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Zika virus, the new kid on the block

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According to Tolstoy, happy families were all alike, whereas unhappy families were each unhappy in their individual ways. So it is with the emergence of new virus infections. Each new virus epidemic brings misery to affected human populations, in unique ways. In the last 15 years, we have experienced the emergence and spread of Severe Acute Respiratory Syndrome (SARS), H5N1 and H7N9 influenza A viruses, pandemic influenza A(H1N1)pdm09, Middle Eastern Respiratory Syndrome (MERS) and Ebola virus disease, and most recently in 2015–16, Zika virus. The wider societal impact that such infectious disease events can cause has been amply demonstrated with Ebola virus in West Africa, which was responsible for over 11,000 deaths and has inhibited economic growth in this war-torn region of the world [1].

Each of the viruses mentioned above occupies a different ecological niche, with diverse impact on the human population (magnitude of the epidemic, disease severity) as a result of transmission characteristics, host immune response and disease pathogenesis. Serious complications and deaths from Zika virus infection have not been common: most infections are asymptomatic or very mild, although there is an association with neurological complications such as Guillain–Barré syndrome. The key issue, however, is the impact of infection on pregnancy.

For most emerging viruses, classical control measures of contact tracing and quarantine will eventually break chains of transmission between humans following zoonotic infection, when human-to-human transmission occurs and infectiousness is related to symptomatic illness. However, when infection is through a vector-borne route and sexual transmission can occur from a minimally symptomatic person, such as with Zika virus infection, additional population-based control measures must be undertaken. Vector control requires sustained and determined efforts to achieve a measurable impact and may involve a range of interventions at a personal level (e.g. avoidance, mosquito nets and insecticide) and at population level (e.g. breeding genetically resistant mosquitoes). Steering towards other rational interventions requires evidence from well-documented individual case studies.

Zika virus disease (ZVD) is a mosquito-borne infection caused by Zika virus, a member of the genus *Flavivirus* and family *Flaviviridae*. It was first isolated from a monkey in the Zika forest in Uganda in 1947. For those with symptoms, Zika virus generally causes a mild, short-lived (2–7 days) disease. Typical symptoms include: rash, itching/pruritus, low-grade fever, joint pain (with possible swelling mainly in the smaller joints of the hands and feet), conjunctivitis/red eyes, headache, muscle pain, lower back pain and eye pain – some of which were described in two of the case studies in this issue of *Eurosurveillance* [2,3]. However, the majority of people infected either do not have symptoms or have a very mild illness, and therefore the identification of symptom-free, but infectious individuals becomes much more problematic, particularly when coupled with consideration of sexual transmission of the virus, as described in the case report on sexual transmission in asymptomatic returning travellers [4]. In Brazil, a country heavily and early affected by the current Zika virus epidemic, an upsurge of cases of ZVD in women has been noted, with the underlying hypothesis that sexual transmission may be more important than hitherto recognised [5]. This may be consistent with modelling estimates that suggest that the R_0 for transmission is lower than calculated for dengue, inferring that modes of infection other than *Aedes aegypti* bites might be involved [6]. The three articles in today's *Eurosurveillance* add to the accumulating body of evidence of persistence of virus in seminal fluids and emphasise the sexual transmission of Zika virus, including sexual transmission between completely asymptomatic individuals [4].

While the exact relationship of detection of viral genome by reverse transcription-polymerase chain reaction (RT- PCR) and virus infectivity and transmissibility from seminal fluid remains uncertain, the current European advice from the European Centre for Disease

Prevention and Control (ECDC) about prevention of sexual transmission of viral infection through the use of safer sex with condoms for several months in returning symptomatic and asymptomatic travellers from endemic areas [7] seems prudent and proportionate. The longest time for recovery of infectious virus from semen occurred at around 19–25 days post illness [8,9], the longest estimated time to sexual transmission following onset of illness is 32 to 41 days [10], whereas detection of viral genome to 60 days or more, previously noted in [8] is also noted in [2]. All of these estimates are subject to some uncertainties, but strongly suggest viral persistence in an immunologically privileged site. The kinetics of antibody response indicate that neutralising antibody will be present by this stage after illness onset. We should not be too surprised by this, following the demonstration of exactly this phenomenon in survivors of Ebola virus disease [11]. Even though these are different groups of viruses with different pathogenesis, both have a viraemic phase with possible replication in vascular and reticuloendothelial tissue. As further data develop about duration of persistence of viral material in the testes, pragmatic public health advice about condom use, for a reasonable period beyond last known documented virus detection, may be a more rational and cost-effective approach than country-specific advice that returning travellers should routinely undergo laboratory testing.

There is scientific consensus that Zika virus is a cause of microcephaly and other congenital anomalies, also referred to as congenital Zika virus syndrome, the full definition for which continues to evolve as case-based information develops. The World Health Organization has thus started coordinating efforts to define congenital Zika virus syndrome and has issued an open invitation to all partners to join in this effort [12]. The combination of widespread asymptomatic infection, seminal fluid infectiousness, transmission from men to women and infection in early pregnancy, with a linkage to congenital anomaly, is an unfolding horror story, which we are ill-equipped to deal with. The current scenario is a true global public health emergency, which requires an urgent raft of measures to minimise the impact. We should be under no illusions – it will take several years to find permanent solutions, such as vaccination.

The current response to Zika virus in mainland European countries is centred on limited numbers of cases of returning travellers. There will be a close watch in Europe in the coming summer season for any autochthonous transmission in areas where *Ae. aegypti* or *Ae. albopictus* is established [13], although the risks are considered to be low [7].

Disease control programmes start with awareness and accurate diagnosis. The symptoms of Zika virus infection can be similar to those of dengue, caused by a related flavivirus, or chikungunya, an alphavirus, which are often co-circulating in affected areas where Zika virus is present. Testing of symptomatic infections for individual case management is most needed in pregnancy. On the other hand, reliance on clinical diagnosis will not be sufficient to provide accurate estimates of disease burden in the affected countries and where laboratory capacity is limited. Laboratory testing of a proportion of all clinical cases is essential for feeding accurate information into predictive transmission modelling. During the first five to seven days after onset of clinical illness during the acute viraemic phase, serum can be used for detection of viral genomic material by real-time RT-PCR. Body fluids such as urine or oral fluid may extend the window for genome detection during acute illness, and urine is already recommended for testing by some public health agencies [14]. These fluids were useful in the limited case studies reported in *Eurosurveillance* [2,3]. As more data become available from cohort studies, information about the reliability of virus detection in different body compartments during the acute phase of illness would be very welcome. Further partial genome sequencing may be helpful to confirm strain variation, and in any case will be important to track the relationship between strain variation and clinical outcome.

Overall laboratory capacity and cost of testing will raise barriers to providing widespread diagnostic support in affected areas, but is less of an issue in Europe. There is an urgent need to develop low-cost, simple point-of-care tests for viral antigen detection, as has been possible for dengue virus. It has taken many decades to realise the potential contribution of self-sampling, using non-invasive body fluids, to support disease control efforts. The detection of substantial amounts of Zika virus in urine and oral fluids suggests that detection of early infection could be attempted from these fluids. If dipsticks or similar simple devices can be developed for antibody detection, with sufficient sensitivity either for capillary blood finger prick testing and urine or for oral fluid sampling, the desirable goal of specific testing linked to self-sampling can provide some additional capacity within severely constrained health systems in affected countries.

Limited data from a small number of cases are currently available on the serological responses to Zika virus. There is antibody cross-reactivity with other flaviviruses, especially dengue virus and yellow fever virus or, less frequently, with West Nile virus. Serology focussing on the detection of viral E antigen, a key viral structural protein involved in virus receptor binding, is likely to demonstrate cross-reactivity between flaviviruses, as there is a high degree of conservation of the human immune response to this viral protein. Tests based around virus neutralisation need to be interpreted with caution and may not be useful as

first-line screening tests, although they may have a role in confirmation of antibody status. The application of serological tests (ELISA or immunofluorescence) to detect specific IgM or IgG against Zika virus can be positive five to six days after the onset of symptoms. In line with classical antibody responses to infection, increased antibody titres are seen in paired samples, with an interval of about two weeks. The results of serological tests are much easier to determine in populations, such as returning European travellers, who mostly do not have a background of exposure to multiple co-circulating dengue subtypes or other flaviviruses. Interpretation of serology in flavivirus-exposed populations will be much more challenging. There is an urgent necessity for Zika virus-specific IgG serological tests that can deliver a very high negative predictive value, and distinguish past infection with other flaviviruses, when applied in sero-epidemiological studies, as this will define the extent of susceptibility in the population, to inform wide-scale control measures. On the other hand, there is a need for serological tests that will deliver a high positive predictive value for Zika virus IgM detection following acute illness, where the narrow window for detection of the viral genome by PCR has been missed. It is particularly challenging to ensure that the correct individual diagnosis is made and pregnant women are not subjected to inappropriate procedures for an infection that they do not have. Considerations of anamnestic response to diverse flaviviruses and original antigenic sin may be relevant in highly exposed populations and may give rise to positive serological test results, following acute illness, as a result of lack of specificity. The complexity of serological responses to Zika virus in light of previous multiple diverse flavivirus infections or vaccinations will require careful elaboration and comparison and pooling of datasets internationally, as well as consideration of whether the risk of congenital anomaly is enhanced or reduced in the presence of pre-existing flavivirus antibody.

It may be some years before we have serology that is highly reliable at both the individual patient and population level. There is a notable gap between the application of serology to carefully studied individual cases, with confirmed genome detection and knowledge of dates of onset of illness, and the application of serology to address the wider disease control questions, at scale, in diverse populations.

The development of serological tools for emerging infections inevitably lags behind the availability of accurate molecular diagnostic tools. The experience of SARS a decade ago, showed that even a year after the first serological tools were developed for detection of this infection, the number of laboratories that could reliably perform serology globally was severely limited [15]. The development of serological tools for MERS is also highly restricted. Urgent attention is required for the development and standardisation of serological tools for Zika virus. It is entirely appropriate that international efforts are directed towards collaborative serological studies, international standards and reagents for serology and study of the relationships between antibody responses to Zika virus and other flaviviruses, to define the protein epitopes involved in cross-reactivity, and address the potential for antibody-dependent enhancement between different groups of flaviviruses.

In summary, it will be crucial to provide support to affected countries to enable the development of robust serological tools, with the goal of ensuring safe pregnancies. Serological tests and algorithms for Zika virus-specific serological testing are in their infancy. Achieving reliability of testing will require sharing of data and reagents, a cooperative working approach to develop international standards and a hierarchy of serological algorithms. The current situation requires that we manage expectations and acknowledge uncertainties to patients, physicians and politicians in this unhappy circumstance. Individual case reports, as published in *Eurosurveillance* today, have an important role in providing evidence to reduce uncertainties.

Conflict of interest

None declared.

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Longitudinal follow-up of Zika virus RNA in semen of a traveller returning from Barbados to the Netherlands with Zika virus disease, March 2016

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We report the longitudinal follow-up of Zika virus (ZIKV) RNA in semen of a traveller who developed ZIKV disease after return to the Netherlands from Barbados, March 2016. Persistence of ZIKV RNA in blood, urine, saliva and semen was followed until the loads reached undetectable levels. RNA levels were higher in semen than in other sample types and declined to undetectable level at day 62 post onset of symptoms.

Case report

In March 2016, a previously healthy man in his 50s, reported to the travel clinic at Erasmus MC, with arthralgia, conjunctivitis, fever (≥ 38.1°C) and rash. The patient had returned eight days earlier from a four day business trip to Barbados. He had been vaccinated according the Dutch guidelines for travellers, including for yellow fever (vaccinated in 2012). He remembered being bitten by mosquitoes during early mornings on the island. Four days after returning to the Netherlands he suffered from staggered joint pain in ankles, elbows and hand joints. Two days after the start of the joint pain he developed fever up to 38.5 °C, a total body macular rash, followed by conjunctivitis. The fever disappeared after three days as well as the joint pain, rash and conjunctivitis. No haematospermia was reported. The patient recovered completely. At the time of his visit in Barbados both ZIKV and chikungunya virus (CHIKV) were circulating on the island.

Laboratory observations

Upon first presentation at the clinic, plasma and urine were collected. CHIKV RNA was not detectable by real-time reverse-transcription polymerase chain reaction (RT-PCR) [1], while ZIKV RNA was detectable for two independent RT-PCR targets in plasma and urine [2], confirming ZIKV disease. The patient agreed to follow-up sampling and ZIKV testing of blood, saliva, urine and semen until the sample type turned negative. Additional urine was collected and found positive for the most sensitive target at 6, 10, 13 and 19 days post onset of illness (dpi) (Figure).

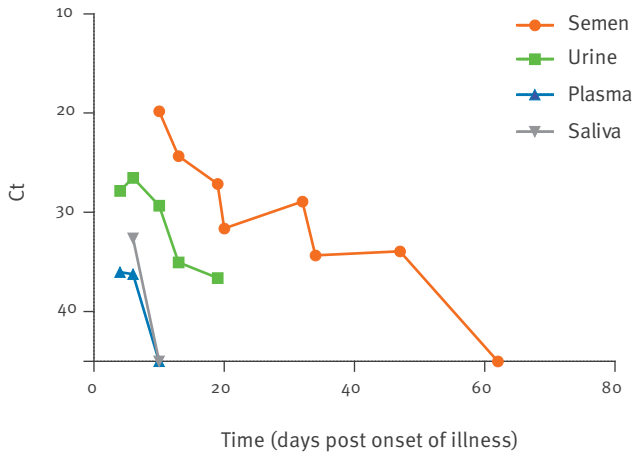
Real-time RT-PCR analysis was validated according to ISO15189;2012 guidelines, briefly total nucleic acids were purified from 200 µL of plasma, saliva, urine or semen sample and eluted in a final volume of 100 µL using MagNaPure LC total nucleic acid isolation kit and the HP200 protocol (Roche Life Science, Almere the Netherlands). Subsequently, 8 µL of eluate in a final volume of 20 µL was used per reaction. Real-time RT-PCR was performed using the primers and probe set 1086/1162c/1107-FAM developed by Lanciotti et al. [2], and which annealed the most sensitive target mentioned above, and Fast Virus 1-Step Master Mix (Life technologies, Nieuwkerk a/d IJssel, the Netherlands) on a LC480-II (Roche), with 5 min 50 °C, 20 s 95 °C, and 45 cycles of 3 s 95 °C and 30 s 60 °C as thermal profile. The real-time RT-PCR was internally controlled for inhibition by addition of phocine distemper virus [3].

Saliva was collected at 6 and 10 dpi and was only positive at day 6. Additional plasma samples were taken at 6 and 10 dpi and ZIKV genome levels turned undetectable at 10 dpi. Semen was collected at 10, 13, 19, 20, 32, 34, 47 and 62 dpi and turned negative at the last sampling point.

Based on the semi-quantitative real-time RT-PCR data for the different sample types, the ZIKV RNA load appeared to be the highest in semen and remained higher in semen than in urine in the follow-up samples. Both urine and semen increased the window of ZIKV detection in clinical samples of this patient, with up to six-fold for semen vs plasma or saliva, and show

Figure

Semi-quantitative kinetics of Zika virus RNA loads in various types of clinical samples according to time post-disease onset, in a Dutch traveller returning from Barbados, March 2016*



Ct: cycle threshold.

a slow decline versus plasma and saliva in ZIKV RNA levels with time.

Attempts to isolate virus from saliva at 6 dpi and from all the semen and urine samples failed. Enzyme-linked immunosorbent assay (ELISA; Euroimmun, Lübeck, Germany) testing on serum collected at 6 and 10 dpi demonstrated the presence of anti-ZIKV IgM in both samples and seroconversion for anti-ZIKV IgG. Virus neutralisation using a ZIKV isolate from Suriname gave a reciprocal neutralising antibody titre of 256 at 47 dpi.

Background

ZIKV is a mosquito-borne flavivirus that has emerged in South and Central America and the Caribbean since 2015, affecting 39 countries in the region with an estimated 270,000 suspected and 40,000 confirmed cases as of 19 May 2016 [3]. Barbados reported its first three autochthonous ZIKV cases on 15 January 2016 [4] and has reported 317 suspected and seven confirmed cases since (status 19 May 2016 [3]). The association of ZIKV infection during pregnancy with microcephaly in newborns and other neurological disorders urged the World Health Organization (WHO) to declare the outbreak of microcephaly-associated ZIKV a Public Health Emergency of International Concern on 1 February 2016 [5]. With the outbreak of ZIKV in some European overseas countries and territories the risk of importation of ZIKV to Europe due to intensive international travel is currently high [6]. In the diagnostic unit at Erasmus MC, 49 confirmed ZIKV patients have been diagnosed in the period between 29 November 2015 and 23 May 2016, including two travellers returning from Barbados in March 2016 (A. van der Eijk, personal communication, May 2016).

Discussion

Aedes aegypti mosquitoes are the main vector for ZIKV transmission. However, the increasing import of ZIKV cases in non-endemic countries has put a previously obscure mode of non-mosquito-borne ZIKV transmission in the limelight: sexual transmission through infectious semen [7,8]. An increasing number of cases describing sexual transmission of ZIKV from symptomatic men-to-women or from symptomatic men-to-men have been reported from areas where *Aedes aegypti* is not present, including Europe [7,9-11]. Although sexual transmission seems to represent the greatest risk for ZIKV transmission in areas without competent mosquito vectors, little is known about the prevalence of ZIKV in semen of symptomatic and asymptomatic infected men, the kinetics of infectious ZIKV and ZIKV RNA in semen in both of these groups, the possible gonadotropism of ZIKV in men and the minimal infectious dose for semen-borne transmission to men and women. Single time point sampling of semen reported the presence of viral RNA at 14 [7], 27 and 62 dpi [12] and isolation of virus 18 and 24 dpi [10].

An important implication of the teratogenicity of ZIKV and semen as source for ZIKV infection [13], is the necessity for protected sexual intercourse at least during pregnancy for men returning from ZIKV endemic areas or when residing in those areas. The European Centre for Disease prevention and Control (ECDC) recommends that male travellers returning from areas with active transmission should consider using a condom with a pregnant partner until the end of pregnancy, but should consider the use of a condom for at least one month upon return to reduce the potential risk of onward sexual transmission with non-pregnant female or male partners [14]. Some national guidelines [15-17] advise condom use for one to two months probably based on the very limited ZIKV isolation data that has been reported for semen up to 24 dpi [7,10].

In line with a report by Mansuy et al. [7], our data show higher ZIKV RNA loads in semen than in other sample types for our patient. Moreover in our study, the viral RNA loads in semen only slowly declined with time to undetectable levels at 62 dpi. Based on this observation and that of others, whereby another study reports a patient's semen still positive for ZIKV RNA 62 dpi, however, with no end-point determination [12], the advice to use a condom with a non-pregnant partner for one month after return from an endemic area might be reconsidered to at least 90 days upon return (including the 62 days [12] and the subsequent maximum incubation time of 13 days [18]) as long as some of the crucial questions mentioned above have not been answered and a fully evidence-based recommendation on condom use is not possible yet. Another implication is the need for review of inclusion criteria for sperm donation.

The higher ZIKV RNA load in semen than in urine samples taken at the same time points found here, has also been previously observed for two other patients [7,10].

This, together with the longevity of ZIKV RNA in semen indicates that semen should be taken into consideration as a sample of choice for sensitive ZIKV diagnostics with a broad detection window in males. While serology is usually the diagnostic method of choice in convalescent and asymptomatic patients with a suspected arbovirus infection [19], flavivirus serology is highly complex due to co-circulation of cross-reactive other flaviviruses, the existence of cross-reactive flavivirus vaccines and the occurrence of original antigenic sin [18]. Therefore molecular testing on semen might have an added value to serology in males up to 2 months dpi.

Conclusions

We describe the longitudinal follow-up of the presence of ZIKV RNA in semen and other sample types in a returning traveller. Semi-quantitative ZIKV RNA levels were consistently higher in semen than in urine in the convalescent phase and slowly declined to an undetectable level at 62 dpi. Testing of semen could have an added value to testing of urine and serology in men up to 2 months dpi. Although ZIKV was not isolated here, the use of condoms and the abstinence of semen donation up to 90 dpi as a precautionary measure to prevent sexual transmission of ZIKV, might need consideration until a fully evidence-based assessment can be made based on data generated within larger patient datasets.

*Erratum

The Figure was corrected on 16 June 2016.

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The patient provided written informed consent to participate in the study and for publication of this case-report.

Conflict of interest

None declared.

Authors' contributions

CR data analysis, figure, writing; SP molecular data generation, data analysis; CGvK, RM serology; JvK tissue culture; TL, MK co-writing; AvdE, medical coordinator; EvG, co-writing, treating physician. All authors reviewed and commented on the manuscript.

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Sexual transmission of Zika virus in an entirely asymptomatic couple returning from a Zika epidemic area, France, April 2016

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The current Zika virus outbreak and its potential severe health consequences, especially congenital fetal syndrome, have led to increased concern about sexual transmission, especially in pregnant women and women of reproductive age. Here we report a case of Zika virus sexual transmission, likely male-to-female, in a totally asymptomatic couple.

Zika virus (ZIKV) is a mosquito-borne flavivirus transmitted by *Aedes* species [1,2]. It is also the first flavivirus known to be sexually transmittable between symptomatic patients [3-6]. We here report a ZIKV sexual transmission in a couple returning from Martinique, whereby both partners were asymptomatic.

Description of cases

A couple wishing to have children was referred to our Assisted Reproduction Treatment (ART) centre for in vitro fertilisation with donor semen (IVF-D), as the male partner had non-obstructive azoospermia. The couple presented for a first consultation in early February 2016 in preparation of an IVF-D cycle scheduled two months later. Until this time, they had planned to spend a two-week holiday in early March in the French overseas department and region of Martinique, which is an epidemic area for ZIKV [7].

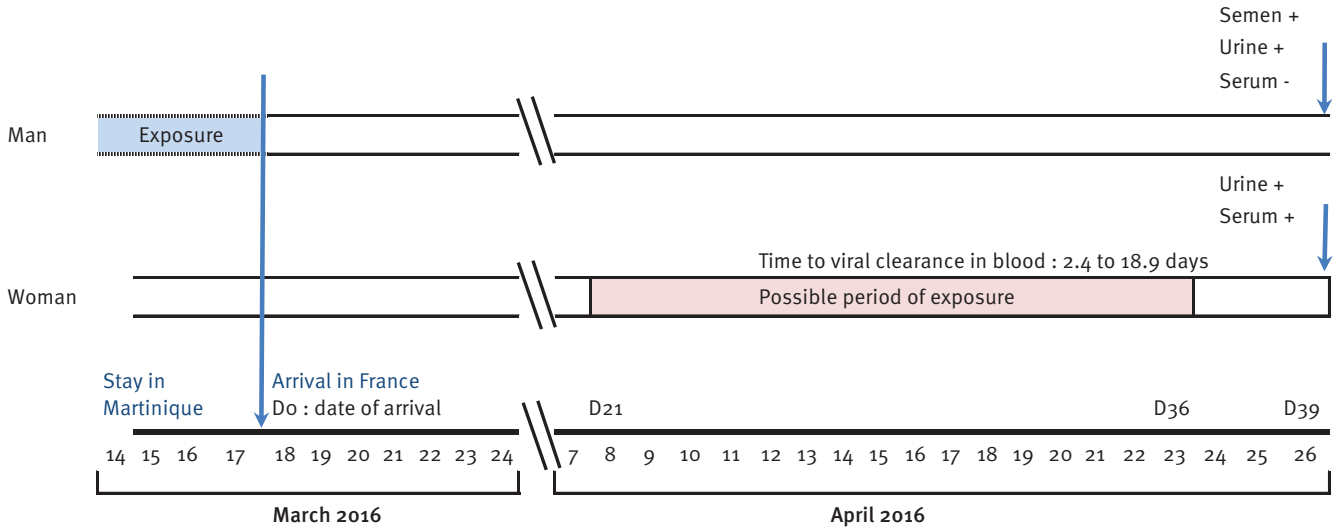
On the day before the first consultation, guidelines issued by the French governmental agency regulating ART, had been released concerning ZIKV. In the case of the couple, these guidelines recommended that both partners be tested for ZIKV RNA by reverse transcription-polymerase chain reaction (RT-PCR) in blood and urine samples at least 28 days after they came back from Martinique [8]. In addition, at the same occasion and according to the guidelines, samples of the man's seminal plasma and sperm cell suspension obtained after sperm preparation on gradient were also to be tested for ZIKV RNA by RT-PCR [8]. The couple was thus informed that the IVF-D cycle would be delayed and an appointment for the ZIKV RNA tests was scheduled 39 days after their return from the holiday (Figure).

Virological diagnosis was performed as previously described [3]. The woman tested positive for ZIKV RNA by RT-PCR in blood (i.e. serum) and urine samples. The man tested negative for ZIKV RNA in blood, but positive in urine and seminal plasma. Sperm cell suspension was not tested for ZIKV, as he was azoospermic. Serological analysis for the man indicated the presence of anti-ZIKV IgM (absence of anti-dengue IGM) and anti-flaviviruses IgG. At the time of the consultation and during the following week, both partners reported having no clinical symptoms of ZIKV infection during and after their stay in Martinique (i.e. no fever, cutaneous manifestation, arthritis, nor myalgia).

As *Ae. albopictus* and *Ae. aegypti* are not established in Brittany where the patients lived, the hypothesis of a local vector-borne infection was excluded. Due to the absence of clinical symptoms, the probable date of exposure to ZIKV could not be determined from the incubation period range. Instead, we used data from a systematic review of the time of viral clearance estimating that 5% of cases will have no detectable virus in the blood by 2.4 days after infection and 95% by 18.9 days [9]. The results of the blood tests, whereby the man tested negative for the virus, while the woman tested positive, pointed to him having been infected before her. The most likely period of exposure to ZIKV for the man was during his stay in Martinique. The woman was found to be viraemic in blood 39 days after her return, which if she had been infected at any time during the holiday trip would correspond to at least 20

Figure 1

Timelines showing the key dates of possible exposure to Zika virus (ZIKV) for two cases of ZIKV infection and results of ZIKV reverse transcription-polymerase chain reaction tests on blood, urine or semen, France, March–April 2016



days longer than the maximal time of viral clearance (Figure). These data, associated with the detection of ZIKV RNA in the man's semen, support the hypothesis of ZIKV sexual transmission from the man to the woman between day 21 and day 36 after their return from Martinique. The couple reported having several unprotected sexual intercourses after their return.

Background

ZIKV is an emerging flavivirus currently responsible for a major outbreak in different areas of the world including for example South America, as well as islands of the Caribbean and the Pacific [7]. In addition to the vector-borne transmission of the virus by *Ae. aegypti* or *Ae. albopictus*, evidence of sexual transmission has been reported between symptomatic patients, from men-to-women or from men-to-men [3-6]. The interval between the onset of symptoms in the man and his partner has been observed to vary from 4 to 44 days [10]. Recently published data show a higher incidence of ZIKV infection in women of reproductive age in Brazil, suggesting a potential role for sexual transmission in the outbreak dynamic [11]. Knowledge on the maximum delay for possible sexual transmission from the time of infection, as well as knowledge of possible transmissions from asymptomatic infected individuals, are of real interest for public health in terms of establishing control measures, improving surveillance to detect the emergence of ZIKV in areas with no current circulation but where *Aedes* is established, and in terms of understanding the outbreak dynamic.

Discussion and conclusions

The finding in our study of a likely man-to-woman sexual transmission of ZIKV between two asymptomatic cases coincided with systematic virological testing in the context of ART. To date, all reported sexual transmissions implicated an index case with symptoms of ZIKV infection, either during a stay in an epidemic area or during the 2 weeks after return from such an area [3-6,10].

As up to 80% of patients infected with ZIKV remain asymptomatic, the level of sexual transmission could play a more important role than expected in the overall dynamic of ZIKV circulation [12]. This unapparent risk of transmission is of concern for pregnant women and women considering pregnancy, and highlights the need to reinforce the counselling and recommendations given to men travelling to epidemic regions and having sex with women of reproductive age. The possible sexual transmission from asymptomatic cases also increases the risk of emergence of ZIKV in Europe in areas where *Ae. albopictus* and *Ae. aegypti* are present.

ZIKV sexual transmission has been reported to occur up to 41 days after the onset of symptoms of the index case and ZIVK RNA has been detected in semen samples at 62 days post-symptom onset [10,13]. Issues related to viral presence and load in semen have been recently highlighted for Rift Valley fever virus and Ebola virus [14,15], whereby questions on the potential consequences have been raised for Ebola virus, as its long-lasting persistence has been shown in semen of survivors [15]. Whether the seminal level of ZIKV RNA follows the same slow decreasing pattern than Ebola virus is not known, but can be expected.

Crucial questions remain to be addressed regarding ZIKV sexual transmission. First, the prevalence of ZIKV persistence in semen needs to be clarified in large epidemiological studies. Second, describing the duration of virus persistence in semen and the dynamics of RNA viral load in semen will help decipher the virus pathophysiological cycle in the male genitourinary tract. These data are of utmost importance in order

to determine the overall probability of ZIKV sexual transmission.

Conflict of interest

None declared.

Authors' contributions

Wrote the manuscript: TF, SM; performed laboratory investigations: MM, IL-G; managed the patients and lead the epidemiological investigation: BH, TF, SM, CS, PB; revised the manuscript: BH, IL-G

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Sexual transmission of Zika virus in Germany, April 2016

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Zika virus (ZIKV), an emerging mosquito-borne flavivirus, causes a mild dengue fever-like illness but has recently been associated with neurological disease and severe birth defects. The virus is currently causing a large epidemic in the Americas. Here, we report a male-to-female sexual transmission of ZIKV in Germany in April 2016, following travel to Puerto Rico of the male patient, demonstrated by subsequent seroconversions and molecular identification of identical virus sequences from both patients.

Case descriptions

On 13 April 2016, the index patient, a 35-year old male German citizen, presented at his general practitioner with signs and symptoms of a common cold. He had returned to northern Germany on 5 April after a one-week stay in Puerto Rico. At the time, a Zika virus (ZIKV) outbreak was ongoing in Puerto Rico [1]. On 6 April, the patient felt weak, but attributed his symptoms to travel-associated exhaustion. One day later, he noticed swollen nuchal lymph nodes. From 8 to 10 April his joints (fingers, knees and ankles) were swollen and painful, such that he could barely walk, and he had a hardly visible but very itchy rash. Neither conjunctivitis nor fever were present. After cessation of these symptoms, the patient developed symptoms of an upper respiratory tract infection lasting for about one week, dominated by a runny nose and persistent sinus headaches. A serum sample of 13 April was negative for Dengue virus (DENV) NS1 antigen, but showed an ZIKV antibody IgM titre of 1:1,280 and an IgG titre of 1:20,480 by indirect immunofluorescence test (IIFT, reference: <1:20) [2]. Results were also positive for anti-ZIKV IgM and IgG in a commercial ELISA (Anti-Zika Virus ELISA, EUROIMMUN, Lübeck, Germany) [2]. Consequently, Zika virus disease (ZVD) was diagnosed (Figure 1).

On 17 April, his 29-year-old female partner who had not been travelling, also developed upper respiratory tract infection. On 20 April, she additionally noted swollen lymph nodes in the neck. The following morning she noticed having unusually red cheeks and a non-itchy rash which spread within one to two hours to her entire body, except the palms of her hands. Fever and conjunctivitis were not noted. On the same day, 21 April, the index patient was informed about his ZIKV infection and his partner was tested for ZIKV infection, too. She was negative for anti-ZIKV IgG and IgM in IIFT and ELISA, as well as DENV NS1-Antigen, however, ZIKV RNA was detected in serum and urine by real time RT-PCR (RealStar Zika Virus RT-PCR Kit 1.0, altona Diagnostics, Hamburg, Germany) and she was diagnosed with ZVD (Figure 1). ZIKV RNA was not detected in saliva. During the next days she developed swollen and painful joints affecting some fingers and the ankles. Symptoms resolved by 25 April.

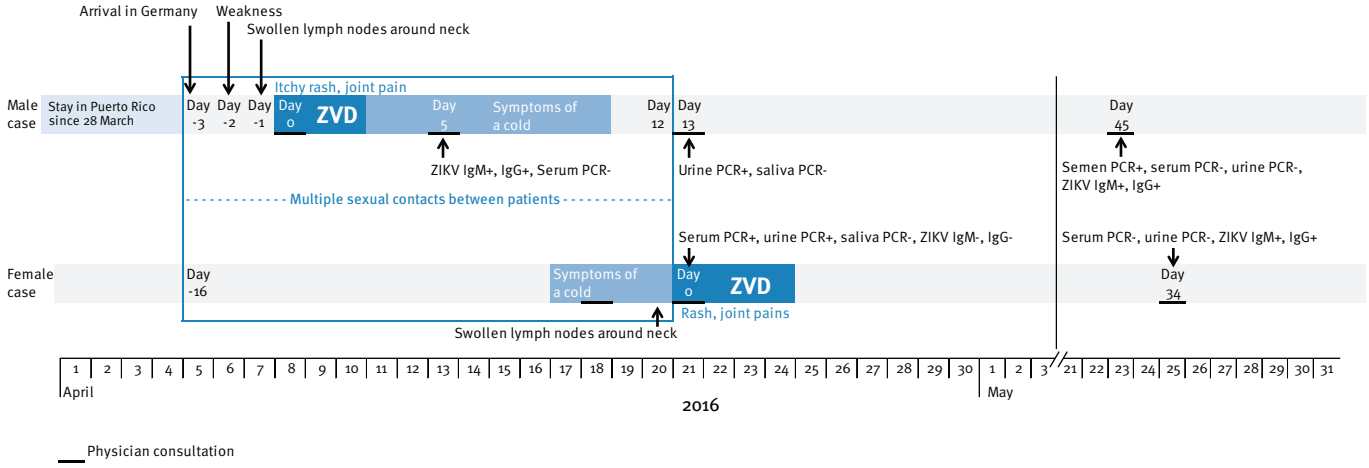
The male patient had ZIKV RNA in urine, but not in saliva and serum 13 days after onset of symptoms. One semen sample taken 45 days after onset of symptoms tested positive for ZIKV RNA and the RNA load was $6x10^4$ copies/mL. In contrast, urine and serum samples were then both negative for ZIKV RNA. ZIKV could not be isolated in cell culture from semen. The female patient tested positive for anti-ZIKV-IgM and IgG in the IIFT and ELISA 34 days after onset of symptoms demonstrating ZIKV seroconversion. Her urine and serum samples then tested negative for ZIKV RNA.

Sequence analysis from urine samples

A fragment of the ZIKV NS5 gene (ca 400 bp, excluding primers) could be amplified from the urine of both patients (GenBank accession numbers KX253994 and KX253995). Sequence analysis showed 100% identity of the samples from both patients (exhibiting only one

Figure 1

Sequence of events in sexually transmitted Zika virus infection from male traveller returning from Puerto Rico, to his female partner, Germany, April 2016



ZIKV: Zika virus; ZVD: Zika virus disease.

synonymous substitution in comparison to the closely related ZIKV strains from GenBank). Based on the phylogeny the two sequences clustered together with the recently detected ZIKV strains from Puerto Rico and other Central and South American countries into the same ZIKV outbreak clade from the Asian ZIKV lineage (Figure 2).

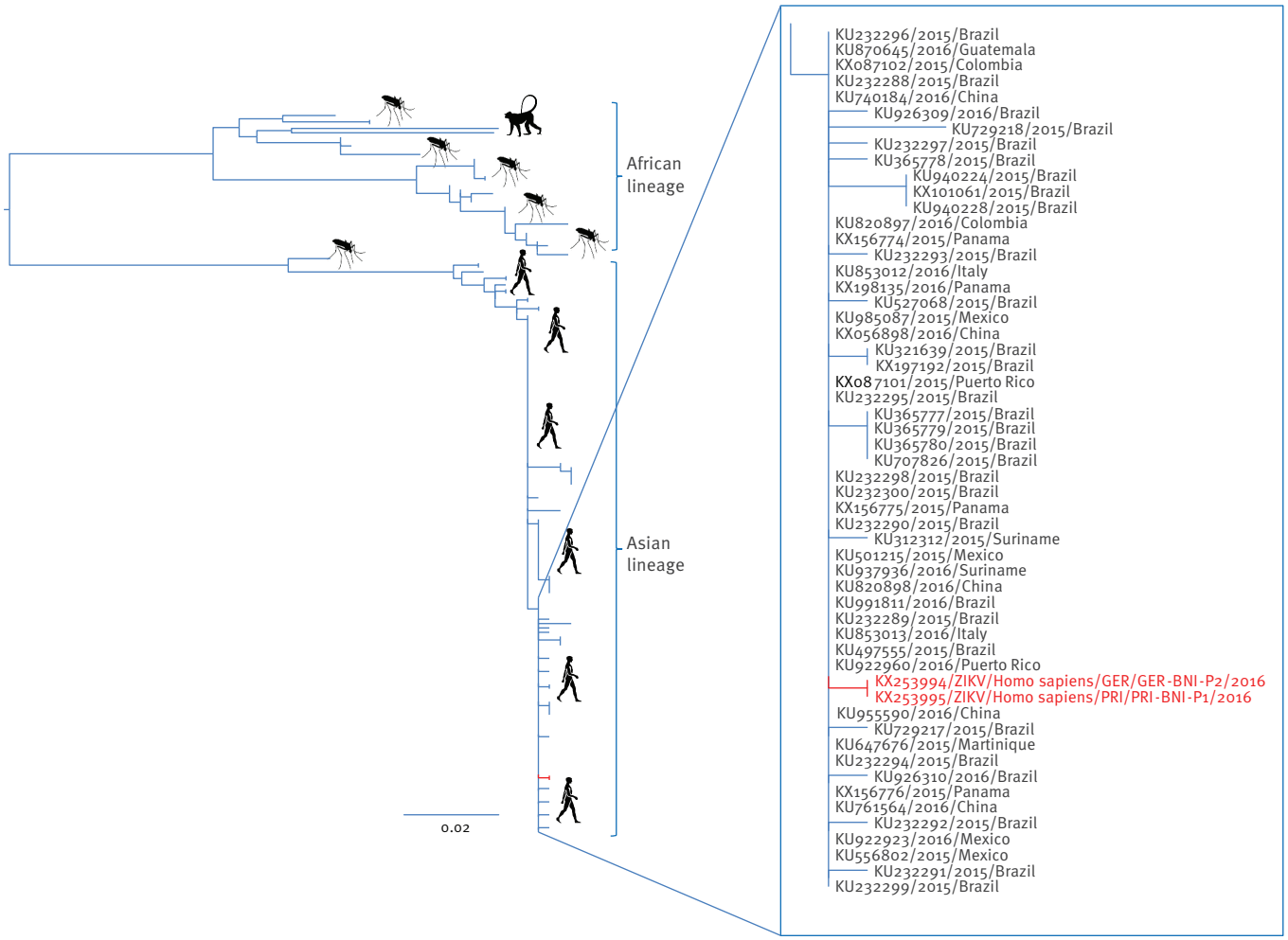
The female patient did not recall any mosquito bites during the previous few weeks, when it was spring. In addition, ZIKV vector mosquito species *Aedes aegypti* or *Ae. albopictus* have not been described anywhere in northern Germany, thus a mosquito-borne transmission was regarded as highly unlikely. She had last been in an area at risk for ZIKV infection between Christmas 2015 and mid-January 2016, when the couple had visited a later ZIKV-affected country together. A male-to-female sexual ZIKV transmission was assumed given the sequence of clinical symptoms in the two patients and subsequent demonstration of seroconversion in the female patient. The molecular analyses and prolonged detection of ZIKV RNA in semen support the assumption. The couple reported multiple sexual contacts (vaginal intercourse without condoms) from the day of his return from travel (5 April) to onset of her rash (21 April), i.e. slightly before and up to 12 days after his onset of ZVD-like symptoms (Figure 1). She is taking oral contraceptives and was not pregnant at the time of infection. He did not report haematospermia or symptoms of prostatitis.

Background

ZIKV is an emerging mosquito-borne flavivirus that causes a mild, dengue fever-like illness termed ZVD [3]. The most common symptoms are rash, arthralgia, and conjunctivitis. ZIKV infection has recently been associated with neurological disease and severe birth defects [4-6]. The Asian genotype of ZIKV is currently causing a large epidemic in the Americas [7]. ZIKV infection has also been detected in returning travellers from endemic and epidemic regions [8,9]. The virus is most likely transmitted through bites of infected *Ae. aegypti* and *Ae. albopictus* mosquitoes [10,11]. Sexual transmission, which had first been assumed in 2008 in the United States (US) in the wife of a man who had previously been to Senegal [12]. Circumstantial evidence suggested possible sexual transmission by vaginal intercourse in the days after the return of the male patient and before onset of symptoms of ZIKV infection and concomitant prostatitis. He had developed haematospermia four days after disease onset. Further potential evidence for sexual transmissibility was seen in 2013 by detection of ZIKV RNA in semen ($2.9x10^7$ and $1.1x10^7$ copies/mL) and urine of a patient from Tahiti with haematospermia some weeks after symptoms compatible with ZVD. ZIKV was successfully cultured from semen and urine [13]. Additional evidence was reported from the United Kingdom by detection of ZIKV RNA from semen 27 and 62 days after onset of febrile illness in a man who had returned from the Cook Islands in 2014, and a prolonged potential for sexual transmission was discussed [14]. A male-to-male sexual transmission after anal sex was reported from the US in January 2016, after travel to Venezuela of the index patient, as shown by positive anti-ZIKV IgM and IgG detection in both patients. Haematospermia and symptoms of prostatitis were absent [15]. Retrospective analyses showing serological evidence of ZIKV infection suggested sexual transmission in an Italian couple after return of the male partner from Thailand [16]. A case series with incomplete results has been published from the US [17], describing infections in women

Figure 2

Maximum likelihood phylogenetic tree, sexually transmitted Zika virus infection from male traveller returning from Puerto Rico, to his female partner, Germany, April 2016



In the entries in red, P represent the two patients described here.

The tree is based on nt sequences of the partial NS5 gene (ca 380 bp). It shows the placement of the ZIKV virus strains that were characterised directly from the urine (obtained from both patients, P1 and P2) in comparison to all available Zika virus (ZIKV) sequences in GenBank. The analysis was performed under the best fit nt substitution model identified as the Tamura-Nei (TN93+Γ) model using jModelTest v2. To assess the robustness of each node, a bootstrap resampling process was performed (1,000 replicates) using the nearest neighbour interchange (NNI) branch-swapping method available in PhyML v3.0. For better visualisation of the position of the ZIKV strains identified in both patients we magnified the area of the American ZIKV outbreak clade which indicates the location of ZIKV from this study (red). The taxon information includes the GenBank accession number, isolation/detection year and country where the virus was isolated/detected. Scale bar indicates mean number of nt substitutions per site.

following sexual contacts with their male partners after these had travelled in Central America and the Caribbean. Recently, sexual transmission was shown in a French woman after unprotected vaginal and oral intercourse with her male partner who had previously travelled to Brazil. ZIKV was detected by PCR and culture in his urine and semen on days 18 and 24 ($2.9x10^8$ and $3.5\ x10^7$ copies/mL in semen) following a period of symptomatic ZIKV infection. He did not report haematospermia [18]. A roughly 100,000 times higher ZIKV load in semen ($8.6x10^{10}$ copies/mL) than in blood was recently demonstrated in a traveller returning to France from Brazil and French Guyana [19].

Conclusions

Here we describe a sexually transmitted ZIKV infection in Germany. The ZIKV RNA load in semen on day 45 post symptom onset was significantly lower than in previous reports [13,18,19]. Sexual transmission of ZIKV is of special concern during pregnancy and specific guidelines for prevention have been published [4,20]. In order to protect the fetus, the European Centre for Disease Prevention and Control (ECDC) notes that male travellers returning from affected areas should consider using a condom with a pregnant partner until the end of pregnancy [4]. In addition, the World Health Organization (WHO) now advises male travellers returning from areas with ongoing ZIKV transmission

to practice safer sex or consider abstinence for at least eight weeks after their return in order to reduce the potential risk of onward sexual transmission. This period extends to at least six months if symptoms of ZIKV infection occur, during or after travel [20]. In contrast to previously described index cases involved in sexual transmission, our case did not notice haematospermia. It will be important to elucidate if haematospermia is associated with a higher ZIKV load in semen and if there is a correlation with the risk of sexual transmission of ZIKV. In addition, experimental data on ZIKV infection of semen are needed to substantiate the epidemiological findings.

Conflict of interest

None declared.

Authors' contributions

Wrote the manuscript: CF, DC, SG, JSC, DT
Performed laboratory or epidemiological investigations: CF, AS, NN, DC, JSC, DT.

Performed data analysis: CF, DC, JSC, DT.

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Outbreak of diarrhoeal illness in participants in an obstacle adventure race, Alpes-Maritimes, France, June 2015

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An acute gastroenteritis (AG) outbreak occurred among participants in an obstacle race in France in the summer of 2015. An investigation in two phases was conducted to identify the source of infection and document the extent of the outbreak. First, a message on a social media website asked racers to report any symptoms by email to the Regional Health Agency of Provence-Alpes-Côte d'Azur. Second, a retrospective cross-sectional study was conducted through an interactive questionnaire for all participants, followed by an analytical study of potential risks factors. Of 8,229 persons registered, 1,264 adults reported AG resolved within 48 hours. Of adults who reported AG, 866 met the case definition. Age group, departure time and ingestion of mud were associated with AG. Twenty stool specimens tested negative for bacteria. All four stool samples tested for viruses were positive for norovirus genogroup I and genotype 2. No indicator bacteria for faecal contamination were found in drinking water but muddy water of ponds tested positive. The outbreak was possibly caused by human-to-human transmission of a norovirus introduced by one or more persons and transmitted through contaminated mud. Risks related to similar races should be assessed and recommendations be proposed to raise awareness among health authorities and organisers.

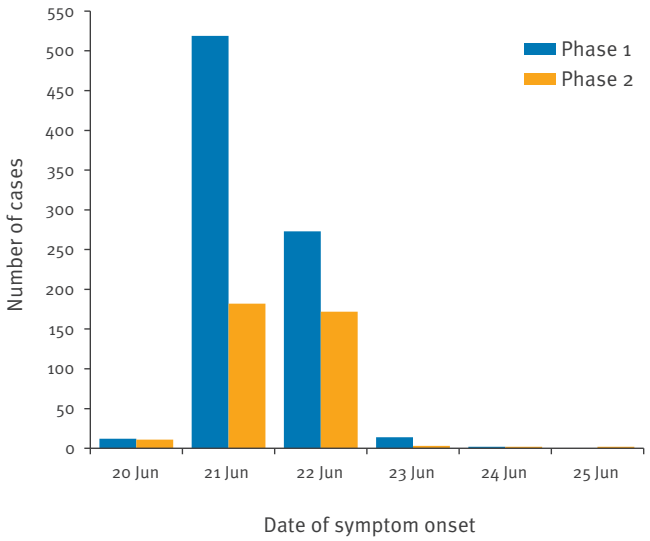
Introduction

Obstacle races are extreme sport events, combining a difficult path and obstacles. On a distance of several kilometres, obstacles such as walls, nets, ice baths, barbed wires, mud mounds, tunnel crawls, dark rooms, live electrical wires are positioned along the path. These sport events have been growing in popularity during the last years. They gather several thousands of participants and are now a worldwide phenomenon.

Racers are heavily exposed to mud and water. They frequently fall, face first, into mud or have their heads submerged in water. Sometimes, there is a different path available for children. Events are usually held in rural areas and often include man-made slurry fields (a mixture of soil and water). In areas commonly frequented by animals, topsoil used to make slurry fields can be contaminated with faeces from ruminants or wild animals [1-7]. Multiple exposures to mud can occur in this kind of sport events [2-9]. Mud is an efficient vehicle for pathogenic microorganisms [7]. Ingestion of mud by racers who unintentionally swallow sufficient numbers of pathogens can cause illness, as described in the literature [2-9]. Over the last few years, outbreaks of acute gastroenteritis (AG) have been reported among participants in an obstacle race (with artificial obstacles) and in other endurance races that take place in nature (mountain bike races, triathlons) [1-6]. *Campylobacter* outbreaks have been reported, such as in the United States (US) in 2012, after an obstacle race among military personnel in Nevada [2], and in Norway in 2011, after a mountain-bike race [3]. In the United Kingdom (UK), a case of *Escherichia coli* 0157:H7 after a mountain-bike race has been described [4]. Other races, such as triathlons, have been linked to cases of leptospirosis, for example on the Reunion Island in 2013 [8]. Skin wounds caused by *Aeromonas hydrophila*, after a 'mud football' have been described in Australia in 2002 [9].

Figure 1

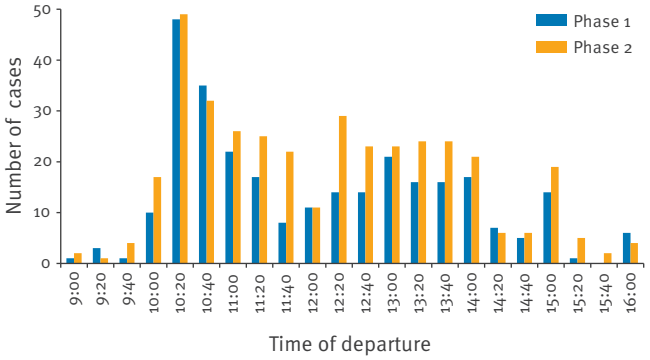
Epidemic curve by onset of signs and symptoms of gastrointestinal disease for phase 1 (n=820) and for phase 2 (n=372) on obstacle adventure race, Alpes-Maritimes, France, June 2015



Self-reported data.

Figure 2

Number of people with signs and symptoms of acute gastroenteritis for phase 1 (n=287) and for phase 2 (n=375) by departure wave on obstacle adventure race, Alpes-Maritimes, France, June 2015



Self-reported data.

On 22 June 2015 at 16:00, a hospital near Nice alerted the Regional Health Agency of Provence-Alpes-Côte d'Azur (ARS PACA) of 22 patients with AG after participation in an obstacle race, in the south of France. On a social media website, many other participants reported also suffering from similar symptoms. An investigation was immediately conducted in order to identify the source of infection and document the extent of the outbreak.

Methods

The ARS PACA, responsible for the outbreak investigation and implementation of control measures, requested support of the Regional Office of the French Public Health Agency (Cire Sud).

Epidemiological investigation

The investigation of the outbreak was conducted in two phases. The first phase started immediately, upon receipt of the outbreak alert, on 22 June 2015. ARS PACA and Cire Sud sent a message on a social media website informing the participants of the obstacle race that an investigation was conducted and asked them to report any recent or current gastrointestinal illness by email to a dedicated address of ARS. This information was also relayed by the organisers of the race and the local press.

The second phase, aimed at collecting additional information, was conducted retrospectively through an interactive application, Voozanoo (a secure web-based platform for hosting personal health data) including sections on socio-demographic characteristics, presence of symptoms or not, and sections on potential exposures (catering, means of contact with mud). This short questionnaire (available from the authors upon request) was intended for all participants of the obstacle race, whether or not they had clinical signs of AG. On 30 June, the company that had organised the event sent the Internet link for this survey by email in a newsletter to all the participants of the obstacle race.

Cross-sectional descriptive studies were conducted in two phases, and were followed by an analytical study of the potential risks factors (in the second phase).

Case definitions

A case of AG was defined as a racer in the obstacle race that took place on 20 June, with self-reported gastrointestinal illness (vomiting and/or diarrhoea) associated or not with other symptoms within eight days from the race.

A secondary case of AG was defined as a person who did not participate in the obstacle race as racer, with gastrointestinal illness following at least 24 hours after a contact with a case of AG among racers.

Microbiological investigation

We recommended all laboratories to test stool specimens from the racers for *Salmonella*, *Shigella*, *Campylobacter* and *Yersinia*, and to send stool specimens to the National Reference Laboratory (NRL) for Enteric viruses in Dijon in order to test for norovirus, sapovirus, rotavirus, adenovirus and astrovirus.

Food and environmental investigations

The company organising the event was contacted on 23 June to collect information about the race (organisation, description of the obstacles, digging works, etc.)

Table 1

Determinants of signs of acute gastroenteritis, univariate analysis of phase2, obstacle adventure race, Alpes-Maritimes, France, June 2015 (N = 745)

Risks factors	AG cases		Non AG cases		Total	OR	95% CI	p value[a]
Total	**(n=375)**	**%**	**(n=370)**	**%**	**(N=745)**	–	–	–
Sex								
Male	200	55	197	54	397	1.05	0.79–1.41	0.73
Female	163	45	169	46	332	Ref	Ref	Ref
Age group (years)								
18–27	141	38	74	20	215	3.19	2.12–4.82	<0.01
28–32	94	25	86	23	180	1.83	1.20–2.79	0.05
33–39	72	19	94	25	166	1.28	0.84–1.97	0.25
40–61	68	18	114	31	182	Ref	Ref	Ref
Department of residency								
Alpes–Maritimes	281	76	305	82	586	Ref	Ref	Ref
Other departments and countries	90	24	65	18	155	1.5	0.78–1.08	0.24
Departure time								
09:00-09:59 (waves 1–3)	7	2	69	19	76	Ref	Ref	Ref
10:00-10:59 (waves 4–6)	98	26	71	19	169	13.61	5.90–31.37	<0.01
11:00-11:59 (waves 7–9)	73	19	61	17	134	11.8	5.05–27.56	<0.01
12:00-12:59 (waves 10–12)	63	17	50	14	113	12.42	5.25–29.40	<0.01
13:00-13:59 (waves 13–15)	71	19	53	14	124	13.2	5.62–31.05	<0.01
14:00-14:59 (waves16–18)	33	9	37	10	70	8.79	3.55–21.80	<0.01
15:00-16:20 (waves 19–22)	30	8	27	7	57	10.95	4.30–27.91	<0.01
Full race								
Yes	368	99	360	98	728	2.04	0.51–8.24	0.34
No	3	1	6	2	9	Ref	Ref	Ref
Race time								
≤2h30	130	35	129	35	259	Ref	Ref	Ref
>2h30	244	66	239	65	483	1.01	0.75–1.37	0.93
Ingestion of mud								
Yes	195	53	140	38	335	1.78	1.33–2.40	<0.01
No	176	47	226	62	402	Ref	Ref	Ref
Inhalation of mud								
Yes	289	77	247	68	536	1.63	1.17–2.26	<0.01
No	84	23	117	32	201	Ref	Ref	Ref
Eating at feed stations								
Yes	337	90	325	89	662	1.23	0.78–1.96	0.37
No	37	10	44	12	81	Ref	Ref	Ref
Eating food from stalls								
Yes	124	33	106	29	230	1.22	0.89–1.67	0.21
No	250	67	261	71	511	Ref	Ref	Ref
Bringing food								
Yes	104	28	94	26	198	1.13	0.81–1.56	0.47
No	266	72	271	74	537	Ref	Ref	Ref

AG: acute gastroenteritis; CI: confidence interval; OR: odds ratio; Ref: reference.

[a] The values in bold are significant.

Self-reported data.

and the catering company, to obtain information about menus and food samples for analysis.

Samples of muddy water were collected on 23 June from the ponds of the race and samples of drinking water from the city distribution system and tested for bacteria indicating faecal contamination (*Escherichia coli*, total coliforms, *Enterococci*, aerobic microorganisms) and for *Salmonella*, enterovirus, *Vibrio cholerae* and *Vibrio parahaemolyticus*, and free-living amoebae

Table 2

Determinants of acute gastroenteritis, final multivariate model of phase 2, obstacle adventure race, Alpes-Maritimes, France, June 2015 (n = 722)

Risk factors	OR adjusted	95% CI	p value[a]
Age groups (years)			
18–27	5.10	2.15–12.12	<0.01
28–32	2.50	1.05–5.95	0.39
33–39	1.95	0.80–4.79	0.14
40–61	Ref	Ref	Ref
Departure time			
09:00–09:59 (waves 1–3)	Ref	Ref	Ref
10:00–10:59 (waves 4–6)	12.99	5.52–30.56	<0.01
11:00–11:59 (waves 7–9)	10.56	4.43–25.17	<0.01
12:00–12:59 (waves 10–12)	11.92	4.93–28.82	<0.01
12:00–12:59 (waves 10–12)	12.96	5.39–31.19	<0.01
14:00–14:59 (waves16–18)	8.18	3.20–20.88	<0.01
15:00–16:20 (waves 19–22)	8.56	3.25–22.51	<0.01
Ingestion of mud			
Yes	1.66	1.21–2.29	0.02
No	Ref	Ref	Ref

CI: confidence interval; OR: odds ratio; Ref: reference.
[a] The values in bold are significant.
Self-reported data.

(*Naegleria*). These samples were tested by a laboratory in Lyon. Water samples were tested for *Campylobacter* and norovirus by the Laboratory for Hydrology of the French Agency for Food, Environmental and Occupational Health & Safety (ANSES,) in Nancy. The University Laboratory of Pharmacy in Lyon tested the specimens for species of *Naegleria* [10].

We provided recommendations on measures to prevent secondary spread during a press conference organised on 22 June, through a weekly report of the Cire Sud, on the ARS PACA website, and we posted prevention messages on a social media website. The outcome of the investigation was also published on all these websites.

Results

Description of the event

The obstacle race took place over one day. A total of 8,229 participants were registered: 7,804 adults and 425 children (aged 7–12 years). Adult racers completed 13 km and 22 obstacles. Departures took place every 20 minutes, from 9:00 to 16:00, by waves of around 400 participants. On the path, dunes had been constructed and artificial ponds dug and filled with water from the city distribution system mixed with the original soil. The children's path was shorter and did not include ponds of muddy water.

Three feed stations were set up along the race course. Food included pre-wrapped cakes, pre-cut bananas, dried apricots, all served in plastic containers from which participants picked them with their bare hands. Drinks included bottled water, energy drinks and beer in cups.

The race was held near a riding centre, and some participants reported spontaneously seeing horse dung on or near the track, or smelling sewage.

Epidemiological investigation

Description of the outbreak

Phase 1 of the epidemiological investigation was conducted from 22 to 27 June; we received 1,370 emails, 81% (1,111/1,370) of which had been sent within 24 hours after the message about the outbreak investigation was published on the social media website. Among the respondents, 1,300 AG cases were reported: 1,264 adults (attack rate: 16%; 1,264/7,804) and 36 secondary cases (not included in this analysis).

Phase 2 was conducted from 30 June to 27 July and a total of 748 questionnaires were completed (for 745 adults and 3 children). Among the participants who filled out the questionnaire, more than half (404/748; 54%) did it the day the newsletter came out and one third (247/748; 33%) the day after. There were 375 AG cases and 373 non cases. Secondary cases reported by respondents rose to a total of 177 cases, not included in this analysis.

The mean age of adult respondents was 33 years (range: 18–61 years). The male/female sex ratio was 1.2. Participants came mostly from Alpes-Maritimes (580/745; 78%), followed by other French departments or other countries.

In both phases, no cases were reported among the child racers. Only adults were included in the analysis.

The median incubation period was one day (ranges: 0–4 days for phase 1 and 0–5 days for phase 2).

Self-reported onset of AG symptoms was available for 820 participants (phase 1) and 372 (phase 2).) Of the symptoms 97% (792/820) and 95% (354/372) occurred on day 1 and 2 after the race for phase 1 and 2, respectively (Figure 1).

In phase 1, the description of symptoms was available for 1,001 (79%) of 1,264 symptomatic participants and 866 (69%) met the definition of an AG case. The most common symptoms besides vomiting and/or diarrhoea, were abdominal cramps (369/866; 43%) and fever (354/866; 41%). Those symptoms were over or being spontaneously resolved in 48 hours.

In phase 2, 69% of participants notified being ill (513/745) and among them, 73% met the case definition (375/513); of these, 67% (251/375) presented fever and 43% (161/375) other digestive symptoms. The other

symptoms were non-specific, such as chills (215/375; 57%), headaches (101/375; 27%), asthenia (322/375; 86%), and muscle ache (262/375; 70%).

Among the 375 AG cases, 52% (n=196) consulted a general practitioner and 4% (n=16) an emergency service. No hospitalisation was reported. The median duration of AG was two days (range: 1-9 days).

The departure time was available for 287 AG cases in phase 1 and 330 in phase 2 (Figure 2). Cases were identified for all departure waves, except one at 15:40 for phase 1. The number of cases was low for the first waves (range: 1–17 cases), and the highest for the fifth wave, with 48 (phase 1) and 49 AG cases (phase 2). The curves of AG cases over time for the two phases of the investigation follow the same dynamic (trend chi-square not significant).

Study of risks factors

Age group, departure time and ingestion of mud were associated with AG at the $p<0.2$ level in the univariate analysis (Table 1).

The final multivariable model included age group, departure time and ingestion of mud (Table 2). Younger participants (aged 18–27) years had a significantly higher risk or AG in the multivariate analysis compared with older participants. The risk of AG was higher in the morning, from 10:00 to 14:00 and still high in the afternoon compared with the first hour of departure waves (9:00–10:00). The ingestion of mud is also a significant risk factor of AG.

Microbiological investigations

We obtained the results of 20 stool samples. Results of bacteriological testing were negative for all samples, except one (positive for *Shigella sonnei)*. Among the four specimens sent to the NRL for enteric viruses, all were positive for norovirus genogroup I and genotype 2 (GI.2).

Food investigation

During the race, the participants did not carry their own food and drinks. However, they consumed beverages and food distributed in feed stations. One third of the cases reported having eaten food sold by the catering company after the race. No AG case was reported among non-participants, organisers and staff of the event. The catering company had no food samples for microbiological analysis.

Environmental investigation

Drinking-water from the city distribution system and environmental water samples from muddy water ponds were collected on 23 June. All three drinking water samples were negative for the indicator bacteria (total coliforms, *E. coli, Enterococcus*), viruses and parasites. Of the environmental water specimens (n=5) taken from five ponds on 23 June, all were found contaminated by aerobic microorganisms at 36 °C (with counts ranging from 86 to 3,200,000 UFC/100 mL) and 22 °C (with counts ranging from 2,500 to 1,400,000 UFC/100 mL), indicating bacterial contamination. All specimens were negative for *Salmonella, Campylobacter*, Enterovirus, *V. cholerae* and *V. parahemolyticus*. All environmental samples were positive for *Naegleria* spp. (with cells ranging from 400 to 280,000/L), but the pathogenic species *N. fowleri* was not detected. Norovirus genogroup I and II were not detected.

Discussion

A large AG outbreak occurred among the participants of obstacle race in the department of Alpes-Maritimes, France, on 20 June 2015. Of 7,804 adult participants, 1,264 were ill and 866 met the case definition of AG. This outbreak occurred from 20 to 25 June and the epidemiological curves during two different phases of the outbreak investigation were characteristic of a point-source outbreak. Moreover, they were similar and showed a higher number of cases on day 1 and 2 after the race. The clinical characteristics reported, the incubation period of the AG, the presence of vomiting, the resolution of symptoms for most cases within 48 hours and the absence of severe clinical forms, were compatible with a viral origin of the outbreak.

The epidemiological investigation enabled us to identify mud ingestion as the main risk factor of developing AG; no other source of infection was identified.

Several arguments support a common source outbreak of human origin related to the ingestion of contaminated mud. In outbreaks such as the one described here, the first hypothesis usually considers food sources. However, the first information reported by the participants did not support a food source, because of the diversity of supplies (feed stations only for participants in the race, food stalls for everyone, food brought by racers). Information gathered from organisers showed that no case of AG had been reported among the 360 members of the organisation and volunteers who received the food which was also sold at food stalls open to the public.

Unfortunately, no food samples were available for laboratory testing. Among participants, 90% of cases and 89% of non-cases (phase 2) consumed food from mass catering served at food stalls. However, the hypothesis that participants could have contaminated one another when dipping their muddy hands into the plastic containers with pre-cut bananas or dried fruits seems plausible. Another argument for contaminated mud as vehicle in the outbreak is that no child has been reported ill. The children's path was different from the adults' and did not have any obstacle with muddy water. This reinforces the idea of a human contamination in the obstacles with muddy water of the adult path. Secondary spread between ill participants and their relatives happened outside of the context of the race. Norovirus GI.2 was identified in the four stool samples. This strain of human origin [11], was possibly

introduced by one person or more of the team preparing the path or by participants in the first waves. In spite of the negative tests for norovirus in the muddy water, we cannot exclude that it was contaminated with such viruses [12]. Indeed, the sensitivity of the norovirus detection method could have been significantly reduced because only low volumes of muddy water were available for testing. Norovirus can persist in the environment, but the viral contamination of the water may have been partly reduced over the three days that preceded the sampling. Last, it is possible that the most contaminated ponds may have not been sampled. We consider it highly probable that the virus had been disseminated in the days preceding the race or early during the race [13].

Although our investigations led us to identify a point source with probable norovirus contamination as cause of the outbreak, there are some difficulties and limitations. The phase 1 of the investigation had to be conducted quickly, but it would have been better to have the time to ask the participants a few specific questions instead of just asking them to report any AG. This would have led to improved data quality. Nevertheless, participants gave us spontaneously detailed descriptions of their symptoms which allowed us to use the data.

Another limitation is that it was not possible to identify the exact number of cases. Phase 1 listed the participants who reported their illness voluntarily. More than 1,000 replies were sent by email within the 24 hours that followed the request for information from participants in the race. But there is a possibility that a greater number of persons was aware of the message but did not take the time to report their illness, in particular if symptoms were mild.

During phase 2, the response rate was lower, which prevented us from estimating the actual number of cases. As the event company had email addresses of participants, it had been agreed with the organisers to go through them to ask the participants to fill out the questionnaire, at the Internet link provided. The information was sent to the event company on 25 June, but was only inserted in a newsletter sent to the participants on 30 June i.e. 10 days after the event and eight days after the peak of the outbreak. It is likely that this newsletter was seen by more participants than those who replied during phase 1, because the message was sent directly to their email address. However, this email did not trigger as many as replies as expected, maybe because the illness was not that severe and therefore the participants did not feel it was useful to answer.

The investigation (phase 2) showed that several hundreds of persons who suffered from AG went to emergency services and consulted general practitioners. We were only able to trace down 20 stool samples and four were sent to the NRL of enteric viruses, despite many calls and emails from ARS PACA. Studies in French general population [14] and in general practitioners [15] also indicate that few stool samples are requested. The study indicates that stool samples are requested five times more often in patients with bloody diarrhoea or with a long duration of illness before consultation. Awareness actions among health professionals are needed.

This major outbreak of AG stands out by the way ARS PACA and Cire Sud quickly reacted to it. The most recent channels of communication were used in its investigation and management; social media and email access were extensively used by the target population of this study.

Sport events that tend to exceed one's self limits have been growing in popularity on an international scale. Mud is often found in this type of events and therefore health risks factors have to be taken into account. Despite the high number of people affected, there were no severe health consequences. However, they could have been more serious, depending of the pathogens involved (*E. coli, Campylobacter, Salmonella,* Leptospira) [1-9]. Fortunately, sports people who participate in this type of events are likely to be in good health.

Recommendations for the organisation of events such as obstacle or endurance races should be proposed. Racers and adventure race organisers should be aware of the potential risk of inadvertent ingestion of muddy, possibly contaminated, water during the race. For instance, persons preparing the path or participating in obstacle races should not have diarrhoea or vomiting in the two days before the race. In general, planners of obstacle adventure races should consider building slurry field challenges where animal faecal contamination is unlikely. The courses should be pre-routed to avoid areas heavily contaminated with animal faeces. No unwrapped food should be served. Facilities for washing hands with clean water should be available.

The risks related to obstacle or endurance races should be further assessed, on the one hand to guide health authorities in their vigilance and their prevention measures, and on the other hand to guide organisers in the choice of materials used and raise awareness of potential risk factors.

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Conflict of interest

None declared.

Authors' contributions

Caroline Six: member of the epidemiologic investigation team, drafting and revising the manuscript.

Samer Aboukais: management of the outbreak; revising the manuscript.

Sandra Giron: member of the epidemiologic investigation team; revising the manuscript.

Jean-Christophe D'Oliveira: analysis of water samples, notification of laboratory results; revising the manuscript.

Françoise Peloux-Petiot: management of the outbreak; revising the manuscript.

Florian Franke: member of the epidemiologic investigation team; revising the manuscript.

Hervé Terrien: management and selection type of analysis of water samples; revising the manuscript.

Fabrice Dassonville: management and selection type of analysis of water samples; revising the manuscript.

Joël Deniau: data manager; revising the manuscript.

Katia Ambert-Balay: analysis of human samples, notification of laboratory results; revising the manuscript.

Thierry Chesnot: analysis of water samples, notification of laboratory results; revising the manuscript.

Raymond Ruimy: analysis of human samples, notification of laboratory results; revising the manuscript.

Michel Pélandakis: analysis of water samples, notification of laboratory results; revising the manuscript.

Patrick Basset: participation in the management of the outbreak; revising the manuscript.

Manuel Munoz Rivero: management of the outbreak; revising the manuscript.

Philippe Malfait: member of the epidemiologic investigation team; comments on the manuscript and revising the manuscript.

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