numbers and the proportion of cases exposed to the suspected vehicle) to inform local health authorities of an appropriate number of control subjects when deciding whether or not to conduct a case–control study.

Discussion

Epidemiological investigations of FBDO, if conducted, seldom go beyond the evaluation of cases' food histories. If the prevalence of consumption of a food in the general population can be assumed to be low (e.g. raw milk in Germany) or is indicated by background data such as consumption surveys (e.g. water cress in the UK [8]), this may provide sufficient descriptive epidemiological evidence to implicate a specific food vehicle, and more targeted investigations (e.g. food sampling) or control measures can be initiated. In these instances, delaying intervention measures because an analytical study has not been conducted or yet completed may put the public at unacceptable risk. More often than not, however, it remains unclear whether a food item frequently consumed by cases implicates the contaminated vehicle or merely reflects the popularity of the food in the source population. For illustration, in Germany in 2010, in 252 of the 399 (63%) FBDO reported with 'weak' evidence, the only reported evidence implicating a food or meal was that 'the majority of cases consumed the implicated food' (a field entry in the electronic outbreak report). Probably most investigations ended inconclusively, no intervention occurred, and nothing could be learnt to inform improvements in food safety. To make matters worse, in some FBDO more than one food may be found to have been frequently consumed by cases. Irrespective of whether there are one or more such vehicles, an analytical epidemiological comparison is necessary to distinguish between popular foods and those associated with being a case.

We have developed a simple data entry and analysis tool for basic epidemiological comparisons in the investigation of point source FBDO. The main advantages of the tool are speed and ease of use. It only requires standard office software and the file is small enough to run on most computers. It does not require knowledge of specific statistical software (e.g. R, SAS, SPSS, Stata), but data can easily be imported by such software if more sophisticated analyses are needed. The software tool has already proven useful in a large norovirus outbreak linked to contaminated frozen strawberries [10], for which studies were completed, including analysis, within one day. In addition to FBDO, the tool can be used for the investigation of other outbreaks, e.g. of vaccine-preventable diseases. However, the tool may not be as suitable for the investigation

Figure 3

Spreadsheet 'Cohort study' with 2x2 tables displaying univariable measures of association for each dichotomous exposure in investigations of foodborne disease outbreaks in Germany

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
7	**Cinnamon rolls**	Ill	Not ill	Total	Attack rate (%)	RR	95%CI			**Spicy sprout salad**	Ill	Not ill	Total	Attack rate (%)	RR	95%CI			
8	Exposed	16	7	23	70	6,26	2,39	16,40		Exposed	6	18	24	25	0,63	0,28	1,40		
9	Unexposed	4	32	36	11					Unexposed	14	21	35	40					
10	Total	20	39	59	34					Total	20	39	59	34					
11	Proportion exposed (%)	80	18	39						Proportion exposed (%)	30	46	41						
12																			
13	**Oysters**	Ill	Not ill	Total	Attack rate (%)	RR	95%CI			**Raw minced pork**	Ill	Not ill	Total	Attack rate (%)	RR	95%CI			
14	Exposed	5	9	14	36	1,07	0,47	2,42		Exposed	13	34	47	28	0,47	0,24	0,92		
15	Unexposed	15	30	45	33					Unexposed	7	5	12	58					
16	Total	20	39	59	34					Total	20	39	59	34					
17	Proportion exposed (%)	25	23	24						Proportion exposed (%)	65	87	80						
18																			
19	**Watermelon**	Ill	Not ill	Total	Attack rate (%)	RR	95%CI			**Food item 6**	Ill	Not ill	Total	Attack rate (%)	RR	95%CI			
20	Exposed	11	19	30	37	1,18	0,58	2,42		Exposed	0	0	0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!		
21	Unexposed	9	20	29	31					Unexposed	0	0	0						
22	Total	20	39	59	34					Total	0	0	0						
23	Proportion exposed (%)	55	49	51						Proportion exposed (%)	#DIV/0!	#DIV/0!	#DIV/0!						
24																			
25	**Food item 7**	Ill	Not ill	Total	Attack rate (%)	RR	95%CI			**Food item 8**	Ill	Not ill	Total	Attack rate (%)	RR	95%CI			
26	Exposed	0	0	0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!		Exposed	0	0	0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!		
27	Unexposed	0	0	0						Unexposed	0	0	0						
28	Total	0	0	0						Total	0	0	0						
29	Proportion exposed (%)	#DIV/0!	#DIV/0!	#DIV/0!						Proportion exposed (%)	#DIV/0!	#DIV/0!	#DIV/0!						
30																			
31	**Food item 9**	Ill	Not ill	Total	Attack rate (%)	RR	95%CI			**Food item 10**	Ill	Not ill	Total	Attack rate (%)	RR	95%CI			
32	Exposed	0	0	0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!		Exposed	0	0	0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!		
33	Unexposed	0	0	0						Unexposed	0	0	0						
34	Total	0	0	0						Total	0	0	0						
35	Proportion exposed (%)	#DIV/0!	#DIV/0!	#DIV/0!						Proportion exposed (%)	#DIV/0!	#DIV/0!	#DIV/0!						
36																			
37	**Food item 11**	Ill	Not ill	Total	Attack rate (%)	RR	95%CI			**Food item 12**	Ill	Not ill	Total	Attack rate (%)	RR	95%CI			
38	Exposed	0	0	0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!		Exposed	0	0	0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!		
39	Unexposed	0	0	0						Unexposed	0	0	0						
40	Total	0	0	0						Total	0	0	0						
41	Proportion exposed (%)	#DIV/0!	#DIV/0!	#DIV/0!						Proportion exposed (%)	#DIV/0!	#DIV/0!	#DIV/0!						
42																			
43	**Food item 13**	Ill	Not ill	Total	Attack rate (%)	RR	95%CI			**Food item 14**	Ill	Not ill	Total	Attack rate (%)	RR	95%CI			
44	Exposed	0	0	0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!		Exposed	0	0	0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!		
45	Unexposed	0	0	0						Unexposed	0	0	0						
46	Total	0	0	0						Total	0	0	0						
47	Proportion exposed (%)	#DIV/0!	#DIV/0!	#DIV/0!						Proportion exposed (%)	#DIV/0!	#DIV/0!	#DIV/0!						
48																			
49					Attack rate									Attack rate					

Information | Linelist | Epidemic curve | Cohort study | Case-control study | 2x2 table RR | 2x2 table OR

of geographically diffuse outbreaks where exposure lists are not readily available and where investigators initially rely on hypothesis-generating interviews with cases to identify exposures of interest that can be further investigated by an analytical study. Most reported FBDO in Europe with strong evidence for a food vehicle involve only a single place of exposure, implying that most of them are investigated by local health authorities. Furthermore, analytical epidemiological studies are seldom conducted in FBDO in Europe, considering the large number of FBDO reported to EFSA. We therefore believe that this starter kit may be useful in other countries of the EU and welcome suggestions and feedback for its further improvement.

As much as we are advocating the use of a simple analysis tool in investigations of local point source FBDO, we advise judicious use and data interpretation, and emphasise the importance of considering analytical epidemiological evidence in the context of all other available evidence, e.g. microbiological evidence and product-tracing evidence. It is important to note that the tool has purposely been kept simple and permits only univariable comparisons. The results are a starting point for further, more targeted, investigations to identify the ingredient of the implicated meal carrying the causal agent. These investigations could include more detailed statistical analysis, further analytical studies, additional food histories, and environmental investigations, as well as food sampling and product tracing. At least some of these investigations are necessary to identify a suspected vehicle and where and when contamination with (or multiplication of) the causative agent occurred.

We acknowledge that analytical epidemiological studies are not a universal remedy, and not all FBDO lend themselves to an analytical investigation. For example, some FBDO are small, which hampers meaningful studies. Among them are those that occur in a single household (often ascertained in the German surveillance system). It is difficult to provide a numeric threshold for when an analytical study should be conducted because the decision depends on many factors, such as the dynamic of the outbreak or severity of illnesses. Nevertheless, it is instructive to note that in 2010, 23 FBDO were reported in Germany, each with at least 20 human cases, but evidence from an analytical study had been reported for only one. This number of outbreaks is likely to be an underestimate because a causative agent is not always identified in FBDO. In such cases, information on the outbreaks is often not transmitted from the local level via the state health department to the RKI. The fact that a causative agent has not (yet) be identified in a FBDO should not hinder the use of the tool; clinical case definitions may be perfectly adequate alternatives to microbiological confirmation [10,11]. Epidemiologically identifying a suspected vehicle enables better targeted searches for the causative agent in food samples. In addition, it may allow estimating the incubation period, which may provide crucial hints at the aetiological agent, thereby informing microbiologic investigation of human cases. Again, the norovirus outbreak linked to frozen strawberries is an excellent example [10].

Investigation of FBDO requires adequate resources, which is challenging in times where budget deficits have already left their footprint on public health surveillance in many countries. However, surveillance of FBDO is necessary for understanding the epidemiology of foodborne diseases [4] and to serve the development of public health policies on food safety [12,13] in a systematic and more unbiased fashion than focussing on published outbreaks [14]. The higher the proportion of FBDO with credibly identified food vehicles, the more valid is the evidence base provided by them.

We hope that by providing a simple software tool and by guiding local health authorities on how and which FBDO to investigate, more analytical studies in FBDO investigations will be conducted, and as a consequence, a suspected vehicle will more often be identified. In the long run, it is also important to strengthen the epidemiological skills of local health authorities so that more detailed epidemiological analyses can be conducted independently.

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Conflict of interest

None declared.

Authors' contributions

DW has developed the strategic concept for epidemiologic investigation of food-borne disease outbreaks in Germany. HB has developed the analysis tool. Both authors have drafted and revised the manuscript.

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Letter to the editor: Diagnosis of a single imported dengue case who had travelled to Japan – how serious is it for travellers?

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To the editor: We enjoyed reading the meticulous clinical report of an imported dengue case in a German traveller returning from Japan [1]. It is, however, unclear what level of risk the 'autochthonous' infection of a single case in Japan represents. By investigating the epidemiological aspects of one imported dengue case, we would like to discuss how serious the implications of autochthonous transmission are for future travellers.

The diagnosed case travelled to Japan in August 2013, during which time the dengue virus infection is believed to have occurred. We would like to estimate how many primary cases there were and how transmissible the dengue virus was.

Let I_t and R_t represent the number of primary cases and the effective reproduction number, respectively, at a generation t (i.e. the mean number of secondary cases generated by a single primary case at generation t). Supposing there were St susceptible individuals who can be infected with dengue virus, the probability of producing $I_{t+1}=k$ secondary cases through a single generation interval of dengue (i.e. the time from infection in a primary human case to infection in a secondary human case caused by the primary case through the mosquito vector) is given by [2,3]: (1)

If the diagnosed German patient represents all infected cases, k=1. However, dengue was not at the forefront of Japanese physicians' attention before the case report. If there were other undiagnosed cases in the same generation, k≥2. As can be seen from Equation 1, the reproduction of k cases in generation t+1 depends on three unknown epidemiological parameters, i.e. I^t, R_t and S_t. The negative loglikelihood of observing k secondary cases reads as follows: (2)

By allocating plausible values for a part of three unknown parameters, we can examine hypothetical situations in which a transmission event in a German traveller could have occurred. The relationship between the effective reproduction number and the number of primary cases with three possible values of k (k=1, 3 and 5) and S_t=50 is shown (panel A of the Figure). Maximum likelihood estimates of R_t were obtained for each I_t. R_t would have to be above 1 for I_t<k, but transmission of dengue virus has not been continuously observed in Japan and R_t is unlikely to be above 1 over several generations. More importantly, for I_t≥k, the large It is consistent with R_t sufficiently below 1. Namely, it is likely that the observed event was caused by a certain small number of primary cases I_t presumably with a small R_t<1. S_t had little impact on both R_t and It (data not shown) as well as the likelihood value, and thus, we fixed it at 50 for the rest of our analysis. The negative loglikelihood value as a function of I_t and R_t (without involving statistical estimation) is simulated (panel B of the Figure). This represents the situation for k=1, but k>1 also yielded qualitatively indistinguishable patterns. As R_t becomes larger (and comes close to 1), the most plausible value of I_t is calculated to be 1. Provided that R_t is as small as 0.2, the minimum negative loglikelihood will be observed at I_t=5.

Two limitations of our analysis must be noted. First, an important technical flaw of our exercise is that our

(1) $$\Pr(I_{t+1}=k;I_t,S_t,R_t)=\binom{S_t}{k}\left[1-\exp\left(-\frac{R_tI_t}{S_t}\right)\right]^k\exp\left(-\frac{R_tI_t}{S_t}\right)^{S_t-k}$$

(2) $$L(I_t,S_t,R_t;I_{t+1}=k)=\sum_{j=1}^{k}\ln j-\sum_{i=S_t-k+1}^{S_t}\ln i-k\ln\left[1-\exp\left(-\frac{R_tI_t}{S_t}\right)\right]+(S_t-k)\frac{R_tI_t}{S_t}$$

arguments start with a rare event (i.e. diagnosis of a single imported case) and thus our results could have over-interpreted the actual risk of dengue in Japan. The actual risk could be even smaller than what has been calculated here, but we decided to use the biased sample, because the over-interpreted risk would still appear to be far smaller than that in dengue-endemic settings (and this notion should be shared with non-experts). Second, due to data limitation, our exercise only extends to the diagnosed dataset of the reported German case. It is hard to take into account unrecognised transmission events at another time and another geographical location.

Despite these limitations, our crude analysis of this diagnostic event indicates the following: (i) the number of primary cases was probably small; and (ii) even with a certain number of primary cases, a large I_t leads to a small R_t, which is substantially below 1. These would not permit dengue virus transmission to continue in the suspected transmission settings in Japan. Of course, demonstrating an autochthonous transmission event is of the utmost importance, because it reflects establishment of a transmission cycle through *Aedes* spp. within Japan. Nevertheless, it should be noted that the diagnosis of an imported case does not directly indicate that the actual risk of infection in Japan is high or that dengue is endemic in that country. Rather, based on the very limited biased data, our exercise indicates that it is very unlikely that dengue is endemic in Japan. Our results and travel history of the diagnosed case are consistent with an exposure near Narita International Airport, where there could be mosquito vectors that have bitten infected travellers from endemic countries. Indeed, there has been a report of 'airport dengue' in Australia [4]. A future seroepidemiological survey could help validate the findings from this short note [5].

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Conflict of interest

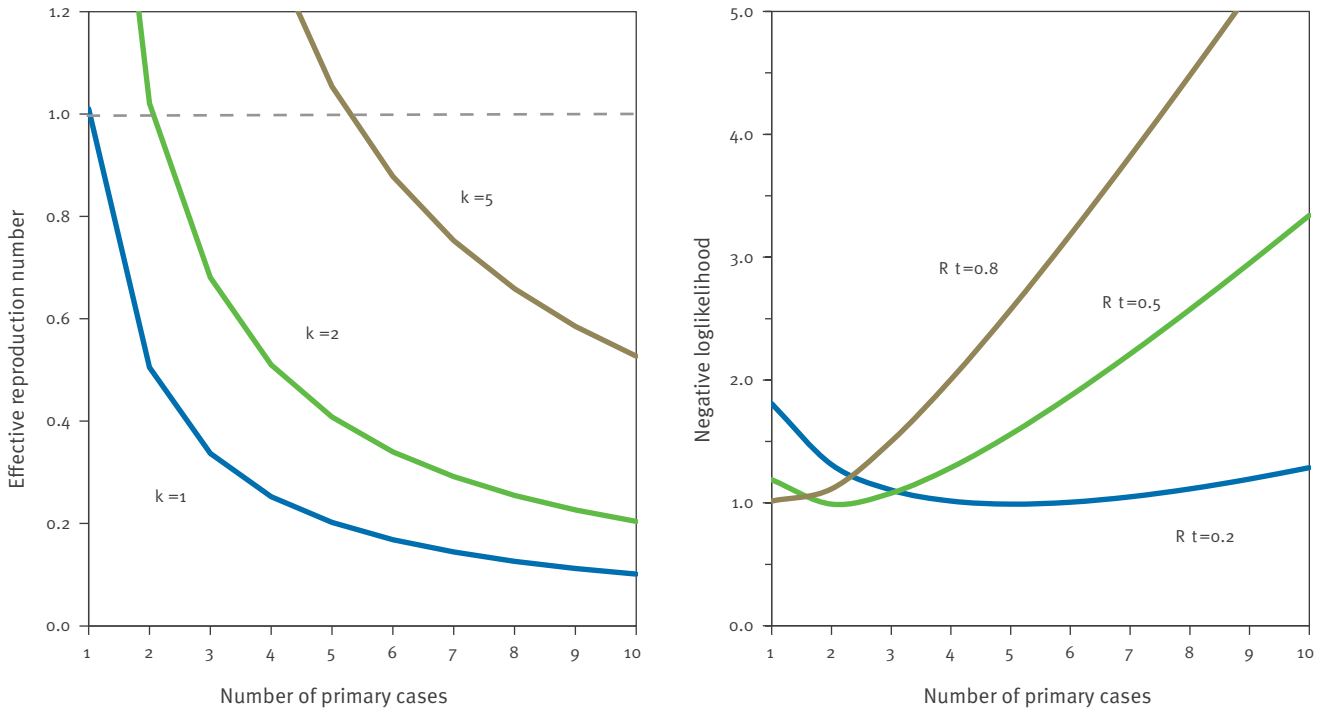
None declared.

Authors' contributions

Ryo Ueno and Hiroshi Nishiura conceived modelling ideas, interpreted the results and revised the manuscript. Hiroshi Nishiura implemented computational analyses and drafted the manuscript.

Figure

Analysis of transmission event data using a chain binomial stochastic model



Panel A. The relationship between the maximum likelihood estimate of the effective reproduction number and the number of primary cases. The number of secondary cases, *k*, has been varied from 1 to 5. The number of susceptible persons has little impact on the results and was fixed at 50. The horizontal dashed line represents the threshold value of the reproduction number, below which the transmission event does not continue through this generation.

Panel B. The simulated negative loglikelihood values as a function of the effective reproduction number and the number of primary cases. The optimal value of the number of primary cases is seen where the negative loglikelihood takes the minimum value. The effective reproduction number, *R*t, is varied from 0.2 to 0.8. The number of secondary cases, *k* has been fixed at 1, but $k>1$ does not yield qualitatively different patterns.

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Authors reply: Diagnosis of a single imported dengue case who had travelled to Japan – how serious is it for travellers?

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To the editor: We would like to thank Ueno and Nishiura for their comments [1] on our paper entitled 'Autochthonous dengue virus infection in Japan imported into Germany, September 2013' [2]. We would like to clarify that we fully agree with our Japanese colleagues regarding the low risk of acquiring dengue virus (DENV) infections in Japan. It was never our aim to create any form of hysteria about the autochthonous DENV transmission in Japan and we do not recommend the issuing of a travel warning based on one reported case. As stated in the manuscript, we rather consider our reported case as a reminder to clinicians to consider dengue fever as a differential diagnosis, both in Japan and in travel clinics worldwide. There are documented cases of misdiagnosed DENV infections in travellers that resulted in a fatal outcome [3].

Nonetheless, we think that the calculations presented by Ueno and Nishiura in their letter to the editor should be interpreted with caution: while the epidemiological concepts behind the formulae seem sound, and thus may lead to arithmetically precise results, which indeed suggest a low risk for DENV becoming endemic or epidemic in Japan, our current assumptions are based on only one case. Thus, the variability of data-based estimates is high. The author's state, 'However, dengue was not at the forefront of Japanese physicians' attention before the case report'. This implies that some dengue cases may have remained undetected. The majority of DENV infections present with few and often non-specific symptoms [4] and it is particularly likely that cases remain undetected if DENV transmission is restricted to only a short period in summer, as has been reported from Croatia and France [5-7].

The detection of one case, with the possibility of other undetected cases, demonstrates that there is a risk for dengue outbreaks in Japan, although we agree with the authors' conclusion that the risk of dengue becoming endemic is small. Dengue outbreaks in Japan were reported between 1942 and 1945 [8]. Certainly transmission may have taken place at Narita International Airport, where the presence of *Aedes aegypti* has recently been demonstrated [9]. However, the information of 'numerous mosquito bites' received while grape picking in Fuefuki, Japan, during the hottest month of the year, suggests the possibility that our patient was infected in the Japanese countryside, with mosquito´s infection originally 'seeded' by an imported infection. Both potential scenarios could be further examined by human serosurveys in the respective prefectures and at the airports and by entomological investigations assessing the abundance of mosquito vectors and their DENV infection rates. Such investigations were immediately performed in Croatia in 2010, after we reported the first autochthonous dengue fever case for Croatia and Europe [5,6]. These investigations revealed important findings, such as the serotype and genotype of the circulating DENV in Croatia [10]. In addition, hospital-based surveillance for dengue fever cases in Japan may strengthen risk assessments and forecasting models. We would highly welcome such investigations by our Japanese colleagues.

Conflict of interest

None declared.

Authors' contributions

JSC and CF wrote a draft response, NGS, BK and KS verified the analysis and provided comments, and all authors approved the final version

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