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Re-emergence of animal rabies in northern Greece and subsequent human exposure, October 2012 – March 2013

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Greece has been rabies-free since 1987 with no human cases since 1970. During 2012 to 2013, rabies has re-emerged in wild and domestic animals in northern Greece. By end March 2013, rabies was diagnosed in 17 animals including 14 red foxes, two shepherd dogs and one cat; 104 subsequent human exposures required post-exposure prophylaxis according to the World Health Organization criteria. Human exposures occurred within 50 km radius of a confirmed rabies case in a wild or domestic animal, and most frequently stray dogs were involved.

Introduction

The last animal rabies case in Greece, dates back to 1987 while the last human case was reported in 1970 [1]. Here we describe the re-emergence of rabies in both wild and domestic animals during October 2012 to end March 2013 in northern and central Greece that was associated with human exposure. We discuss significant public health implications of the situation.

Rabid fox

On 15 October 15 2012, a red fox (Vulpes vulpes) exhibited aggressive behavior during daytime, threatening inhabitants of a west Macedonian village in the area of Kozani. The animal was destroyed and transported to the National Reference Laboratory (NRL) for Animal Rabies at the Centre of Athens Veterinary Institutions Virus Department, of the Ministry of Rural Development and Food as part of a wild animal surveillance program for rabies organised and implemented by the Ministry of Rural Development since April 2012 because of documented presence of lyssavirus in neighboring Balkan countries. Four days later on 19 October, the brain samples tested positive for lyssavirus by fluorescence antibody test (FAT) and molecular techniques i.e. real-time RT-PCR and RT-PCR followed by sequencing.

Rabid shepherd dog and exposure of humans and domestic animals

On 10 November 2012, in west Macedonia, near the Greek-Albanian border in the area of Ieropigi, Kastoria, a shepherd dog, belonging to a herdsman, bit the thigh of a passing-by hunter unprovoked. Two days later, on 12 November, the dog developed an aggressive behaviour attacking other dogs and sheep of the herd. It was consequently destroyed and brain tissue samples investigated at the NRL in Athens were positive for lyssavirus both by FAT and molecular techniques on 16 November.

Tracing of exposed humans and animals and first control measures

An epidemiological investigation was initiated on 16 November by the Emergency Response Center of the Hellenic Centre for Diseases Control and Prevention (KEELPNO), Athens, in order to identify all individuals who had had contact with the dog and possible exposure to the lyssa virus. Seven people possibly exposed were interviewed. Besides the hunter and the shepherd, three relatives of the latter reported close exposure according to the World Health Organization exposure category III [2] i.e. dog bite and/or mucous membrane exposure to the rabid dog. All five including

Figure

Geographical location and type of animals with rabies identified through the animal surveillance program for rabies, Greece October 2012 to end March 2013 (n=17)

the veterinarian that had sampled the animal received human rabies immunoglobulin along with rabies immunisation series. None of the exposed individuals has developed any symptoms of human rabies so far. Two further possible contacts did not fulfill the WHO criteria for post-exposure prophylaxis.

Testing of brain samples from one exposed dog gave negative results. Hunting was prohibited in the affected area within a radius of 5km for 15 days and all shepherd dogs (approximately 800) were vaccinated against rabies during the next 15 days.

Results from regular rabies surveillance November to March 2013

In addition to the two animal cases described, and through the enhanced surveillance instituted by the veterinarian authorities, since November 2012 until end March 2013 we have identified additional 13 red foxes, one shepherd dog (20 December 2012) and one domestic cat (28 February 2013) with laboratory confirmed rabies (Figure). No dead wild or domestic animals were found except for the aforementioned domestic cat that was free to circulate outside the owner's house and was found dead. One other domestic animal (shepherd

Table

Administered rabies post-exposure prophylaxis by exposure category, Greece October 2012 - 19 March 2013

Post-exposure prophylaxis	Exposure category I[a]	Exposure category II[a]	Exposure category III[a]	Exposure undetermined[b]	Total
Vaccine only	1	14	36	5	**56**
Vaccine and anti-rabies immunoglobin	0	7	39	2	**48**
Total (%)	**1 (1)**	**21 (20)**	**75 (72)**	**7 (7)**	**104 (100)**

[a] Exposure categories according to WHO criteria [2]. Category I: touching animals, licks on intact skin; Category II: nibbling of uncovered skin, minor scratches or abrasions without bleeding; Category III – single or multiple transdermal bites or scratches, contamination of mucous membrane with saliva from licks, licks on broken skin, exposures to bats.

[b] Undetermined category of exposure refers to cases with insufficient information.

dog) suspected of infection due to aggressive behavior was destroyed and found positive for rabies.

In total 104 human exposures (category I: 1; 1%; category II: 21; 20; category III: 75; 72% and 7;7% unknown) have been reported to KEELPNO resulting in the administration of post-exposure prophylaxis according to the WHO criteria [2] (Table). All but 14 (six for each domestic rabid dog and two for the rabid cat) were exposures to potentially rabid animals and occurred in areas/prefectures within 50km radius of a confirmed rabies case in a wild or domestic animal. Sixty-six per cent (68 of 104) of the exposures were reported in males and the mean age of the exposed persons was 34 years (range 6-83 years).

In most instances (95 of 104; 91%) dogs were the animals involved. In 82 incidents dogs were involved, in five incidents cats, in one a weasel and in one the animal was not identified by the exposed individual. In the dog incidents, stray dogs represented the majority (n=63; 77%) followed by domestic dogs (n=15; 18%) and shepherd dogs (n=4; 5%). In each incident a mean of 1.2 persons were exposed (range 1-6 individuals).

Rabies situation in the Balkans countries

Although Greece was declared rabies-free in 1987, reports of rabies in wild and domestic animals exist for the neighboring countries [3-7]. In fact rabies appears to be prevalent in a number of reservoir species in southeastern Europe and in countries north and east of Greece [6]. Recent phylogenetic analyses have shown a westward movement of rabies via the movement of wild animals from Bulgaria to other Balkan countries suggesting that this is a local event unrelated to the circulation of phylogenetically distinct viral strains in Turkey [6]. In addition, in a previous study a distinct group of viruses identified in foxes in Serbia provided evidence for southward movement of rabies from Hungary, Serbia and Romania into Bulgaria [4]. In another report that compared the nucleoprotein sequence among animal rabies isolates from three Balkan countries, including recent isolates from the years 2011-12, all strains belonged to the eastern European group implicating wildlife movement in the transmission of rabies across the region [7]. However, more information is necessary regarding the circulation of the virus and more genotypic data will assist in establishing a pattern for the spread of disease. Only one autochthonous human rabies case was reported in 2009 in the European Union, in Romania,a person bitten by a fox [8].

Public health measures in Greece

Following the report of the first domestic animal case of rabies in the area of Macedonia, northern Greece, several measures were undertaken by the Greek National Authorities (Ministry of Rural Development and Food and Ministry of Health).

Discussion

The reported cases of confirmed and possible human rabies exposure after domestic or stray animal contact raise important public health concerns: first, there is an urgent need for a prevalence estimate of the virus circulation in wild animals in the area of northern Greece. Such information will help guide immediate vaccination efforts targeting wild animals that are reservoirs for the virus. It is likely that the virus circulates largely in populations of red foxes as red foxes are considered as the most important wild animal reservoir [2]. Second, there is an urgent need for an immunisation program for wild animals. Experience from other countries has shown that rabies elimination cannot solely rely on measures that include farm animals or domestic pets such as compulsory vaccination and/or the control of stray animal population [9]. Reducing population density through culling or sterilisation of the main wildlife reservoirs such as foxes has been the most important factor in rabies elimination in these countries [9]. Successive oral vaccination campaigns (supported by the European Community) using bait vaccines have

Box

Measures taken by the Greek national authorities to prevent and control the re-emergence of rabies, April 2013

Rabies alert issued to local veterinary and public health authorities, the forestry services and hunting associations.
Enhancement of existing rabies animal surveillance program and inclusion of the entire country.
High priority given to rabies immunisation of all stray cats and dogs in the affected prefectures[a] together with strict enforcement of the national law on compulsory rabies vaccination for pet animals.
Publication on the KEELPNO website (www.keelpno.gr) of educational material on rabies as well as information regarding pre- and post- exposure prophylaxis i.e guidance for healthcare workers on the response to human cases with potential exposure.
Creation of a network of reference hospitals for each of the seven administrative health districts of the country to respond to a potential human case along with setting up a stockpile of human rabies immuneglobulin and vaccine.
Creation of an inter-sectoral group at the Ministry of Health, consisting of epidemiologists, veterinarians, infectious disease and other public health professionals to provide continuous evaluation of the situation, scientific advice and risk assessment.
Prioritisation of preventive vaccination for specific high risk groups e.g. game wardens and veterinarians participating in the rabies surveillance program in animals.

[a] Prefectures with confirmed rabies in animals in October 2012 to end March 2013: Kozani, Kastoria, Kilkis, Pella, Trikala.

been successful in this regard in recent elimination efforts in some European countries for example Estonia but not in others e.g. Latvia and Lithuania [9]. Third, all domestic and stray animals especially in areas where sylvatic rabies is prevalent should be vaccinated; since the majority of the bites originated from stray dogs they should be targeted first. Unofficial information about illegal importation of unimmunised hunting dogs justify the implementation of strict border control, hygiene and immunisation checks and appropriate quarantine during the importation process of such animals according to relevant EU legislation [10]. In Greece, all imported dogs are checked for rabies immunisation with appropriate documentation together with antibody titers and if negative, entry to the country is not permitted. Other strategies pertaining to hunting animals such as the prohibition of hunting with dogs have not been discussed yet; nevertheless, the obligation to keep dogs on a leash is recommended. Fourth, the public needs to be aware about the potential for rabies exposures in areas where the virus circulates in wild animals. Pre-exposure vaccination for high risk groups is a priority in our targeted initial interventions. The travel health department of KEELPNO is advising for preventive measures e.g. avoiding contact with wild and domestic animals, special attention for children exposure and pre-exposure prophylaxis only for high risk groups (e.g. game wardens, hunters, veterinarians working in the field) travelling to the affected areas; it also encourages the use of post-exposure prophylaxis according to the WHO guidelines. Currently, the risk of rabies to travelers to Greece remains extremely low and so far only the northern part of the country is affected. Fifth, healthcare workers need to carefully evaluate each human exposure from a potentially rabied animal and take the appropriate actions. Since the human rabies immunoglobulin is expensive, a risk assessment as proposed by WHO [2] should guide a cost-effective approach in its administration.

Last but not least and since the disease was likely introduced to Greece by rabid wild foxes crossing borders in the north of the country, close collaboration with the neighboring countries is of paramount importance especially with regards to control measures in wild animals.

Rabies remains an infectious disease with almost 100% fatality. The reappearance of rabies in Greece highlights the importance of rabies control measures not only in our country and its neighbors but throughout Europe. Italy recently reported the re-appearance of rabies in wild animals and implemented an extensive array of measures [11-13]. The institution of appropriate measures through a cross-sectoral collaboration between veterinary and human health authorities, together with an educational campaign for the public, the veterinary community as well as healthcare workers, will be crucial and potentially effective in preventing the re-emergence of human cases in Greece and other European countries.

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Authors' contributions

Sotirios Tsiodras and Georgios Dougas prepared the first draft of this manuscript. Georgios Dougas, Agoritsa Baka, Theano Georgakopoulou, Asimina Balaska, Georgios Rigakos, Dimitrios Iliopoulos and Spyros Sapounas provided the surveillance and human exposure and prophylaxis data. Spyros Doudounakis, Charalambos Billinis, Vassileios Kontos, Konstantia E. Tasioudi, Myrsini Tzani, Paraskevi Tsarouxa, Peristera Iliadou and Olga Mangana-Vougiouka were part of the response team in the veterinary section and provided the animal rabies data. Spyros Doudounakis was responsible for the animal virological data. All authors were members of national or local committees taking the several measures regarding rabies response and reported data on taken measures and actions. All authors read and critically revised the first as well as the subsequent and final drafts of this manuscript.

Conflict of interest

None declared.

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Outbreak of listerosis due to imported cooked ham, Switzerland 2011

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From 24 April to 31 July 2011, nine cases of listeriosis were registered in the cantons of Aargau, Basel-Land and Zurich, Switzerland. In six of the cases, infection with *Listeria monocytogenes* was laboratory confirmed, while three remained suspected cases. The suspected cases were family members of confirmed cases with identical or similar symptoms. All confirmed cases were infected with a *L. monocytogenes* strain belonging to serovar 1/2a: all had an indistinguishable pulsotype by pulsed-field gel electrophoresis (PFGE). The same strain was detected in samples of cooked ham that were on sale from a particular retailer. Two samples of ham tested contained 470 and 4,800 colony-forming units (CFU) *L. monocytogenes* per gram respectively. Data of shopper cards from two confirmed cases could be evaluated: both cases had purchased the contaminated ham. The outbreak initiated a product recall and alert actions at national and European level, through the Rapid Alert System for Food and Feed (RASFF). Following the RASFF alert, the company producing the contaminated ham was inspected by the responsible authorities. Their investigations showed that the ham was not contaminated in the production plant, but in the premises of a company to which slicing and packing was outsourced.

Introduction

Infections with *Listeria monocytogenes* in animals have been known since the first studies in this field by Murray et al. in 1926 [1]. Soon after, the first sporadic infections in humans were detected [2]. In Switzerland, they have been reported mandatorily since 1975. At the beginning of the 1980s, the first outbreaks due to foods contaminated with *L. monocytogenes* were recognised in the United States: the foods concerned were coleslaw, pasteurised milk and Mexican-style soft cheese [1]. In Switzerland, beginning in 1983, an increase in the number of listeriosis cases was observed. After extensive outbreak investigations, an artisanal soft cheese (Vacherin Mont d'Or), produced in the winter months in the western part of the country, was identified as the source of infection [3].

The annual incidence of listeriosis in Switzerland has fluctuated over the last two and half decades, with a minimum of 0.3 cases per 100,000 inhabitants in 1990 and a maximum of 1.0 case per 100,000 inhabitants in 2006. The annual incidence in 2011 was 0.6 cases per 100,000 inhabitants [4].

During 1990 to 2008, the most prevalent serotypes were 1/2a and 4b. In contrast, other serotypes (1/2b, 1/2c, 3a, 4d) were comparatively rarely represented; only 1/b reached a prevalence of 15%, in 2007 [4].

In Switzerland, most cases of listeriosis are sporadic. An evaluation of patient data in the 1990s showed that in 115 (52%) of the 222 cases, an underlying disease was reported, 48 (22%) were mother–child cases, and in 59 (27%) of the cases, no underlying condition was reported. For pregnant women with the disease (n=28), the following symptoms were observed: abortion (n=8), endometritis (n=2), septicaemia (n=1), amnionitis (n=1) and minor symptoms such as gastroenteritis (n=8). Cases who were newborns (n=20) showed septicaemia (n=11), meningitis (n=3), pneumonia (n=3) and granulomatosis infantiseptica (n=2). For one newborn, there was no indication of symptoms. For patients who were neither pregnant women nor neonates (n=174), the following symptoms were reported: meningitis and meningo-encephalitis (40%, n=70), septicaemia (14% , n=25), pneumonia (11%, n=19), wound and joint infections (3%, n=5), endocarditis (2%, n=4), peritonitis (2%, n=3) and aggravation of general condition (2%, n=3). For 14 (8%) of the patients, minor symptoms such as diarrhoea occurred and in 31 (18%) of the cases, no symptoms were indicated [5].

Outbreaks of *L. monocytogenes* infections in Switzerland are rare and, until the outbreak described

in this report, had occurred only twice since 1983 [3,6]. Here, we describe an outbreak of listeriosis in 2011 due to contaminated imported cooked ham. The first notification of a possible ongoing outbreak was obtained through the mandatory reporting system for infectious diseases. The subsequent investigation was conducted mainly by the responsible food-control authorities, supported by other institutions. As outbreaks of listeriosis are almost exclusively food-borne [1], the aim of the investigation was to identify as quickly as possible the contaminated food-stuff that was the infection source, interrupt the infection chain and by taking adequate measures thereby re-establish food safety.

Methods

Outbreak case definition

A confirmed case was defined as person whose infection – reported to the Federal Office of Public Health – was laboratory confirmed as due to *L. monocytogenes* serotype 1/2a matching the outbreak pulsed-field gel electrophoresis (PFGE) pattern, with a test date between 24 April and 31 July 2011 in the cantons of Aargau, Basel-Land and Zurich, Switzerland.

A suspected case was a clinically compatible case of *L. monocytogenes* infection who had an epidemiological link to a confirmed case (family member), with a date of symptom onset between 24 April and 31 July 2011 in the cantons of Aargau, Basel-Land and Zurich, Switzerland.

The cases were detected through the mandatory reporting system for infectious diseases.

Patient interviews and evaluation of shopper cards

The occurrence of a cluster with four patients infected with *L. monocytogenes* 1/2a led the health authorities of canton Zurich to the decision to undertake patient interviews as soon as possible. Telephone interviews with seven patients (three couples and a single person), based on a standardised questionnaire, were carried out by a microbiologist of the local authority of food control in the canton of Zurich. The patients were asked which locations with collective catering (restaurant, party, etc.) they had visited in the two months before onset of symptoms. Furthermore, they were asked about their consumption habits during this time period, concerning categories of foods at major risk for the transmission of *L. monocytogenes* (raw milk, soft cheese, raw meat dishes, cured and fermented raw meat products and smoked fish). If high-risk products had been consumed, the interviewer tried to find out which brands had been purchased and from where. The patients were interviewed within 8 to 18 days after symptom onset.

In addition, data on shopper cards (client cards) of two of the couples were available for evaluation.

Microbiological tests

In the investigation, as a consequence of the interviews with the patients, samples of salami and samples of cooked ham were analysed. Testing for *L. monocytogenes* was done using the mandatory methods for official laboratories of food control based on the International Organization for Standardization (ISO) 11290-2 for quantitative detection [7] and ISO 11290-1 for qualitative detection [8]. Rapid detection was carried out by enrichment in half Fraser broth followed by real-time polymerase chain reaction [9]. Samples that were *L. monocytogenes*-positive by PCR were confirmed with culture tests according to ISO [8].

Serotyping was performed using a commercial set of Listeria O-factor and H-factor antisera from Denka Seiken (Pharma Consulting, Burgdorf, Switzerland).

Rapid alert system for food and feed (RASFF)

RASFF is a platform of the European Union (EU), used by the member countries for the exchange of information concerning foods and feeds that do not comply with the law. On the basis of a veterinary agreement, Switzerland is a part-member of RASFF and runs two official border inspection points at Zurich and Geneva airports and has therefore full access to border rejection notifications. Other notifications, such as alerts, are only distributed to the Swiss national RASFF contact point if Switzerland is directly concerned. This applies when a product has been delivered from an EU country to Switzerland or has been produced by a Swiss company.

PFGE genotyping and analysis

Basically, a previously described protocol was used [10] with the following minor modifications: use of SeaPlaque agarose instead of SeaKem agarose Gold; use of an additional 200 units (U) of achromopeptidase in the lysis mix; overnight lysis and DNA cleavage with 200 U of ApaI, 50 U of AscI or 50 U of SmaI. The PFGE was run at 14 °C for 20 hours with 6 V/cm under a linear ramp from 4 to 40 seconds using an angle of 120°.

For pattern comparison, BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium) was used. Pairwise similarities between the ApaI and AscI PFGE patterns were calculated using the JACCARD similarity coefficient. Clustering was based on the unweighted pair-group method with averages (UPGMA), setting tolerance and optimisation at 1% each. We used *Xba*I-digested DNA from *Salmonella* serovar Braenderup strain H9812 (ATCC BAA 664) as a fragment-size reference.

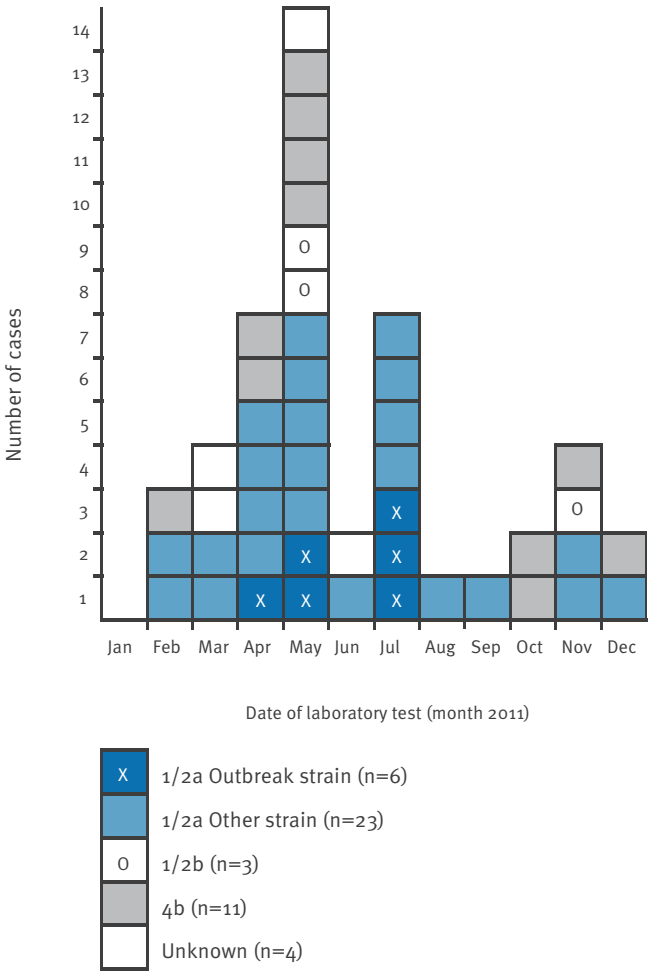
Results

Recognition of the outbreak

In May 2011, the Cantonal Ministry of Health in Zurich reported four laboratory-confirmed cases of listeriosis – occurring between 25 April and 5 May – in whom the *L. monocytogenes* isolate belonged to the serotype

Figure 1

Serotype distribution of listeriosis cases registered at the Federal Office of Public Health by date of laboratory test, Switzerland, 1 January–31 December 2011 (n=47)



1/2a. This represented a clear increase in the number of cases, compared with the normal epidemiological situation in the canton, where a mean of 0.42 listeriosis cases with *L. monocytogenes* serotype 1/2a were observed per month from January 2007 to December 2011 (unpublished data from the mandatory reporting system).

Analysis of all laboratory-confirmed listeriosis cases in Switzerland in 2011 showed that the dominant serotype that year was 1/2a (Figure 1).

On 6 June 2011, the national reference laboratory reported three of the four serotype 1/2a isolates to be indistinguishable by PFGE. This finding indicated a possible ongoing outbreak. Subsequent PFGE typing of all 1/2a isolates received by the reference laboratory allowed the detection of three more confirmed cases belonging to the outbreak.

Characteristics of patients

The infection of the six listeriosis patients was laboratory confirmed as *L. monocytogenes* 1/2a in April, May and July (Figure 1). The spouses of three of the confirmed cases (Cases 1–3) developed simultaneously the same or very similar symptoms as their partners. Case 1 experienced vomiting, diarrhoea and syncope (partner: vomiting and diarrhoea), Case 2 and partner had vomiting, diarrhoea, fever and shivering, and Case 3 had abdominal pain, diarrhoea, fever, headache and pneumonia (partner: nausea, abdominal pain, diarrhoea, fever, headache and vertigo). The partners of Cases 1–3 were suspected cases since *L. monocytogenes* was not isolated.

Five of the six laboratory-confirmed cases were aged 65 years or older; two of these elderly patients had underlying conditions (type-2 diabetes, asthma, heart disease, macrocythemia).

Outcome of patient interviews

Due to the unusual increase in the number of cases with *L. monocytogenes* 1/2a in April to May 2011 in Zurich, patients were interviewed. These interviews, with three confirmed cases and their spouses (suspected cases) and a single person, identified the following common behaviour: they purchased foods in shops of a particular retailer and consumed soft cheeses and meat products such as salami. All seven reported having eaten salami of a well-known brand that has a large presence on the Swiss market.

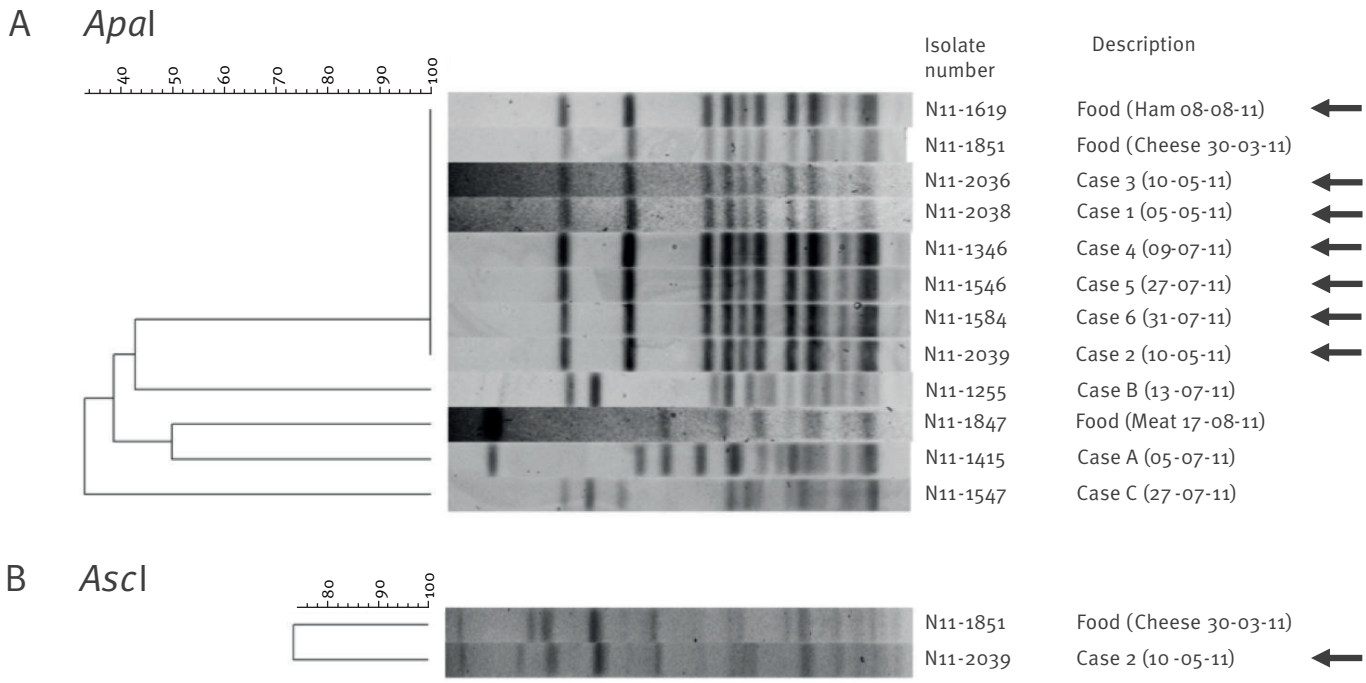
Outcome of bacteriological tests

Given the results of the interviews of the patients, 11 different salami varieties of the suspected producer were sampled at retail and tested by enrichment for the presence of *L. monocytogenes* in 25 gram of salami. None of these analyses revealed a positive result.

On 2 August, a retail company reported to the relevant local authority the finding of an *L. monocytogenes* isolate from a sample of cooked ham imported from Italy (Type A). The laboratory analysis, which revealed 4,800 colony-forming units (CFU) of *L. monocytogenes* per gram, was conducted as part of the retailer's routine quality control practices. On 3 August, the cantonal laboratory of food control took official samples of type A ham at retail. On 4 August, *L. monocytogenes* was demonstrated by rapid detection, and subsequent quantitative analysis revealed 470 CFU/g. On the basis of the analytical evidence, on the same day, recall action was undertaken and information for the public was issued by the retailer on the Internet and by press release. Also on the same day, the food control authority of the Ticino – the canton where the importer of the contaminated product was based – was informed and investigations were immediately undertaken. They ascertained the types and amounts of products that had been imported and also identified the distribution

Figure 2

Relationship of PFGE patterns generated with *Apa*I (panel A) or *Asc*I (panel B) of *Listeria monocytogenes* isolates from clinical (n=9) and food (n=3) samples, Switzerland, 30 March–17 August 2011



PFGE: pulsed-field gel electrophoresis.

Isolates belonging to the outbreak strain are marked with an arrow. The dates in parentheses are the dates of strain isolation.

network. It was found that the contaminated ham was produced exclusively for the Swiss retailer in question.

As part of the inspection of the importing company, samples of the suspected ham (Type A), of 'mortadella' (scalded sausage from pork originally produced in the region around Bologna, Italy) and another type of cooked ham (Type B) of the same producer were also taken and analysed for the presence of *L. monocytogenes*. These tests confirmed the known contamination of the type A ham and also demonstrated *L. monocytogenes* in Type B cooked ham (no quantitative data available). As a result of these findings, legal measures were enacted in order to stop the importation of the relevant products.

Evaluation of shopper cards

Since all the interviewed patients (n=7) reported being regular customers of the particular retailer, an evaluation of shopper cards (client cards) was carried out. This action required legal clarification and the agreement of the patients and the retail company. On 10 August, the data of two such cards became available, showing that two couples had purchased cooked ham of type A. Case 1 and partner had purchased the product on 21 April (onset of symptoms: 25 April) and Case 3 and partner on 28 April (onset of symptoms: 2 May). This added further evidence that cooked ham of Type A was the vehicle of the infection.

Reporting to the Rapid Alert System for Food and Feed (RASFF)

On 4 August, the Federal Office of Public Health reported the isolation of *L. monocytogenes* from the Type A ham to RASFF, which subsequently sent out an alert on 5 August. Further bacteriological findings were also reported to RASFF and initiated a follow-up alert (9 August) and a notification (18 August).

As a result of reporting the outbreak investigation data to RASFF, the producer in Italy carried out extensive investigations to find the source of the contamination. It was shown that the production processes and facilities conformed to legal requirements and that *L. monocytogenes* was not detectable in food or environmental samples. Further investigations finally traced the *L. monocytogenes* source to a company that, as an outsourced service, sliced and packed the meat products.

Typing of *L. monocytogenes* isolates

PFGE patterns of the L. monocoytogenes 1/2a strains isolated from six laboratory-confirmed cases of the outbreak (Cases 1–6) and from cooked ham are shown (Figure 2, panel A). For comparison, we also analysed several strains of *L. monocytogenes* 1/2a that were isolated between April and August and sent to the national reference laboratory for typing. The PFGE patterns of these strains, isolated from patients not involved in the outbreak (Cases A, B and C) and from a meat sample (N11-1847) were clearly different from that of the outbreak strain, showing relative relationships of less than 50%.

One strain, however, isolated from cheese (N11-1851) showed an ApaI profile indistinguishable from that of the outbreak strain. Therefore, the isolate from the cheese together with all isolates (clinical and from the ham) that belonged to the outbreak strain were subjected to additional PFGE analyses using the restriction endonucleases AscI and SmaI. All patterns generated by SmaI, including that of the cheese isolate, were indistinguishable (data not shown). However, the AscI pattern of the cheese isolate turned out to be different from the outbreak strain (Figure 2, panel B). The pattern for the isolate from Case 2 is shown as an example, but all isolates belonging to the outbreak strain were indistinguishable using AscI (data not shown).

The seventh patient interviewed was shown by PFGE not to be part of the outbreak (data not shown).

Discussion

Not surprisingly, the patients could not remember precisely where all their food products had been purchased several weeks before symptom onset. However, several food types known to be a risk in the context of listeriosis could be excluded and some common behaviours were identified. Notably, those interviewed purchased food mostly from one particular retail company and all stated having consumed meat products such as salami of a particular brand. As cooked ham was not considered a major risk for infection with *L. monocytogenes*, a question relating to ham consumption was not included in the questionnaire. This was a limiting factor and in future investigations, a questionnaire of higher discriminatory power should be used. Other limiting factors were that not all cases of the outbreak were interviewed and it is also not known whether those who bought the ham actually ate it.

In the case of food-borne outbreaks, the main objective is always rapid identification of the infectious source, thereby allowing the responsible authorities to re-establish food safety for consumers, often using a combination of epidemiological and microbiological techniques. In this outbreak, laboratory techniques played a key role as the isolation of *L. monocytogenes* from a quality-control sample of ham gave the crucial information. If a food company identifies contaminated products that may pose a risk for the consumers, the finding has to be reported to the authorities in charge, as decreed in Switzerland by the Ordinance on Foods and Utility Articles [11]. Analysis of the isolate from a cooked ham sample enabled the identification of the source of infection and origin of contamination. PFGE typing of an initial series of *L. monocytogenes* strains indicated that there was an ongoing outbreak. Use of three restriction enzymes increased the discriminative power of PFGE permitting the identification of small differences.

Evaluation of information from shopper cards has already been used in outbreak investigations [12]. In the outbreak reported here, the cooked ham that was suspected to be contaminated with *L. monocytogenes* could be identified on the cards of two couples. However, this information was only available when the source of the outbreak had already been identified. The reason for this delay was that this was the first time such cards had been used in a Swiss outbreak investigation and several aspects, such as legal questions, had to be clarified. The experience gained should help to speed up the procedure in future outbreak investigations in the country. We consider that evaluation of shopper cards is a powerful instrument, which should be a basic element of all outbreak investigations where commercial products are suspected to be the source of infections.

The data on the cards also allowed us to conclude that the incubation period for two confirmed cases and their partners must have been rather short (Case 1: purchase of ham on 21 April and onset of symptoms on 25 April; Case 3: ham purchased on 28 April and symptom onset on 2 May). For listeriosis, the incubation period is in the range of 3 to 70 days, with the median estimated to be 21 days [13]. The short incubation time and the more or less simultaneous onset of symptoms in the cases' partners indicates that the cooked ham may have been heavily contaminated with *L. monocytogenes* at the time of consumption. Cases 1 and 3 purchased cooked ham within a period of seven days. From the information on the shopper cards, it is not possible to say whether the purchased packs of ham belonged to the same lot. However, the short shelf life of cooked ham suggests that a particular lot, or two consecutive lots, were concerned.

In situations such as this, in which an imported meat product was found to be the source of the infectious agent, national authorities can decree certain measures that the importer should carry out, but have no jurisdiction over the producer. However, following the RASFF alert, relevant authorities in the country where the producer was based took certain actions and decreed that risk management measures be carried out. This allowed the identification of a company that sliced and packed the cooked ham as the origin of the contamination. The producer immediately stopped working with the company concerned, awarding the contract to another firm. After this measure, exportation to Switzerland was

possible again. Concerning contaminated foods and feeds, RASFF makes the rapid exchange of information between European countries possible. In the case of certain outbreak investigations, even closer cross-border information sharing (for example, exchange of bacterial isolates) would be useful. Currently, such a form of cooperation is not institutionalised in Europe and depends on the goodwill of the participating institutions and authorities.

In total, nine outbreak cases were detected over a period of around three months. This could indicate that the concerned company had a persistent hygiene problem in their facilities. However, the rather low number of human cases might suggest that the cooked ham was not contaminated at high levels throughout the entire period of the outbreak. This assumption is supported by the quantitative testing, which revealed 4,800 CFU of *L. monocytogenes* per gram in one sample of ham and only 470 CFU/g in another. In an earlier listeriosis outbreak in Switzerland, due to contaminated soft cheese, counts up to 32,000 CFU/g of *L. monocytogenes* per gram were found. This cheese caused 12 human cases of listeriosis in a shorter period, of about two months [6]. There are no dose-response data on *L. monocytogenes* infections in humans; however, a risk assessment showed that the vast majority of cases of listeriosis are associated with the consumption of foods that do not meet current standards for *L. monocytogenes* in foods (maximum 100 CFU/g) [14].

Meat products are known to be an important source of *L. monocytogenes*, leading to human infections [1]. With regards to ham, a retrospective case–control study in England, with people aged over 60 years, identified this product as a risk factor for listeriosis [15]. Furthermore, 'rillettes' – a spread prepared with ham and cooked with grease – was found to be the vehicle of a listeriosis outbreak in France [16]. To the best of our knowledge, the outbreak we describe here is the first in which of the vehicle of a listeriosis outbreak was shown to be sliced and pre-packed ham. In our opinion, the slicer exclusively processed for a large retailer in Switzerland and this was probably the reason why cases were not picked up elsewhere.

The slicing of meat products is a critical step in food production. It had been shown experimentally that *L. monocytogenes* from an inoculated slicer blade could be found on up to 30 slices of un-inoculated products such as turkey breast or salami [17]. *L. monocytogenes* is known to be a psychrotrophic microorganism, and strains particularly adapted to low temperatures are known [18]. In ready-to-eat salads, including smoked ham salad, growth rates of more than 0.5 log10 in 48 hours were demonstrated at storage temperatures of 7 °C [19]. For frankfurters kept at 8 °C, a 2 log10 increase of *L. monocytogenes* counts was demonstrated within 4 to 13 days of storage [20]. These facts illustrate the need for a proper evaluation of storage conditions (time and temperature) for products at risk. In households, eating perishable products within shelf-life dates and having correctly operating refrigerators are essential. Furthermore, persons with a compromised immune system and pregnant women should refrain from eating foods known to be at risk of contamination with *L. monocytogenes*. In hospitals, ready-to-eat meat products should only be served when the absence of *L. monocytogenes* can be guaranteed by an adequate quality control [21].

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Outbreak of leptospirosis among canyoning participants, Martinique, 2011

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Two gendarmes who participated in canyoning activities on 27 June 2011 on the Caribbean island of Martinique were diagnosed with leptospirosis using quantitative real-time polymerase chain reaction (qPCR), 9 and 12 days after the event. Among the 45 participants who were contacted, 41 returned a completed questionnaire, of whom eight met the outbreak case definition. The eight cases sought medical attention and were given antibiotics within the first week after fever onset. No severe manifestations of leptospirosis were reported. In seven of the eight cases, the infection was confirmed by qPCR. Three pathogenic *Leptospira* species, including *L. kmetyi*, were identified in four of the cases. None of the evaluated risk factors were statistically associated with having developed leptospirosis. Rapid diagnostic assays, such as qPCR, are particularly appropriate in this setting – sporting events with prolonged fresh-water exposure – for early diagnosis and to help formulate public health recommendations. Participants in such events should be made specifically aware of the risk of leptospirosis, particularly during periods of heavy rainfall and flooding.

Introduction

Over the past few decades, sporting events in tropical areas have become increasingly popular among travellers and athletes. At the same time, leptospirosis outbreaks following exposure to leptospires during recreational water activities, such as swimming, canoeing, kayaking or rafting, have been increasingly reported [1-5]. In Martinique – a Caribbean island and French overseas department and region with a tropical climate – we previously reported a leptospirosis outbreak among participants in a race that took place in the tropical forest after unusually heavy seasonal rainfalls [6].

Leptospirosis is a bacterial zoonosis, distributed worldwide, whose incidence is higher in the tropics, where transmission conditions are favourable. Many wild and domestic animals serve as reservoirs for pathogenic *Leptospira* strains. Humans are usually infected through abraded skin or mucous membranes in contact with water or soil contaminated by the urine of animal *Leptospira* reservoirs and, less frequently, by direct contact with infected animals or their urine. Heavy rainfall and flooding strongly increase human exposure to leptospires-contaminated water [7-9]. After a mean incubation period of 10 days (range: 2–30), clinical manifestations are protean and the spectrum of symptoms ranges from subclinical or mild, anicteric, febrile disease to multiorgan involvement associated with high mortality [8,10]. Asymptomatic infection is common and may occur in up to 60–70% of infected individuals, as was reported for serologically identified infections during a 1995 epidemic in Nicaragua and a 1999 outbreak in Thailand [11,12].

At present, although the microscopic agglutination test (MAT) and culture are the gold standard for diagnosis, only direct detection methods using polymerase chain reaction (PCR) can provide a rapid diagnosis during the early, acute stage of the disease [6,8]. Although the potential benefit of antibiotic treatment for leptospirosis has not been fully established by randomised placebo-controlled trials, antibiotics may at least decrease the duration of illness and they are commonly used to treat laboratory-diagnosed or even clinically suspected leptospirosis, whether mild or severe [8,9,13,14].

Outbreak detection

During 10 to18 July 2011, six adults presented at Trinité Hospital, on the east coast of Martinique, with fever associated with chills, headache, myalgia, elevated liver-enzyme levels, leukocytosis and

thrombocytopenia. During that period, two of them were diagnosed with leptospirosis, which was confirmed by quantitative real-time polymerase chain reaction (qPCR) based on the amplification of pathogenic *Leptospira* spp. in blood samples. The six patients were part of a group of 45 gendarmes, from a branch of the French Armed Forces, who had participated in a series of three canyon-rescue training exercises along the Absalon River between 27 and 30 June 2011. This river, located in the tropical forest, is very popular for canyoning activities.

Military medical officers notified the regional office of the French Institute for Public Health Surveillance (Institut de Veille Sanitaire, InVS) of these cases on 19 July. Immediately, an investigation was initiated in cooperation with the Department of Infectious Diseases of Fort-de-France Hospital, with the aim of: (i) informing all the participants of the potential risk of leptospirosis and the need to seek medical care if ill; (ii) assessing the magnitude of the outbreak; and (iii) identifying the risk factors and protective measures to be taken in order to make relevant public health recommendations. We report on the results of this investigation and discuss recommendations for preventing leptospirosis.

Methods

Epidemiological investigation

A list of all participants in the training exercises was obtained from the military medical officers. By the end of July, a letter, sent to all participants, informed them of their possible exposure to leptospirosis during their recent canyoning activities. They were advised to seek medical attention should they develop a fever (temperature greater than 37.8 °C) associated with two or more of the following symptoms or signs: chills, headache, muscle aches, joint aches, conjunctivitis, cough, diarrhoea or haemorrhaging. Accompanying the letter was a standardised questionnaire designed to obtain socio-demographic information (symptoms, specific activities and behaviour during canyoning – e.g. length of stay in the canyon, skin lesions, swallowing any river water, type of protective clothing worn during canyoning), possible exposure since 1 June (e.g. exposures associated with leisure or professional activities, contact with animals, gardening and any type of freshwater exposure) and previous antibiotic use. Moreover, the participants were asked if they had any knowledge of the risk of aquiring leptopirosis before their involvement in the activities. For those who sought medical attention, more detailed clinical information was obtained by reviewing medical records.

Outbreak case definition

A suspected case of leptospirosis was defined as a person having participated in one of the three canyoning activities and who reported a fever during 27 June to 30 July 2011, along with two or more of the above-mentioned symptoms.

A laboratory-confirmed case of leptospirosis required at least one of the following criteria: (i) positive qPCR; (ii) culture isolation of *Leptospira*; or (iii) positive MAT.

In this report, we use the term ‘cases’ to include suspected and laboratory-confirmed cases.

Non-cases were asymptomatic or pauci-symptomatic participants.

Laboratory investigation

Serum samples were obtained from participants who met the suspected case definition and who underwent a physical examination. Laboratory studies were performed by the Microbiology Unit of Fort-de-France Hospital (qPCR, culture and environmental investigation), and the National Reference Center and the WHO Collaborating Center for Leptospirosis at the Institut Pasteur, Paris, France (enzyme-linked immunosorbent assay (ELISA), MAT and genomic characterisation of *Leptospira* spp.).

Blood samples collected in ethylenediaminetetraacetic acid (EDTA) tubes obtained from patients during their first week of symptoms were tested by qPCR. After first concentrating the bacteria, DNA was extracted using the QIAamp DNA Mini Kit (Qiagen SA, Courtaboeuf, France). Then qPCR was performed on an iQTM5 real-time PCR detection system (Bio-Rad Laboratories, Marnes-la-Coquette, France) using the DNA-binding dye technique (SYBR Green). The primer set consisting of LFB1-F (5’ CATTCATGTTTCGAATCATTTCAAA 3’) and LFB1-R (5’ GGCCCAAGTTCCTTCTAAAAG 3’) used target DNA from pathogenic leptospires and amplified a 331 base pair (bp) fragment [15]. To control for DNA extraction and to detect the presence of PCR inhibitors in DNA extracts, we amplified a fragment of the hypoxanthinephosphoribosyl-transferase (HPRT), as an internal control. A PCR was considered as negative when we were not able to detect the target PCR product but able to detect the PCR internal-control product.

A partial sequence of the *Leptospira rrs* gene from positive blood samples was amplified by nested-PCR using Taq polymerase and primers A/B, then C/RS4 [16,17]. Sequencing was performed at the Genotyping of Pathogens and Public Health Platform (Institut Pasteur, Paris, France) and sequences were aligned in GenBank using the Basic Local Alignment Search Tool (BLAST) to identify the species.

Blood samples were tested at the Institut Pasteur with an in-house IgM ELISA and MAT using 24 antigens. Serovars included in the MAT screening panel were based on prior knowledge of regional epidemiology (Table 1). For MAT, a titre greater than 100 against any of the pathogenic antigens was considered positive if a patient’s origin was from a non-endemic area. For patients living in an endemic area, such as the

Table 1

Panel of *Leptospira* serogroups used in microscopic agglutination testing, leptospirosis outbreak among canyoning participants, Martinique, 2011

Leptospira species	Serogroup	Serovar	Strain
L. interrogans	Australis	Australis	Ballico
L. interrogans	Autumnalis	Autumnalis	Akiyami A
L. interrogans	Bataviae	Bataviae	Van Tienen
L. interrogans	Canicola	Canicola	Hond Utrecht IV
L. borgpetersenii	Ballum	Castellonis	Castellon 3
L. kirschneri	Cynopteri	Cynopteri	3522 C
L. kirschneri	Grippotyphosa	Grippotyphosa	Moskva V
L. interrogans	Sejroe	Hardjobovis	Sponselee
L. interrogans	Hebdomadis	Hebdomadis	Hebdomadis
L. interrogans	Icterohaemorrhagiae	Copenhageni	Wijnberg
L. noguchii	Panama	Panama	CZ 214 K
L. biflexa	Semaranga	Patoc	Patoc 1
L. interrogans	Pomona	Pomona	Pomona
L. interrogans	Pyrogenes	Pyrogenes	Salinem
L. borgpetersenii	Sejroë	Sejroë	M 84
L. borgpetersenii	Tarassovi	Tarassovi	Mitis Johnson
L. interrogans	Icterohaemorrhagiae	Icterohaemorrhagiae	Verdun
L. weilii	Celledoni	ND	2011/01963
L. interrogans	Djasiman	Djasiman	Djasiman
L. borgpetersenii	Mini	ND	2008/01925
L. weilii	Sarmin	Sarmin	Sarmin
L. santarosai	Shermani	Shermani	1342 K
L. borgpetersenii	Javanica	Javanica	Poi
L. noguchii	Louisiana	Louisiana	LUC1945

ND: Not determined.

Caribbean, a titre of 400 was used, given possible past exposure.

Leptospira spp. were cultured by inoculating 1 ml of heparinised plasma into 10 ml of Ellinghausen-McCullough-Johnson-Harris (EMJH) media, which were incubated at 30 °C and observed weekly for growth for two months.

Environmental investigation

The Absalon River receives run-off from several houses and some family-owned livestock farms. Surface water samples collected from the river, three and seven weeks after the last training exercise were passed through 0.22 micron filters to remove potential environmental contaminants and were cultured in EMJH media for the presence of leptospires. The filters were crushed and incubated overnight in distilled water. DNA was extracted from the supernatant and qPCR were performed using the protocol described above.

Weather data, including the amount of rainfall during June 2011 in Martinique, were obtained from Météo France, the French national meteorological service.

Statistical analysis

Exposure information and clinical data and laboratory results, when available, were entered into EpiData version.3.1 and analysed with Stata version 9.0 software (College Station, Texas, United States). Categorical variables were expressed as numbers (%) and continuous variables as medians (range).

The relationships between case status (cases versus non-cases) and risk factors were tested in univariate analyses using Fisher's exact test and the Mann–Whitney U test. A p value ‹0.05 defined significance. The effect of each exposure variable was quantified by estimating the relative risk (RR) and its respective 95% confidence interval (CI).

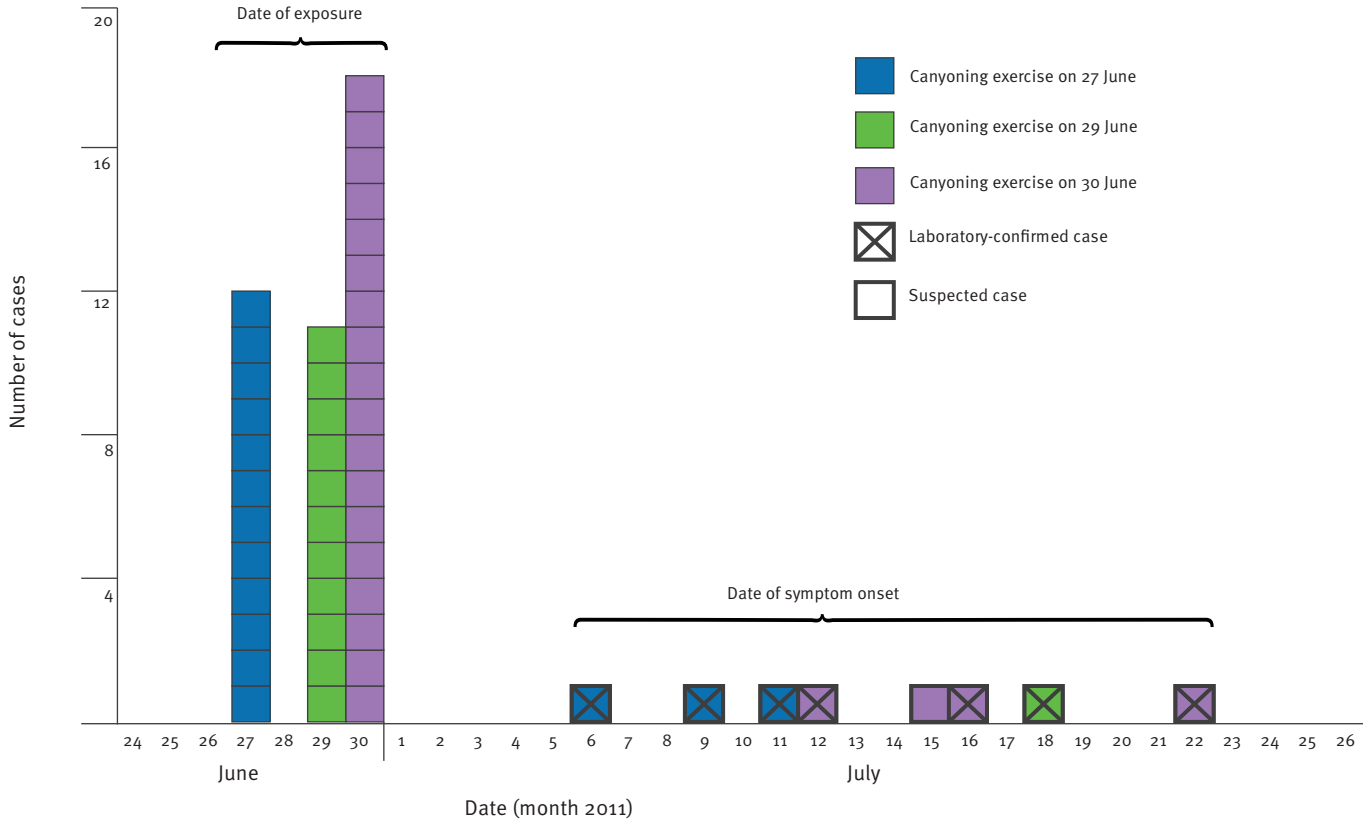
Results

Epidemiological investigation

Among 45 participants in the canyon-rescue training exercises, 41 returned a completed questionnaire. Of these, 39 were male; their median age was 33 years (range: 20–53).

Figure

Training exercises[a] and symptom onset for cases (n=8), leptospirosis outbreak among canyoning participants, Martinique, 2011



[a] Information from 41 participants who completed a questionnaire.

All participants who returned a completed questionnaire were French: 30 lived in Martinique and 11 came from a metropolitan region in mainland France. The median length of time spent in Martinique, for the 26 respondents for whom the information was available, was 1.27 years (range: 0.15–6.03). Of the 45 participants, 16 had participated in the training exercises on 27 June, 11 on 29 June and 18 on 30 June 2011; four had participated in two of the exercises (on 27 and 30 June).

Among the 41 persons who returned a completed questionnaire, eight (attack rate 20%) were suspected cases; their median age was 35.5 years (range: 23–53). All eight were men; seven lived in Martinique and one in a metropolitan region in mainland France. We did not identify any participants who partly met the outbreak case definition (e.g. had fever but no other symptoms). Cases and non-cases were comparable by age, sex, place of residence and length of time spent in Martinique (data not shown).

The eight cases participated in only one of the three training exercises: three in that held on 27 June, one in that on the 29th and four in that on the 30th (day-specific attack rates of 19% (3/16), 9% (1/11) and 22% (4/18), respectively).

Of the eight cases, fever onset was clustered from 6 to 22 July (Figure). The median incubation period, determined from each participant's specific day of canyoning activities, was 14.5 days (range: 9–22). The eight cases sought medical attention at a median of 1.5 days (range: 0–6) after symptom onset. Beta-lactam antibiotics were prescribed for all eight; two were hospitalised for three days. No severe manifestations of leptospirosis were reported. The most common symptoms reported by the eight patients were: fever (n=8), asthenia (n=7), chills (n=7), headache (n=6), muscle aches (n=6), nausea (n=4), joint pain (n=3) and conjunctival suffusion (n=1). At admission, three had elevated liver enzyme or bilirubin levels, two were thrombocytopenic, one had leukocytosis and serum creatinine was elevated in one. C-reactive protein, with a median of 79 mg/L (range: 12–237), was high in all eight patients (norm: 1–10 mg/L).

Table 2

Univariate analysis of investigated factors associated with confirmed (n=7) or suspected (n=1) cases, leptospirosis outbreak among canyoning participants, Martinique, 2011

Risk or protective factor	Number who responded to the particular question[a]		Number who answered yes		Relative risk (95% CI)	P value
	Cases n=8	Non-cases n=33	Cases n=8	Non-cases n=33		
Swallowed river water	4	23	3	10	3.23 (0.38–27.3)	0.326
Had skin abrasions	6	28	3	12	1.27 (0.30–5.40)	1.000
Wore neoprene suit[b]	8	33	0	4	–	0.569

[a] A total of 41 participants, including the eight cases, completed the questionnaire.
[b] Complete neoprene diving suits.

None of the eight cases reported any other canyoning or other potential exposure during leisure or professional activities during the month preceding the training exercises. In a univariate analysis, none of the potential risk or protective factors investigated was significantly associated with leptospirosis (Table 2). Finally, only 15 of the 33 respondents who answered the particular question declared having some knowledge of leptospirosis before the canyoning exercises.

Laboratory investigation

Serum samples were obtained from the eight participants who met the suspected case definition, with a median of two days (range: 0–6) after symptom onset. Acute- and convalescent-phase serum specimens were obtained from only two patients (Cases 4 and 7).

Leptospira infection was confirmed by qPCR in seven of the eight cases (Table 3). ELISA was also positive for three cases (Cases 1, 2 and 7). The acute-phase serum specimen from Case 6 had a negative ELISA and MAT.

Leptospira cultures were negative for two patients tested (Cases 4 and 8). For the other patients, by the time we had the diagnosis, they had received antibiotics, after which point culture is usually not possible.

Sequencing of the *Leptospira* 16S rDNA gene indicated that four cases (Cases 2, 3, 7 and 8) were infected with three genomospecies: *L. santarosai* (two cases), *L. kmetyi* and *L. kmetyi*-like. MAT of Case 1's isolate showed agglutination with the serogroup Celledoni.

Table 3

Laboratory results for leptospirosis outbreak among canyoning participants, Martinique, 2011 (n=8)

Case number	Date of sampling in 2011 (number of days after symptom onset)	qPCR	ELISA titre	MAT titre (serogroup)	Genomic species
1	10 July (4)	Positive	6,400	400 (Celledoni)	ND
2	10 July (1)	Positive	400	50 (Patoc)	*L santarosai*
3	13 July (2)	Positive	NT	NT	*L. kmetyi-like*[a]
4	18 July (0)	Positive	0	0	ND
5	18 July (2)	Positive	NT	NT	ND
6	19 July (4)	Negative	0	0	NT
7	18 July (6)	Positive	1,600	800 (Patoc)	*L. kmetyi*[b]
8	23 July (1)	Positive	0	0	*L santarosai*

ELISA: enzyme-linked immunosorbent assay; MAT: microscopic agglutination test; ND: not determined; NT: not tested; qPCR: quantitative real-time polymerase chain reaction.

[a] Sequencing of the 279 nucleotide 16S rRNA PCR product showed similarity to *L. kmetyi* (273/279 nucleotides) and *L. kirschneri* (272/279 nucleotides).
[b] The 279 nucleotide 16S rRNA PCR product was identical except for two mismatches (277/279 nucleotides) to the corresponding variable region of the 16S rRNA sequence of the *L. kmetyi* reference strain

Environmental investigation

We obtained two sets of water samples from the river where the canyoning took place. All the cultures and the qPCR assays were negative or could not be analysed because of the presence of inhibitors, despite numerous filtrations to remove environmental contaminants.

According to Météo France, the total rainfall recorded in the main lowland of Martinique (which is the nearest place for rainfall recording) during the first six months of 2011 was the highest since 1981 [18]. Moreover, heavy rainfall was recorded in late June. At the same time, the temperatures recorded in June 2011 were the highest since 1971, with a mean maximum of 32.1 °C in the lowland [18].

Discussion

Epidemiology and risk factors

On the basis of our epidemiological investigation, exposure to Absalon River water was the most likely source of *Leptospira* infection. The attack rate of 20% could be explained by a combination of factors, such as: prolonged immersion in water, causing softening and wrinkling of the skin; unavoidable skin and mucous membrane exposure; and probably a higher concentration of leptospires in the river after the unusually heavy rainfall and high temperatures at the end of June. The investigation was conducted as part of a public health response and therefore laboratory analysis was carried out only on samples from symptomatic participants. However, these patients might represent only a small percentage of the infections, as in the majority of persons infected with leptospires, the infection remains subclinical and undiagnosed [8,11]. In previous leptospirosis outbreaks following exposure during recreational water activities, observed attack rates ranged from 12% to 42 % [1,2,4,5].

Although none of the potential risk or protective factors investigated was significantly associated with leptospirosis, it should be noted that: (i) the response rate for some questions was low; (ii) those who reported having swallowed river water had three times the risk of developing leptospirosis compared with those who did not, although it remained not significant; (iii) 15 of 34 respondents reported having skin wounds during the canyoning exercises; and (iv) only four participants reported wearing complete neoprene suits that protected their arms and legs: none of them developed leptospirosis.

Together with being submerged in water, swallowing water potentially contaminated by leptospires was demonstrated to be a risk factor for leptospirosis among military personnel participating in several sporting events, in Okinawa, Japan, and persons drinking water from an Italian fountain [2,4,5,19,20]. In the outbreak reported here, *Leptospira* might also have been contracted via exposure of the conjunctivae and wrinkled, softened skin, as described in other water sports [3].

Although it has been described in recreational settings, leptospirosis in military personnel is mainly an occupational disease and outbreaks have been reported after training exercises in various areas, such as the high jungle rainforest of Peru, Japan, and northern Israel near the Jordan River [21-23]. As reported for other outbreaks related to military training exercices or sports events, young men in the Martinique outbreak reported here were predominantly affected [2,4-6]. However, the numbers of persons potentially exposed were low, comprising only a few women. None of those who wore protective clothing were women.

The presence of inhibitors in the PCR and cultures, or a bacterial load below the detection threshold, could explain the negative results obtained for environmental samples. Environmental investigations of other leptospirosis outbreaks (in which lake, creek and swamp water were sampled) in Illinois and Florida in the United States, samples tested negative, despite epidemiological evidence of widespread *leptospira*l contamination of the water and the surrounding environment [2,5]. As reported by Morgan et al., results of screening large bodies of freshwater for leptospires should not guide public health authorities in making decisions regarding the safe recreational use of water [2].

The end of June, 2011, when the canyoning exercises took place, was characterised by unusually heavy rainfall and high temperatures. In the Caribbean, as in other parts of the world, leptospirosis outbreaks have been reported after periods of heavy rainfall, flooding, and hurricanes [24,25]. In tropical areas where leptospirosis is endemic, it is thought that heavy rain or flooding saturates the potentially contaminated soil and the rising water level carries pathogenic leptospires to the surface of the land and rivers [7]. Outbreaks specifically associated with endurance and water sports events following periods of heavy rainfall have been reported in Costa Rica, the United States, Malaysian Borneo and Martinique [1,2,4-6].

Laboratory aspects

Blood samples for qPCR-based diagnosis were collected after a median of two days after symptom onset. As the qPCR assay used can be completed in around five hours (data not shown), information about the specific risk of leptospirosis was given to all participants within one week after confirmation of the first cases. In the context of an outbreak, MAT confirmation can be impeded by the absence of convalescent-phase samples, the need for reference laboratories, or the lack of sensitivity for region-specific serovars not represented in the test's antigen battery.

As we previously reported for a sporting event in Martinique, direct qPCR allows an unequivocal diagnosis based on a single specimen during the early acute

phase of illness – when treatment is most effective – and before serological and/or culture results become available [6,7,26,27]. Real-time PCR assays are being used increasingly to diagnose leptospirosis [28]. A recent study using a well-characterised cohort of laboratory-confirmed leptospirosis patients in Sri Lanka demonstrated not only the strength of the qPCR assay for early diagnosis, but also that the qPCR window of positivity ranged from day 2 to day 15 after symptom onset [29].

In the same samples used for qPCR-based diagnosis, we identified three *Leptospira* species. The identification of three different *Leptospira* species by sequencing could suggest multiple exposures in a risky environment, reflecting the diversity of potential wild and domestic reservoir animals in tropical areas. During 2010 to 2011, MAT on human serum samples showed that the most prevalent *Leptospira* serogroups in Martinique were Icterohaemorrhagiae (36%), Sejroe (16%), Ballum (12%), Canicola (12%), and Pyrogenes (8%) (data from the National Reference Center for Leptospirosis, France). In our study, blood samples were collected during the first week of the disease, and only two cases' convalescent samples were available for conclusive testing. Therefore, despite the availability of a regionally optimised MAT panel, only one serogroup was identified.

As culture usually has a low sensitivity, it was not surprising that the results were negative. Moreover, the 1:10 dilution we used for culture may have impaired the technique.

Prevention

Following this leptospirosis outbreak in Martinique, health authorities informed all the organisers of canyoning activities on the island about the risk of leptospirosis and the need to inform participants about preventive measures [30]. Considering the inevitability of water exposure during canyoning activities and the higher risk of leptospirosis in tropical areas, all participants should be informed about individual preventive measures [1,3]. Preventive measures for participants in high-risk water activities in military or civilian settings are summarised in the Box. In our study, known risk factors, such as swallowing river water or skin abrasions, were not significantly associated with leptospirosis, but we may not have had sufficient data to detect significance. We can only hypothesise that the use of protective neoprene suits during canyoning might be protective. After a leptospirosis outbreak in workers who participated in cleaning a pond in Thailand, an investigation found that clothing was protective, while the presence of skin wounds was associated with infection, confirming that breaks in the skin serve as portals of entry for the leptospires [12].

None of the leptospirosis vaccines currently available is associated with protection against the wide diversity of serovars encountered in the tropics. The vaccine available in France confers protection only against the serovar Icterohaemorrhagiae. The role of such immunisation to prevent leptospirosis in people who participate regularly in water activities in endemic areas warrants further study. To the best of our knowledge, this could be the first identification of *L. kmetyi* infection in humans, although this finding needs confirmation by genomic identification based on a positive culture.

For residents and travellers who visit endemic areas participating in recreational activities with prolonged water exposure, leptospirosis could theoretically be prevented by antibiotic chemoprophylaxis. The potential protective effect of antibiotics could not be assessed in our study because none of the participants reported any ongoing or recent antibiotic treatment. To date, three randomised clinical trials evaluated whether the use of doxycycline can prevent leptospirosis, either as pre-exposure (weekly administration) or post-exposure (single dose) prophylaxis. Taken together, the data does not support such prophylaxis in all cases, although short-term travellers with high-risk exposure may be helped [31-34]. To date, neither prophylaxis schedule has been evaluated in the setting of sporting events in the tropics. On the basis of the high leptospirosis attack rate among athletes who participated in an endurance race in Borneo in 2000, the United States Centers for Disease Control and Prevention recommended that all ill athletes be treated empirically with doxycycline and that asymptomatic athletes discuss the possible merits of a single doxycycline dose for post-exposure prophylaxis [4]. In the outbreak in Martinique reported here, no such chemoprophylaxis was offered, but participants were advised to seek medical attention and testing with rapid diagnostic assays to confirm the diagnosis and hence treat leptospirosis early during the disease course.

Box

Recommendations regarding high-risk water activities in places such as rivers, lakes or swamps: what to do before, during and after the activities in order to prevent and promptly treat leptospirosis

Before
When possible, avoid any water activities the days following heavy rainfall and/or flooding.

During
Prevent skin abrasions by wearing appropriate clothes (e.g. protective neoprene suits during canyoning), avoid mucous membrane exposure when possible and avoid swallowing the water.

After
Rinse any abrasions or lacerations with clean water and antiseptics. Seek medical attention if fever occurs during the three weeks following the water activities.

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Déjà vu: *Ralstonia mannitolilytica* infection associated with a humidifying respiratory therapy device, Israel, June to July 2011

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Following a bloodstream infection in June 2011 with *Ralstonia mannitolilytica* in a premature infant treated with a humidifying respiratory therapy device, an investigation was initiated at the Hadassah Medical Centres in Jerusalem. The device delivers a warmed and humidified mixture of air and oxygen to patients by nasal cannula. The investigation revealed colonisation with *R. mannitolilytica* of two of 15 patients and contamination of components of five of six devices deployed in the premature units of the Hadassah hospitals. Ten isolates from the investigation were highly related and indistinguishable from isolates described in an outbreak in 2005 in the United States (US). Measures successful in containing the US outbreak were not included in user instructions provided to our hospitals by the distributor of the device.

Introduction

In June 2011, we encountered a case of bloodstream infection due to a Gram-negative bacillus in a premature infant who failed to respond to the initial antibiotic combination of ampicillin, cefotaxime and gentamicin, and later to meropenem. The organism was identified by molecular analysis as *Ralstonia mannitolilytica*. It proved to be resistant to all the above antibiotics and the child recovered after the treatment was changed to co-trimoxazole. The child was treated with a respiratory therapy device for three weeks, during which time the bloodstream infection developed. *R. mannitolilytica* won some notoriety in an outbreak associated with the use of this device in the United States (US) in 2005 [1]. After an initial investigation by the Centers for Disease Control and Prevention (CDC) revealed deficiencies in the reprocessing of the device at that time, it was recalled by the manufacturer in January 2006 and reintroduced a year later with a revised procedure for decontamination and reprocessing approved by the US Food and Drug Administration (FDA).

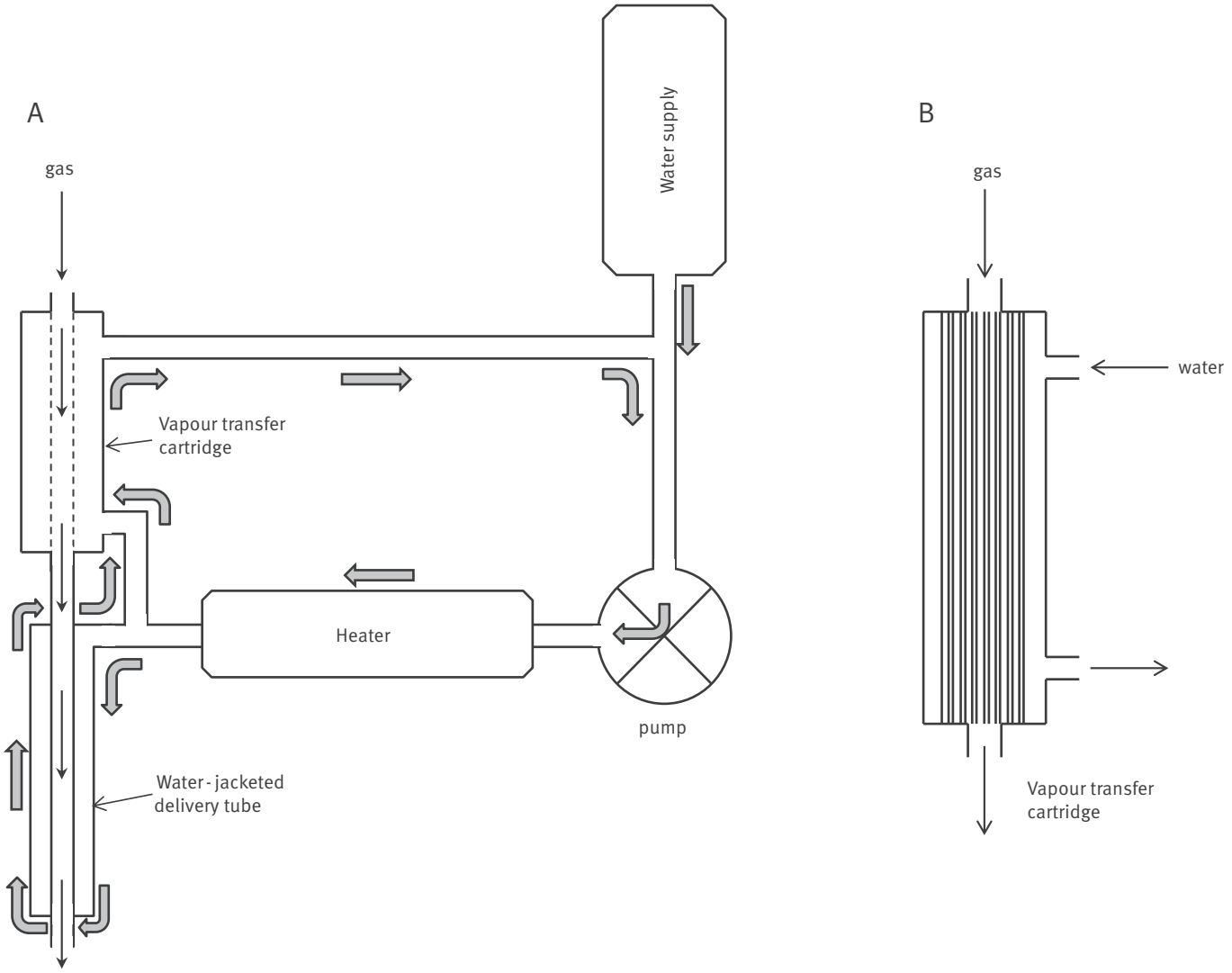
Organisms of the genus *Ralstonia* [2] are aerobic Gram-negative bacilli commonly found in water and other moist environments [3], but isolated infrequently from clinical material, and then mostly in connection with healthcare-associated infections with environmental sources [4]. In recent years *Ralstonia* spp. have been recognised increasingly in sputum from cystic fibrosis patients [5,6]. The species *R. mannitolilytica* [7] has rarely been reported as a cause of human disease, but has been implicated in clusters related to suspected contamination of medical solutions or equipment [1,8-10] as well as among the *Ralstonia* spp. in sputum of patients with cystic fibrosis [7,11,12].

The Vapotherm 2000i (Vapotherm, Stevensville MD, US) is a system for delivering a warmed and humidified oxygen-air mixture to patients, mostly infants, via nasal cannulae (Figure 1). The conditioning of the gases occurs in a vapour transfer cartridge. The system was introduced at our medical centre in January 2007, contemporaneously with its reintroduction to the US market following the outbreak of contamination with *R. mannitolilytica* [1,8,13].

The Hadassah Medical Centre comprises two hospitals, one at Ein Kerem, with approximately 750 beds, and the other at Mount Scopus with about 300 beds. Both have Premature Baby Units, with the primary facility located at Mount Scopus. As soon as the identity of the blood culture isolate was established, and in light of the US experience, the four Vapotherm devices in use at Mount Scopus and the two deployed at Ein Kerem were withdrawn from use. An investigation was initiated to determine whether an environmental source could be found for the organism and whether more infants had been colonised or infected.

Figure 1

Flow diagram of the Vapotherm 2000i system (A) and section of vapour transfer cartridge (B)



Panel B illustrates the hundreds of hollow fibers through which the gas passes surrounded by the humidifying water.

Methods

Patient samples

Fifteen premature infants, including the index patient, were present in the Mount Scopus unit when the aetiological diagnosis of the bloodstream infection was confirmed. Nine of these had been treated at various times with the Vapotherm 2000i. Blood cultures are taken frequently from premature babies, but *R. mannitolilytica* was recovered from blood only from the index patient. Cultures were taken from the nares and oral cavity of all 15 infants.

Blood cultures were performed using the Bactec 9240 system (Becton Dickinson, Sparks MD, US). Nasal and oral cultures were collected from the 15 premature infants hospitalised at Mount Scopus using synthetic sterile swabs transported in Amies's medium (Copan, Brescia, Italy), plated on MacConkey agar and tryptic soy agar (Novamed, Jerusalem Israel), incubated initially at 37° C overnight and then at room temperature.

Environmental samples

Maintenance and reprocessing of the system, including disinfection of the cartridges, were carried out according to instructions provided in Hebrew by the local distributor. Sterile water for the humidification and warming functions of the system was provided in semi-rigid plastic containers manufactured by the Hadassah Pharmacy Service. Vapour transfer cartridges were disinfected between patients or discarded after two months of use. Cartridges that were used on patients with clinical infection were discarded. All tubing was replaced for each new patient. External cleaning and

decontamination of the devices was carried out in accordance with local procedures.

Methods for culture of components of the Vapotherm 2000i system were adapted from those employed by the CDC during the US investigation [1]. External surfaces of the device were not sampled for culture, the focus being placed on the tubing and the humidifying cartridges. Cultures were taken from the Vapotherm delivery tube in use on the index patient. The gas line was sampled under aseptic conditions by flushing with 45 mL of sterile water into a sterile filtration device designed for microbiological sampling (Millipore Microfil system, Millipore, Billerica MA, US). The filter membrane was placed aseptically on tryptic soy agar (Novamed, Jerusalem, Israel) and incubated as above. The water from the outer sleeve of the delivery tube was removed aseptically and filtered and cultured in the same way.

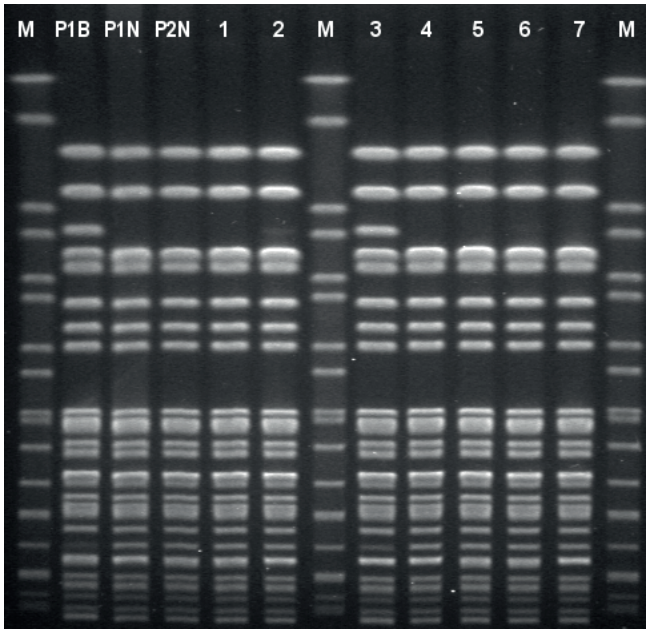
All six vapour transfer cartridges in use at the time when the devices were removed from service (four from Mount Scopus and two from Ein Kerem) were sampled as for the gas line. The cartridges had been in use for periods of time varying from a few days to three weeks. In addition, three unopened cartridges remained in stock, all from the same batch. A sample from one of these was cultured and the remaining two were aseptically dismantled and attempts made to extract bacterial DNA from their contents for 16S rRNA analysis. The sterile water used for the cultures was the same product used for humidification in the operation of the Vapotherm 2000i. This water was produced at that time in the hospital's pharmacy production facility and provided in sterile 1 L plastic containers. The contents of two of these containers were cultured during the investigation, by passing the whole fluid volume through the filters.

Identification and typing

Identification of the index patient's blood culture isolates and the initial environmental isolates from the delivery tube of the index case was attempted using the API 20NE kit (BioMerieux, Marcy l'Etoile, France). Identity was established for all isolates by 16S rRNA gene sequencing [14] followed by sequence comparison using the Basic Local Alignment Search Tool (BLAST) and non-redundant (nr) database from NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). These and all other isolates were then characterised at the Molecular Epidemiology Laboratory of the Israel Ministry of Health by pulsed-field gel electrophoresis (PFGE) using SpeI, according to the PulseNet protocol [15]. Comparison with the isolates of *R. mannitolilytica* from the US outbreak in 2005 was performed bilaterally by the Ministry of Health laboratory and the US CDC, using the BioNumerics 6.5 software package (Applied Maths NV, Sint-Martens-Latem, Belgium) with dice coefficients, a 1% position tolerance and optimisation values. Cluster analysis was performed by the unweighted pair-group mean analysis (UPGMA).

Figure 2

Pulsed field gel electrophoresis of *Ralstonia mannitolilytica* isolated from patients and Vapotherm devices, Israel, June–July 2011 (n=10)



M: Size marker; P1B: blood culture isolate from index case; P1N: nasal isolate from index case; P2N: nasal isolate from second infant; 1: isolate from gas line of delivery tube; 2: isolate from water jacket of delivery tube; 3-6: isolates from four used vapour transfer cartridges from the Mount Scopus hospital; 7: isolate from a used vapour transfer cartridge from the Ein Kerem hospital.

Results

Microbiological investigation

The isolates recovered from the index patient's blood cultures were oxidase-positive and colistin-resistant. All had an API 20NE profile at 48 h of 0045555, giving low probability identifications of *Pseudomonas fluorescens* or *Ralstonia pickettii*, with *Ochrobactrum anthropi* as an additional possibility. BLAST analysis of a 730 bp 16SrRNA gene sequence revealed that the isolate was a *Ralstonia* species, most similar to *R. mannitolilytica*, and the organism was indeed mannitol-positive. This species had not previously been identified at our hospitals. Subsequently, the identity of the isolates was further confirmed by MALDI-TOF mass spectrometry (Vitek MS, BioMerieux, Marcy l'Etoile, France) as *R. mannitolilytica* (99.9%).

Nine of the 15 patients including the index case had been treated with the Vapotherm 2000i at different times during their hospital stays. *R. mannitolilytica* was isolated from the blood and nose of the index case and the nose of one additional child who had been treated with the device (each child had their own cartridge and

tubing, which were kept until the treatment course was completed). In addition to the three patient isolates, *R. mannitolilytica* was obtained from seven environmental samples: from the index patient's delivery tube (both from the gas line and the water jacket), from all four vapour transfer cartridges from Mount Scopus and from one of the two from Ein Kerem. Culture of the unopened cartridge and of the sterile water supplied by the hospital was negative. No bacterial DNA was recovered from the contents of the remaining two unopened cartridges (of the same batch).

Eight of the 10 patient and environmental isolates from the two hospitals were found identical by PFGE (Figure 2) using accepted criteria [16], while two showed the same single-band difference, namely one of the two isolates from the index patient and one of the cartridge isolates from Mount Scopus.

Comparison with the 2005 outbreak in the United States

Apart from the use of an unfiltered instead of a filtered sterile needle to vent the sterile water containers, no significant deviations from the instructions were identified in the reported reprocessing procedure. Examination of the dates of manufacture of the cartridges revealed that at least two of the recently used lots were manufactured in October 2005, before the recall of the system in the US.

Inspection of the PFGE image published in the report of the 2005 US outbreak suggested some similarity between our isolates and those of the US outbreak [1] (Figure 3). Since standardised PulseNet protocols had been used in both investigations, a comparison was therefore undertaken by the Israel Ministry of Health Molecular Biology Laboratory in collaboration with investigators at the CDC who had studied the US event. The comparison included 16 representative US 2005 outbreak-associated isolates and four unassociated isolates. The analysis showed that the eight identical isolates from Israel 2011 were indistinguishable from the US 2005 outbreak isolates, which originated from 22 hospitals in 13 US States, while the two single-band variants showed more than 97% similarity with these (Figure 3). Those isolates in the US study that were unassociated with the outbreak were unrelated, with less than 80% similarity.

Discussion

We have described a cluster of *R. mannitolilytica* isolates that were detected following a bloodstream infection in a premature infant, associated with colonisation of an additional patient and contamination of components of the Vapotherm 2000i device. The outstanding finding of this study was the possibility that the same organism was responsible for two events of contamination separated geographically and temporally by several years (Figure 4).

Although no final conclusion was reached in the US investigation as to the origin of the organism, our combined data strongly suggest that the organisms isolated from both events had the same source. Nevertheless, the question as to what this source was will probably remain unresolved.

While in the CDC investigation in 2005, *R. mannitolilytica* was not isolated from 26 unopened vapour transfer cartridges representing 13 lots, the organism was isolated from such cartridges at two US hospitals [1]. At one hospital, *R. mannitolilytica* was recovered from three unused cartridges from the same lot; at the other, it was grown from three of 10 unused cartridges from different lots [1].

The phenomenon of the reappearance in a different country of a single strain of such a clinically rare organism, in association with the same equipment and after several years, refocuses on the possibility that some cartridges may have been contaminated, perhaps during a calibration process during manufacture that was referred to in the US report. This process used tap water, which suggested that the cartridges might be the leading candidate as a potential source. Cultures of tap water from the Irish plant that calibrated the cartridges, performed at the CDC during that investigation, did not confirm this [1].

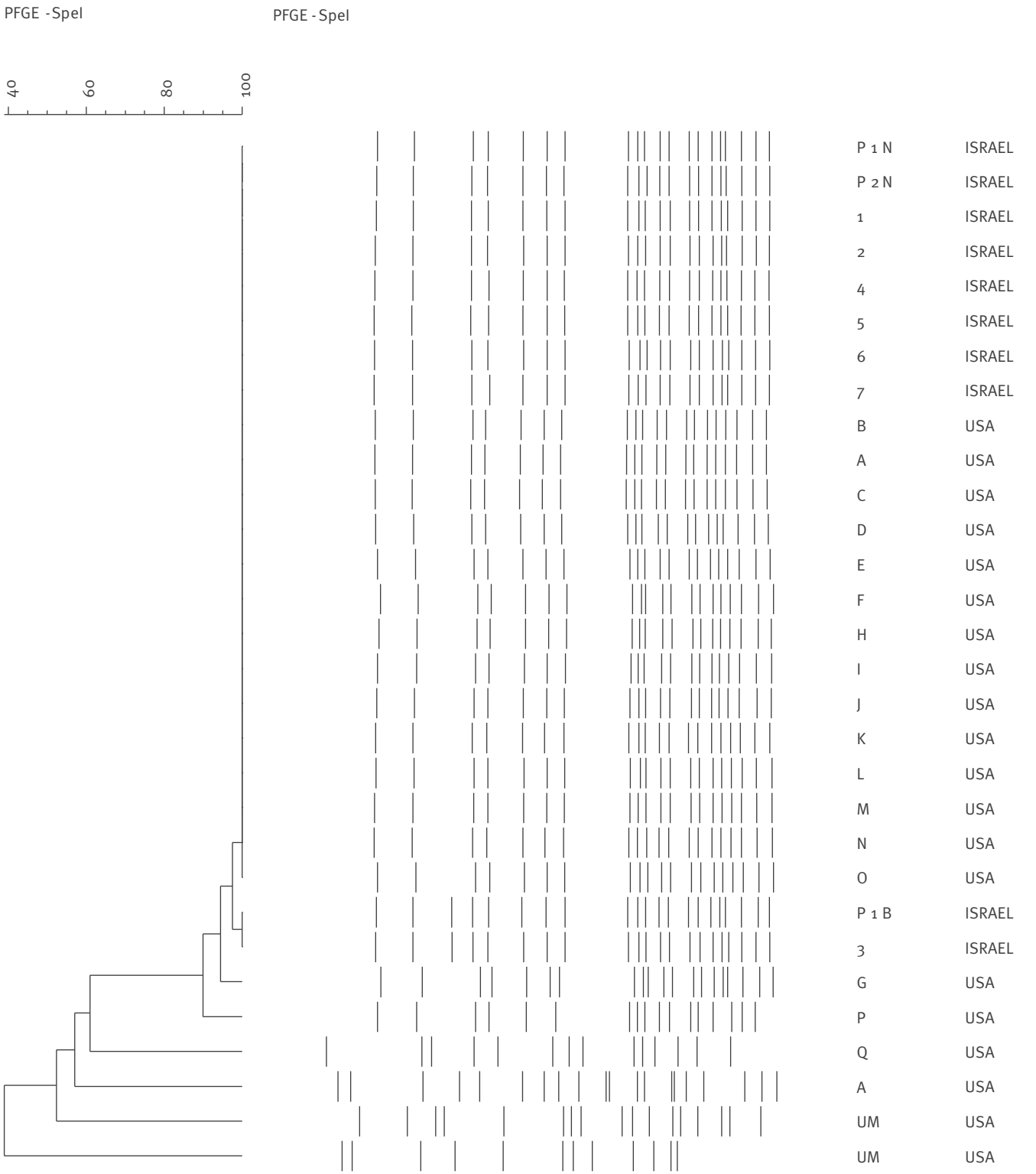
The apparent tenacity of the organism is also a testament to the survival capacity of this strain, or perhaps even the species, which might spur further investigation of this ability. An incentive to study this phenomenon was provided by an informal observation in our laboratory. Colonies of our isolates of *R. mannitolilytica* usually survived no longer than seven or eight days on culture plates, whereas subcultures from suspensions in sterile water maintained at room temperature for over six months produced rapid rich growth of the organism.

To explain the incident in Jerusalem, understanding the solution of the problem adopted in the US in 2006–07 is crucial. The measures introduced included two key changes regarding the cartridges, i) sterilisation of the cartridges during manufacture to ensure their sterility prior to use and ii) discarding the cartridges before decontamination of the water and gas circuits (i.e. single use only, with no reprocessing) [1,17]. These steps would ensure that if contamination of the cartridges were either intrinsic to their manufacture or their calibration, or occurred by some process during use, the chances of transmission of the organism to one or more patients would be minimised or eliminated. Neither of these conditions was met in the instructions provided to us by the local distributor in Israel.

Furthermore, as part of the US solution, the kit provided for routine cleaning (CK-101) before the outbreak, which included disinfection of the cartridge, was replaced by a new kit for disinfection (DK-301) that was

Figure 3

Dendrogram of pulsed-field gel electrophoresis results showing per cent similarity of *Ralstonia mannitolilytica* isolates from the United States in 2005 (n=20) and from Israel in 2011 (n=10)



P1N: index case, nasal isolate; P2N: second infant, nasal isolate; P1B: index case, blood culture isolate; 1-7: environmental isolates from the present investigation. All other isolates were from the United States: those with greater than 80% similarity were associated with the 2005 outbreak and those with less than 80% similarity were unrelated strains isolated previously, including two reference strains from the University of Michigan (UM).

Figure 4

Timeline of the *Ralstonia mannitolilytica* events in the United States, 2005, and in Israel, 2011

BSI: Bloodstream infection of the index patient.

intended for use after discarding the single use cartridge and bridging the resulting gap in the tubing with the cartridge bypass tube provided in the DK-301 kit. This latter kit was, to the best of our knowledge, not made available to Israeli customers, although the local distributor offered an annual high-level disinfection service for which the DK-301 would be deployed (information provided by the local company and the manufacturer). The instructions provided to our institution included reprocessing the cartridges with the proprietary solution of a mix of quaternary ammonium compounds provided with the CK-101 cleaning kit (Control III, Maril Products Inc., Tustin, CA, US). US instructions for use of the Vapotherm 2000i after 2006 state that this solution is not approved for cartridge disinfection [18].

The unopened cartridges we examined for growth or for bacterial DNA (VT01-B type) were produced in 2005. While they bore the CE mark, they were neither marked as sterile, nor for single use. The containers of the cartridges that had been in use and that were manufactured in 2005, 2009 and 2010, including those with positive cultures, were not available for inspection in this regard.

After reintroduction of the device in the US in 2007, customers were invited to have their old VT01-A and VT01-B cartridges replaced by the new devices VT01-AS and VT01-BS as specified in a company document [19] and the associated FDA document [20].

The exact time and manner of introduction of *R. mannitolilytica* in our institution cannot be determined. This was the first time that this taxon was identified at our laboratory. Our data, with the corroborative comparison with the US isolates, indicate that the organism isolated at both our hospitals probably had its source in one or more items of equipment used in the Vapotherm 2000i device. In view of the extremely high similarity of the isolates from our two hospitals and from the US event, we consider it unlikely that venting the sterile water containers with unfiltered needles were responsible for the problem.

Users of the Vapotherm 2000i system should be aware that reprocessing of cartridges regardless of the disinfectant used should not be practiced as this might constitute a possible risk of infection. It should be remembered that our situation was revealed by a bloodstream infection in a premature infant. This raises the question of the desirability of microbiological checks of potentially hazardous equipment or procedures.

In a commentary on the CDC investigation of the US outbreak, Saiman emphasised the importance of correctly classifying patient care equipment in terms of the level of disinfection required to keep that equipment safe for use on patients [21]. It is regrettable that this principle, so admirably applied in the US, does not seem to have been promoted elsewhere.

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Conflict of interest

None declared.

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