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Editorials

Enteroaggregative, Shiga toxin-producing *Escherichia coli* O104:H4 outbreak: new microbiological findings boost coordinated investigations by European public health laboratories

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In the past weeks, we witnessed the unfolding story of one of the largest ever reported outbreaks of haemolytic uremic syndrome (HUS) and bloody diarrhoea caused by Shiga toxin-producing *Escherichia coli* (STEC), also commonly referred to as verocytotoxin-producing *E. coli* (VTEC) and enterohaemorrhagic *E. coli* (EHEC) [1]. This outbreak has caused considerable suffering and resulted in a strain on healthcare and public health systems in parts in Germany. It has shown a number of striking features: an unusually large proportion of HUS cases as compared with diarrhoea cases [1]. Furthermore, whereas usually HUS triggered by STEC infection predominantly affects young children, the great majority of cases in this outbreak are adults and two thirds are women. Between 2 May and 14 June 2011, 3,332 STEC cases, including 818 cases of HUS, were reported from 13 European Union(EU)/European Economic Area (EEA) Member States and 36 patients have died [2]. Over 95% of STEC cases have been reported from Germany and the vast majority of cases reside in or have a history of recent travel to northern Germany. Additional cases related to the outbreak have been reported from Switzerland, the United States and Canada [3]. However, since 10 June, there has been a clear signal that the number of newly reported HUS and STEC cases is gradually decreasing, which suggests that we may finally be reaching the tail end of the outbreak.

The search for the source and vehicle of the outbreak has been a long and arduous process. Initial epidemiological findings pointed to raw vegetables and salads consumed in northern Germany as likely vehicles of infection and consequently led to the recommendation to abstain from eating these vegetables raw in northern Germany [1]. Extensive investigations implicated an organic sprout farm in Lower Saxony near Hamburg. Sprouts produced at this farm had been distributed to many of the incriminated restaurants and catering facilities, and were thus identified as a likely vehicle of infection. On 10 June, German public health and food safety authorities issued a joint statement recommending people to abstain from consuming sprouts [4].

Initial laboratory analysis of clinical isolates from outbreak cases performed at the German National Reference Centre for Salmonella and other Bacterial Enteric Pathogens at the Robert Koch Institute, in Wernigerode, quickly revealed that the epidemic agent was an STEC strain of rare serotype O104:H4, with production of Shiga toxin 2 [1]. Moreover, it was further atypical in that it lacked the attaching/effacing pathogenicity island of virulent STEC strains, as indicated by negative PCR results for the intimin (*eae*) and haemolysin (*hly*) genes. All outbreak-related clinical isolates were found to be multidrug resistant and displayed indistinguishable genomic macrorestriction profiles by pulsed-field gel electrophoresis (PFGE) analysis.

In this issue of *Eurosurveillance*, a collaborative group of investigators, led by the WHO Collaborating Centre for Reference and Research on Escherichia and Klebsiella, report several intriguing and important new findings on the nature and possible origin of the epidemic strain [5]. Firstly, using well- validated genotyping methods, Scheutz et al. provide convincing evidence that the STEC strain causing the outbreak in Germany is in fact not a typical virulent STEC strain, but instead is a much rarer hybrid pathotype that harbours the phage-mediated Shiga toxin determinant with an enteroaggregative *E. coli* (EAggEC) background, more precisely described as enteroaggregative, Shiga toxin/verotoxin-producing *E. coli* (EAggEC STEC/VTEC). Secondly, they also identify in this strain the presence of the receptor for iron-chelating aerobactin, known to be a virulence factor associated with the extra-intestinal *E.*

coli pathotype. Thirdly, they provide new data attesting to a close genetic relatedness of the German outbreak strain to previously described similar EAggEC STEC/VTEC strains. These findings are relevant for identifying the ecological reservoir and evolutionary origin of the epidemic agent, gaining a better understanding of the biological determinants of unusual disease severity and clinical complications seen in outbreak cases and the design of specific diagnostic tools for detection and treatment of STEC cases, and identification of the epidemic strain for accurate outbreak monitoring.

So what do the findings tell us about the reservoir and origin of the pathogen causing this outbreak? EAggEC is a common pathogen causing diarrhoea in travellers and persistent diarrhoea in infants and young children living in countries with poor sanitation [6,7]. In contrast to STEC strains that have an animal reservoir, mostly ruminants, EaggEC strains have a human reservoir. Little is known about the pathogenic role and epidemiological features of infections caused by strains of the hybrid EAggEC STEC/VTEC pathotype. One HUS outbreak caused by a strain of this mixed pathotype, but associated with a distinct serotype, had been previously reported from France in 1998 [8]. Scheutz et al. report that seven previously reported cases of diarrhoea or HUS worldwide caused by EAggEC O104:H4 have been identified: from Germany in 2001, France in 2004, South Korea in 2005, Georgia in 2009 and Finland in 2010 [9,10]. By PFGE analysis of EAggEC O104:H4 strains that are positive and negative for the Shiga toxin (stx) gene, the authors further demonstrate that, in contrast to the diversity seen within this serotype, isolates from the 2011 German outbreak cases exhibit a level of genetic similarity, which is also seen in the EAggEC STEC/VTEC O104:H4 strain from an unpublished outbreak of HUS in Georgia, which was investigated jointly by the United States Centers for Disease Control and Prevention (CDC) and Georgian public health authorities in 2009. However, no epidemiological link between these two outbreaks has been reported as yet and therefore the meaning of this finding remains elusive. Additional comparison of genomic relatedness of the German 2011 epidemic strain with other previously detected STEC O104:H4 strains causing sporadic HUS cases in other parts of the world should provide a more complete understanding of the potential reservoir and possible origin of the 2011 epidemic strain.

Another fascinating development stems from comparative genomics, available in real time, to elucidate the ancestral origin of the 2011 outbreak strain. On 2 June, further information on the nature of the hybrid EAggEC STEC/VTEC pathotype of this strain came from whole genome sequence information generated by two groups of German academic investigators [11]. Sequence information from a third isolate from a patient was subsequently generated at the Health Protection Agency, United Kingdom. The data sets from these sequencing initiatives were instantly released for public access, resulting in data analysis among bioinformaticians and other researchers around the world. Results from these preliminary analyses have been rapidly communicated via blogs, Twitter and private web pages, outside the standard peer-reviewed scientific publication route. These initiatives have confirmed the microbiological characterisation of the outbreak strain made in the public health laboratories by targeted genotyping and phenotyping of facultative *E. coli* virulence genes. Most importantly, among compared *E. coli* genome sequences, the genome of the 2011 outbreak strain clustered closest to an EAggEC strain isolated in 2002, with the addition of stx2 and antibiotic resistance genes.

How do these microbiological findings help clinical and public health laboratories detect and confirm cases in a timely and reliable manner? Further to key information provided by the Robert Koch Institute on strain screening and characterisation, Scheutz et al. also propose an alternative simple laboratory screening tool for detecting the 2011 German outbreak strain: a bacterial cell slide agglutination assay with cross-reacting antiserum against the capsular K9 antigen. This test, depending on reagent availability, can be used for the primary laboratory detection of *E. coli* O104:H4 in faecal specimens from suspected cases. Therefore, this assay enhances the potential capability of microbiology laboratories to detect and report cases accurately to clinical practitioners treating the patients and to public health authorities investigating the outbreak.

In summary, from a scientific perspective, the major findings reported in this issue by Scheutz et al. shed light on the unusual pathogenic features, prior occurrence in human pathology and likely natural reservoir of the *E. coli* strain causing the ongoing HUS and diarrhoea outbreak in Germany. More studies are needed to understand which and how these biological features of the bacterium actually determined the unique clinical and epidemiological disease manifestations in this outbreak.

Furthermore, from a public health perspective, it should be emphasised that the microbiology findings and technical recommendation presented were immediately shared by the authors through EU and international public health and food safety laboratory alert networks. This timely dissemination of key data to those who need to know has included posting technical information on the European Centre for Disease Prevention and Control (ECDC)-supported Epidemic Intelligence Information System (EPIS) rapid exchange platform. The EPIS links together all EU/EEA public health laboratories in the Food- and Waterborne Diseases and Zoonoses network (FWD-Net). In parallel, the European Union Reference Laboratory for Verotoxin-producing *E. coli* rapidly developed a real-time PCR method to detect O104 somatic- and H4 flagellar antigen-associated genes in food samples and shared it with the EU veterinary and food safety reference laboratory network.

This approach illustrates how seamless collaboration between food and public health laboratories, as well the power of harnessing advanced molecular typing technology and electronic communication, can build the laboratory capacity needed to respond appropriately to the cross-border spread of a highly virulent food-borne pathogen.

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Characteristics of the enteroaggregative Shiga toxin/verotoxin-producing *Escherichia coli* O104:H4 strain causing the outbreak of haemolytic uraemic syndrome in Germany, May to June 2011

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The *Escherichia coli* strain causing a large outbreak of haemolytic uraemic syndrome and bloody diarrhoea in Germany in May and June 2011 possesses an unusual combination of pathogenic features typical of enteroaggregative *E. coli* together with the capacity to produce Shiga toxin. Through rapid national and international exchange of information and strains the known occurrence in humans was quickly assessed. We describe simple diagnostic screening tools to detect the outbreak strain in clinical specimens and a novel real-time PCR for its detection in foods.

Sequence of events

Having received the first Early Warning Response System (EWRS) alert issued by the Robert Koch Institute (RKI) in Germany on 23 May about an increase in the number of patients presenting with haemolytic uraemic syndrome (HUS) and bloody diarrhoea caused by Shiga toxin-producing *Escherichia coli* (STEC) with more than 30 possible cases reported since the second week of May, the World Health Organization Collaborating Centre (WHO CC) for Reference and Research on Escherichia and *Klebsiella* at Statens Serum Institut (SSI) in Denmark issued an alert to the Danish *E. coli* network of regional hospitals on the same day. On 24 May, Hvidovre University hospital reported a German patient who had been diagnosed with Shiga toxin/verotoxin-producing *E. coli* (STEC/VTEC) and referred the strain to SSI. The WHO CC found that this first isolate was of serotype O104:H4 and produced Shiga toxin (Stx)/verotoxin (VT) as also reported by RKI. Referral from other regional hospitals identified the German outbreak strain in further patients in Denmark during the next days. This information was immediately shared by postings on the Urgent Inquiry Network (UIN) Epidemic Intelligence Information System (EPIS) hosted by the Food- and Waterborne Diseases and Zoonoses (FWD) Surveillance Network of the European Centre for Disease Control and Prevention (ECDC), and emails to FWD, the European Union Reference Laboratory for *E. coli* (EU-RL) and the two associated networks including public health (ECDC) and food safety (EU-RL) reference laboratories, the Global Food-borne Infections Network (GFN), Food Safety, WHO Geneva and the WHO Regional Office for Europe, and PulseNet at the United States (US) Centers for Disease Control and Prevention (CDC).

Having verified the specific characteristics of eight of the Danish outbreak strains, the WHO CC sent the index strain and the reference strain for the O104 antigen to the EU-RL in Rome. The strains were received on 31 May and tested positive by the EU-RL using a novel real-time PCR developed at the EU-RL and its network for detection of *E. coli* strains of serotype O104:H4. Thus, within a week, screening tools and a novel PCR protocol for detection of the outbreak strain in clinical specimens and in foods were developed, tested and shared with national as well as international networks. In return, members of the networks contributed with their existing knowledge of *E. coli* strains of serotype O104:H4, thereby collectively adding to the existing knowledge of this pathogen and describing the relevant characteristics of the reported strains for public health investigation.

The outbreak strain

The Danish isolates were PCR-positive for the *aggR* gene, which is typical of enteroaggregative *Escherichia coli* (EAggEC). Further analysis showed that the outbreak strain (first eight isolates from Danish patients)

were also positive for the following genes: *sigA, sepA, pic, aatA, aaiC, aap*, as well as *aggA*, which encodes the major component of the AAF/I adhesin. AAF/I is a fimbrial organelle usually associated with a strong ability to form biofilms and haemagglutination with human erythrocytes. Preliminary testing at WHO CC showed that the isolates were moderate to good biofilm producers particularly in Dulbecco´s minimum essential medium (DMEM) supplemented with 0.45% glucose, which is typical and defining for EAggEC strains. The outbreak strain was a typical *E. coli*: lactose-positive, sorbitol-fermenting and beta-glucuronidase-positive. Furthermore, the strain was positive for iutA encoding an aerobactin receptor found in 80% of extraintestinal pathogenic *E. coli* isolated from urosepsis [2] and negative for the STEC-associated adhesin (saa) and cytotoxin subtilase (subAB).

Taken together, these data indicate that the outbreak strain is indeed a typical EAggEC strain that has acquired the bacteriophage encoding Stx/VT. Using a novel protocol for subtyping of *stx/vtx* genes [3], we have shown that the gene encoding Stx/VT is *stx2a/vtx2a*.

Sequence analysis of the published *stx2a/vtx2a* sequence (SRX067313 on http://www.ncbi.nlm.nih.gov/sra) showed 100% amino acid identity of the holotoxin to Stx2a/VT2a from *E. coli* O157:H7 EDL933 isolated from Michigan ground beef in 1983 (accession number X07865 [4]) but differed by one nucleotide at position 867 (C instead of T), making the nucleotide sequence identical to the sequence found in sorbitol-fermenting O157 strains from Germany in 2002 and 2005 (accession numbers AY143336 and AY143337, unpublished), DQ231589 and DQ231590 [5], and Scotland in 2006 (EU526759) [6]. This sequence variant of *stx2a/vtx2a* has also been detected in isolates from seagulls (accession number AB030484, unpublished) and human isolates of different serotypes: *E. coli* O121:H19 from Canada (DQ143182 and DQ143183) [7] and Idaho, US (EF441611) [8], and O111:H8 also from Idaho, US (EF441606) [8].

These findings could explain the unexpectedly high level of virulence in a STEC/VTEC strain negative for the attaching/effacing pathogenicity island. It is indeed conceivable that the enteroaggregative adherence phenotype could have allowed these *E. coli* O104 strains to colonise the intestinal mucosa of the affected patients as efficiently as the typical eae-positive STEC/VTEC strains. The different mechanism of adhesion might also explain why this strain is more likely to cause severe disease in adults rather than in children, as would be usual for typical HUS-associated STEC/VTEC: adults and children might differ in their susceptibility to the adherence and/or colonisation properties of this type of EAggEC strain. Obviously, elucidating this aspect requires dedicated studies and we cannot exclude that the different rates of HUS between adults and children observed in the current outbreak just reflect a difference in the exposures.

Screening for the outbreak strain

Plating clinical samples on extended-spectrum beta-lactamase (ESBL) plates, such as commercially available Tryptone Bile X-Glucuronide (TBX) medium will allow growth of the outbreak strain and inhibit the majority of other *E. coli* strains. Excellent growth of the index strain (only one of the strains has been tested so far) from the outbreak has also been observed as light red colonies on cefixime tellurite sorbitol MacConkey (CT-SMAC) plates at 37 °C, 41.5 °C and 44 °C (Jeppe Boel, personal communication, 3 June 2011). Since cefixime belongs to the class of cephalosporins, it seems likely that the strain can overcome the cefixime concentration in CT-SMAC, but apparently it is also able to overcome the tellurite concentration.

For quick screening of clinical samples, K9 antiserum for live slide agglutination can be used in both primary and secondary testing laboratories. This is because the O104 O antigen is identical to the K9 capsular antigen [9]. The K9 antiserum is readily available from SSI Diagnostica, Hillerød, Denmark (ivdorders@ssi.dk) and described on the SSI website [10]. At SSI, we have agglutinated culture from confluent growth but pools of 5 to10 individual colonies can also be agglutinated. Immediate positive reactions indicating the presence of *E. coli* O104 have all been confirmed by conventional serotyping of O and H antigen, presence of stx2a/vtx2a and lack of the eae gene. Based on our observations so far, all weak reactions have turned out to be negative for the outbreak strain. The strain can also be detected by a number of methods targeting the stx2/vtx2 gene by PCR, RT-PCR or commercial Stx/VT detection kits. The strain must also be negative for the eae gene and confirmed for O104.

Food samples should be enriched in Buffered Peptone Water (225 ml for 25 g test portion) and incubated for 18 to 24 h at 37 °C ± 1 °C. DNA extracted from a 1 ml aliquot is purified and tested for the presence of *stx/vtx* genes (first step of the real-time PCR procedure described in the ISO/TS 13136:2011(E) method [11]).

Samples positive for *stx/vtx* genes (regardless of the presence of the eae gene) are tested for the O104-associated gene (*wzx*O104) [12]. The *wzx*O104-positive enrichment cultures are plated onto two media: (i) MacConkey agar, or TBX, or any other medium suitable for *E. coli* isolation, and (ii) a more selective medium containing an antibiotic supplement. Colonies positive for *stx/vtx* genes are identified for the O104 antigen-associated gene *wzx*O104 and the gene encoding the H4 flagellar antigen, *fliC*H4 [12]. Conventional serotyping can be performed by standard methods [13]. Other markers can be tested by either conventional or real-time PCR for further characterisation.
DNA from an outbreak strain provided by the Robert Koch Institute to be used as positive control in the PCR assays can be obtained from Istituto Superiore di Sanità (ISS) in Rome (crl.vtec@iss.dk).

To the best of our knowledge, this unusual combination of virulence factors of STEC/VTEC and EAggEC has rarely been described in humans. A strain of serotype O111:H2 [14] caused a small outbreak of HUS in France in 1995, but the episode involved children, as is typical for STEC/VTEC [15]. As in the present outbreak in Germany, the association of the French strains with severe disease (HUS) supports the view that this unusual combination of virulence factors might confer a very high degree of virulence.

Serotype O104:H4

Sporadic cases of stx2/vtx2-positive *E. coli* serotype O104:H4 have been reported. These reports include two isolates from patients with HUS in Germany in 2001 [16], one in France in 2004 (data from the dedicated EU surveillance network Enter-net; not including clinical information), one from a case of HUS in Korea in 2005 [17], two HUS cases in the Republic of Georgia in 2009 (unpublished information provided via PulseNet, US CDC), and one uncomplicated case of diarrhoea in Finland in 2010 (reported to FWD on EPIS). The isolates from Germany 2001, Finland 2010 and the Republic of Georgia 2009 were EAggEC and STEC/VTEC.

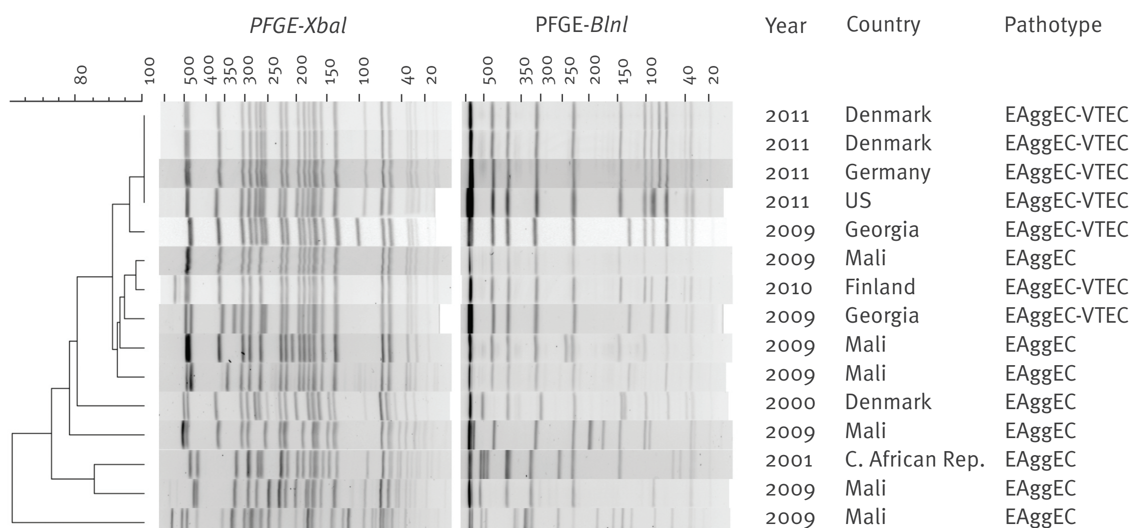
The strain from the Republic of Georgia had the following characteristics: serotype O104:H4, Shiga toxin subtype stx2a, eae-negative, haemolysin-negative, *aatA*-positive (EAggEC marker), susceptible to ceftriaxone (unlike the current outbreak strain), sorbitol-, lactose-, and beta-glucuronidase-positive, biochemically consistent with *E. coli*, Shiga toxin production on the low end of the spectrum, similar to that of the German strain (Peter Gerner-Smidt, personal communication 7 June 2011 from PulseNet, US CDC, and the Georgian team of investigators). At this time, we do not have further information on the remaining O104:H4 STEC/VTEC isolates from France and Korea.

In general, we have limited knowledge on EAggEC of this serotype: The archetype isolate for the aggregative adherence fimbriae type III (AAF/III, encoded by the *agg3A* gene) is strain 55989, which was isolated during a study of EAggEC as a cause of persistent diarrhoea in African patients infected with human immunodeficiency virus (HIV) [18,19]. In a recent study of childhood diarrhoea in Mali, we identified Stx/VT-negative EAggEC O104:H4 in three children with moderate to severe diarrhoea and from three healthy controls (unpublished data). The three EAggEC strains isolated from these cases were PCR-positive for different combinations of *aggR, aatA, aaiC, aap, astA, sepA, pic, sigA, aggA, agg3C* and *agg3A*.

We have compared the pulsed-field gel electrophoresis (PFGE) profiles of the available *E. coli* O104:H4 isolates to elucidate the diversity within this serotype, irrespective of the virulence profile. PFGE typing using the enzymes *XbaI* and *BlnI* showed that the serotype O104:H4 is diverse (Figure). For *XbaI*, a high similarity of >95% was seen for the 2011 German outbreak isolates (isolated in Denmark, Germany and the US) and one of the isolates from Republic of Georgia. A large cluster of isolates with >90% similarity included the German outbreak strain, the two Georgian cases from 2009, the isolate from the Finnish patient (all stx2a/vtx2a and EAggEC) as well as three of the *stx/vtx*-negative EAggEC isolates from patients in Mali. The profiles of five of the *stx/vtx*-negative EAggEC isolates showed

Figure

PFGE profiles (*XbaI* and *BlnI*) of *Escherichia coli* O104 compared with four isolates from the outbreak of haemolytic uraemic syndrome in Germany, May to June 2011



C. African Rep: Central African Republic; CDC: Centers for Disease Control and Prevention; PFGE: pulsed-field gel electrophoresis; RKI: Robert Koch Institute; US: United States.

PFGE profiles (*XbaI* and *BlnI*) using the *E. coli* non-O157 PulseNet protocol (www.pulsenetinternational.com). Dendrogram based on analysis of the *XbaI* profiles. All isolates are EAggEC O104:H4 with and without *stx2/vtx2* gene. German outbreak isolates are from patients infected in May 2011 in Germany and diagnosed in Denmark, the US (profiles provided by PulseNet, US CDC) and Germany (strain provided by RKI, Germany). O104:H4 isolates from Mali are from children with and without diarrhoea.

major differences from the outbreak strain (Figure). The 11 Danish PFGE-typed isolates related to the German outbreak had indistinguishable *XbaI* profiles. One isolate from a case infected in Germany and diagnosed in the US had a minor variation in the *BlnI* profile (profile provided by PulseNet, US CDC) (Figure).

General characteristics of EAggEC

EAggEC is a pathotype of diarrhoeagenic *E. coli* defined as *E. coli* that do not secrete the heat-stable or heat-labile toxins of enterotoxigenic *E. coli* (ETEC), and by its characteristic aggregative or 'stacked brick' pattern (AA) of adherence to HEp2-cells in culture [20]. This property is usually due to the presence of aggregative adherence fimbriae (AAF), whose expression is regulated by the *aggR* gene, located on the large EAggEC virulence plasmid termed pAA [21]. EAggEC infections are usually associated with watery diarrhoea, which is often persistent [20]. Illness results from a complex interaction between pathogen and host, which implicates the initial adherence of the bacteria to the epithelium of terminal ileum and colon, by virtue of the aggregative adherence fimbriae (characteristic aggregative pattern), followed by a damage/secretion stage manifested by cytokine release, mucosal toxicity, intestinal secretion and induction of mucosal inflammation [22-26].

EAggEC is best known for its role in persistent diarrhoea (>14 days) in infants and children in developing countries. Studies in Mongolia [27], India [28], Brazil [29,30], Nigeria [31,32], Israel [33], Venezuela [34], Congo [35] and many other countries, have identified EAggEC as a highly prevalent (often the most prevalent) *E. coli* pathotype in infants. Further, the role of EAggEC as an important pathogen in AIDS patients continues to develop, and EAggEC now ranks among the most important enteric pathogens in this population group [36,37]. In a recent review of all published studies of traveller's diarrhoea, EAggEC was in aggregate second only to ETEC as the most common pathogen [38].

The first reported EAggEC outbreaks occurred in Mexico City before 1993 (year unpublished) where persistent diarrhoea was reported. Five of the infected children died as a consequence of the diarrhoea. Both outbreaks occurred in the malnutrition ward of a paediatric hospital [39], demonstrating that EAggEC is not exclusively a disease of infants under the age of 12 months [40]. Itoh et al. described a massive outbreak of EAggEC diarrhoea among Japanese children in 1993 affecting nearly 2,700 patients [41]. Another EAggEC outbreak was reported in a Serbian nursery in 1995 [42] in which 16 newborn babies (duration of illness 3–9 days) and three infants (18–20 days) developed diarrhoea accompanied by pyrexia and weight loss. Outbreaks have also been reported among adults in the United Kingdom [43] and a small outbreak of EAggEC serotype O92:H33 was reported in Italy in which pecorino cheese (unpasteurised milk) was epidemiologically implicated [44]. As these outbreaks suggest, EAggEC is capable of causing diarrhoea in adults and children, even in the absence of Stx/VT. We believe that this pre-existing diarrhoeagenic and outbreak potential, coupled with the highly virulent Stx/VT, has resulted in a hypervirulent strain currently circulating in Germany. It should also be noted that EAggEC are common in all populations of the world, industrialised and developing, but that no animal reservoir has been described. This observation suggests the startling possibility that this new O104 stain may have the capacity to persist among human populations, perhaps indefinitely.

Conclusions

The rapid exchange of information, strains and DNA fingerprints within existing national and international public health and food safety networks has been vital in the quick and alternative assessment of the public health significance of the strain causing the outbreak of HUS in Germany in May and June 2011. The combined contributions have resulted in major findings including:

- the characterisation of an unusual combination of pathogenic features typical of EAggEC combined with the capacity to produce Shiga toxin in the outbreak strain;
- recommendations for simple diagnostic screening tools for primary laboratory detection of the outbreak strain in clinical specimens;
- a novel real-time PCR protocol for detection of E. coli O104:H4 in foods;
- presentation of the known occurrence and clinical presentation in humans and the likely reservoir.

We hope that this report will help to strengthen existing networks, inspire the development of new networks and improve food safety in the future when new or emerging bacterial pathogens may occur in the food chain.

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Enhanced surveillance during a large outbreak of bloody diarrhoea and haemolytic uraemic syndrome caused by Shiga toxin/verotoxin-producing *Escherichia coli* in Germany, May to June 2011

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Germany has a well established broad statutory surveillance system for infectious diseases. In the context of the current outbreak of bloody diarrhoea and haemolytic uraemic syndrome caused by Shiga toxin/verotoxin-producing *Escherichia coli* in Germany it became clear that the provisions of the routine surveillance system were not sufficient for an adequate response. This article describes the timeline and concepts of the enhanced surveillance implemented during this public health emergency.

On Thursday, 19 May 2011, the Robert Koch Institute (RKI) was informed about a cluster of cases of haemolytic uremic syndrome (HUS) due to Shiga toxin/verotoxin-producing *Escherichia coli* (STEC/VTEC) O104:H4 in the area of Hamburg, Germany. An RKI investigation team visited the affected area the following day. In the face of rapidly rising case numbers, a need for enhanced surveillance was identified on 23 May. We describe here the timeline and concepts of the enhanced surveillance implemented during this massive outbreak of bloody diarrhoea and HUS in May and June 2011 in Germany.

Routine surveillance system

In Germany, STEC/VTEC and HUS have been statutorily notifiable since 2001 according to the Protection against Infection Act (Infektionsschutzgesetz, IfSG [1]). While STEC/VTEC surveillance is based on laboratory analyses, HUS surveillance relies on physicians. Heads of laboratories and physicians must report cases to the local health authorities within 24 hours. The incoming data is validated by the local health authorities and documented electronically. Cases fulfilling the surveillance case definition as issued by RKI [2] are transmitted in anonymous form to the state health authorities by the third working day of the following week. The state health authorities again validate incoming cases and transmit the data to the RKI within the following week. Hence, transferring information on a case from the local to the national health authority may take from a few days up to 16 days.

Epidemiological information is fed back from RKI at least weekly to the stakeholders, e.g. responsible authorities, physicians and laboratories. Information exchange includes teleconferences, reports in the RKI's weekly *Epidemiological Bulletin* and the internet database SurvStat [3].

Enhanced surveillance system

In the context of the outbreak it became immediately clear that the provisions of the routine surveillance system were not sufficient for an adequate response. Hence, the following amendments were implemented:

- Centralising the epidemiological information exchange,
- Accelerating the data flow to the national level,
- Implementing a syndromic surveillance system for bloody diarrhoea in emergency departments,
- Assessing the capacities for HUS-treatment in Germany,
- Initiating active laboratory surveillance.

An overview of routine and newly implemented surveillance systems is given in Figure 1.

Centralising the epidemiological information exchange

On 23 May 2011, the 'Lagezentrum' at the RKI was activated as a central emergency operations centre. A large number of RKI staff was involved in coordinating the collection of epidemiologic information and organising the public health response. From 23 May onwards, teleconferences were conducted almost daily with the responsible state, national and international authorities. Starting on 24 May, epidemiological reports were distributed daily to the responsible authorities, physicians and laboratories to feed back relevant information. Several outbreak-related articles were published in *Eurosurveillance* [4,5] and the German *Epidemiological Bulletin*. The public was regularly informed about the outbreak situation via the RKI website starting on 23 May, press releases were issued on 3 and 10 June. The Federal Centre for Health Education (Bundeszentrale für Gesundheitliche Aufklärung, BZGA), has provided outbreak-related public health advice to the public since 24 May.

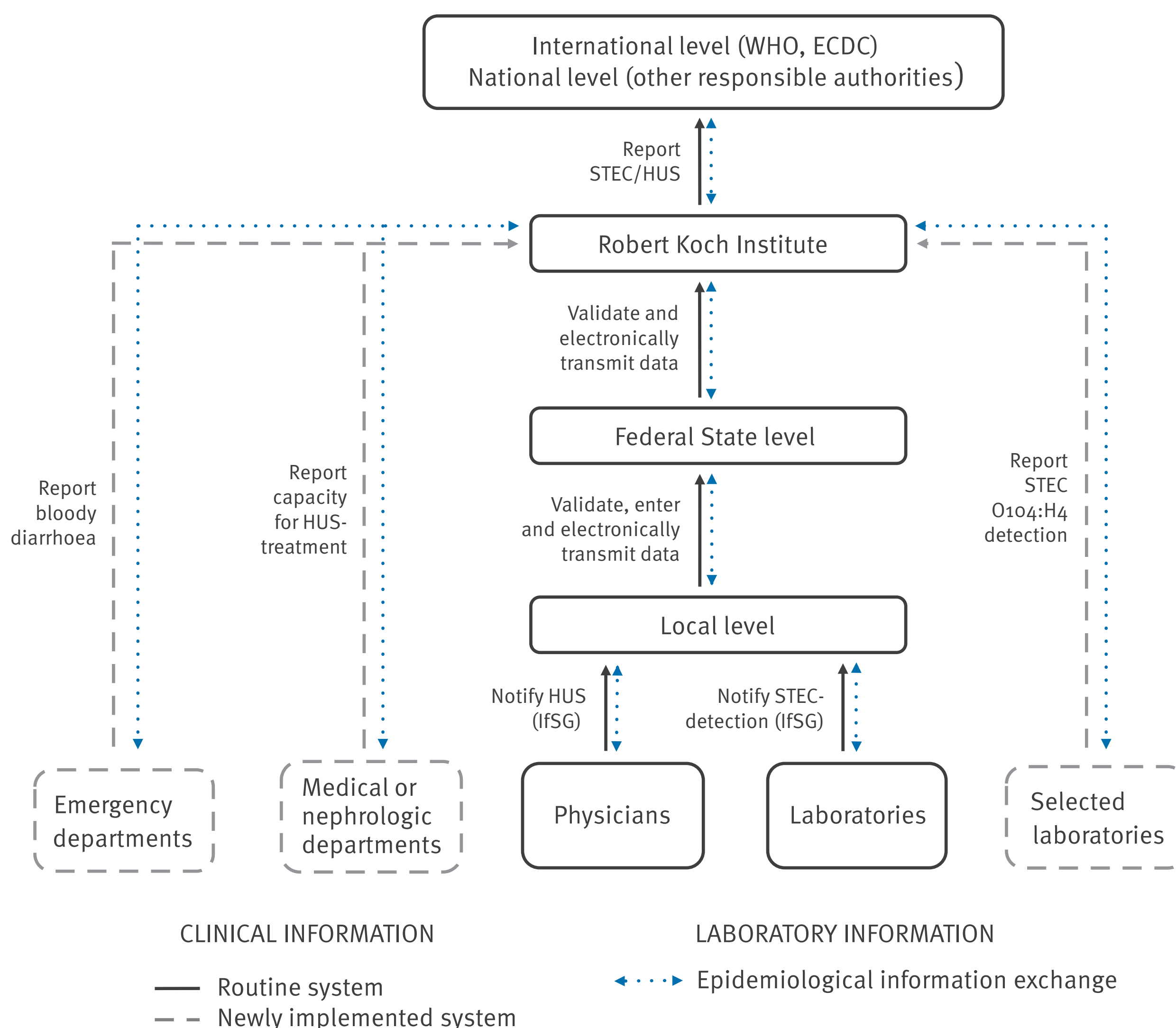
Accelerating the data flow to the national level

From 23 to 27 May 2011, state health authorities were asked to transmit aggregated data via email on a daily basis to the RKI. Concurrently, health authorities were urged to enter and transmit the IfSG data via the electronic surveillance system daily, so that case by case reporting could overtake the aggregated reporting on 27 May. A specific reporting form was published on 26 May to facilitate notification of HUS cases by physicians.

In addition, the existing RKI surveillance case definition was adapted to the outbreak situation to ensure systematic data collection. Modifications included

Figure 1

Data and information flow to and from the Robert Koch Institute during the period of enhanced surveillance, STEC/HUS outbreak, Germany, spring 2011



ECDC: European Centre for Disease Prevention and Control; HUS: haemolytic uraemic syndrome; IfSG: German Protection against Infection Act; STEC: Shiga toxin-producing *Escherichia coli*; WHO: World Health Organization.

limitations of time (onset of disease from 1 May 2011), place (epidemiological link to Germany) and person (e.g. consumption of a food item that was acquired in Germany) concerning exposure as well as inclusion of suspected cases [6].

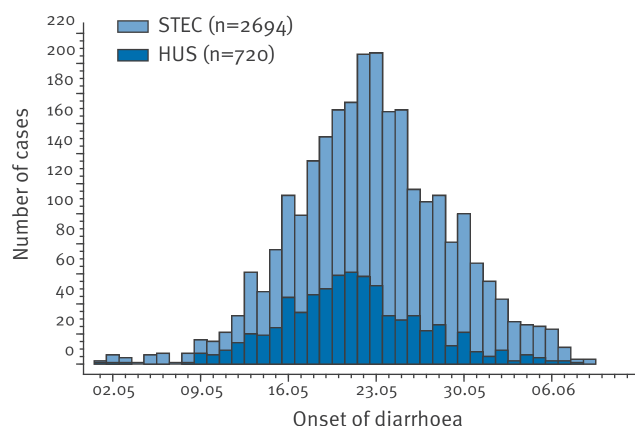
One challenge was counting outbreak-related cases of STEC/VTEC O104:H4 separately from other STEC/VTEC cases, of which a mean of 992 cases annually had been reported to the RKI between 2001 and 2010. In the absence of comprehensive laboratory data for a majority of reported cases, the case definition was revised in a way that listed as exclusion criteria all specific laboratory test results that were not consistent with the characteristics of the outbreak strain.

As of June 12, a total of 3,228 STEC/VTEC and HUS cases in Germany have been associated with the outbreak (Figure 2). The majority of cases (51%) fell ill between 18 and 25 May. The place of exposure was suspected to lie in north-western parts of Germany for most cases (Figure 3). Of the 781 reported HUS cases, 69% were female and 88% were 20 years of age or older. Overall, 22 notified HUS ca¬ses have died. Among all 2,447 STEC/VTEC cases, 59% were female and 87% were 20 years of age or older. Thirteen notified STEC/VTEC cases have died.

Figure 4 shows the transmission delay in days from the local to the national level during the STEC/HUS outbreak period among HUS cases. Among the 740 HUS cases (96%) with known date of notification to the local health authorities, the median transmission delay was two days (25th–75th percentile: 1–4 days, minimum–maximum: 0–18 days). The first HUS-case was reported to the RKI through the electronic surveillance system on 18 May. Another three HUS cases were reported on 23 May. Thereafter, the accelerated data flow became evident, for instance, 47 HUS cases were reported to the RKI on 24 May, 50 HUS cases on 25 May, 100 HUS cases on 26 May and 116 HUS cases on 27 May.

Figure 2

Reported STEC/VTEC and HUS cases, by date of onset of diarrhoea[a], Germany, May–June 2011 (n=2,694)



ED: Emergency department; HUS: haemolytic uraemic syndrome; STEC: Shiga toxin-producing *Escherichia coli*.

[a] Only cases with a notified date of onset since 1 May 2011.

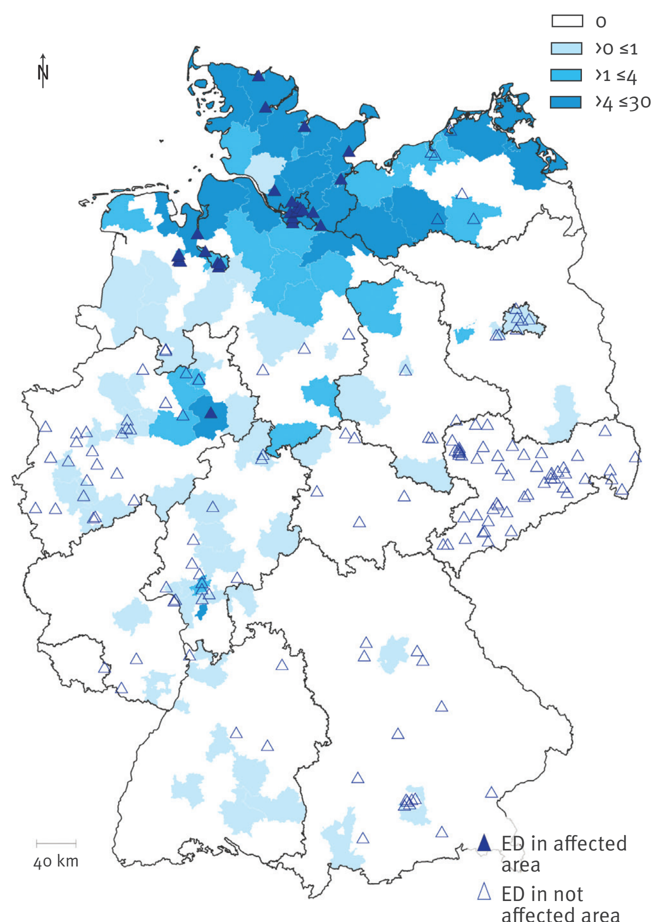
Implementing a syndromic surveillance system for bloody diarrhoea in emergency departments

Since STEC patients often present with bloody diarrhoea, emergency departments (ED) constitute appropriate facilities for the assessment of the temporal trend of an STEC-outbreak. We implemented the surveillance of patients with and without bloody diarrhoea in ED on 27 May.

Participating ED were located in all federal states of Germany, both in areas affected and not affected by the STEC/HUS outbreak (see Figure 4). Data collection covered the total number of new patients in participating ED and the number of patients presenting with bloody diarrhoea by sex and age group (<20 years, ≥20 years). The data were transferred to the RKI by email or fax every day.

Figure 3

Cumulative incidence of HUS cases per suspected county of exposure and emergency departments actively participating in the syndromic surveillance system, Germany, May–June 2011

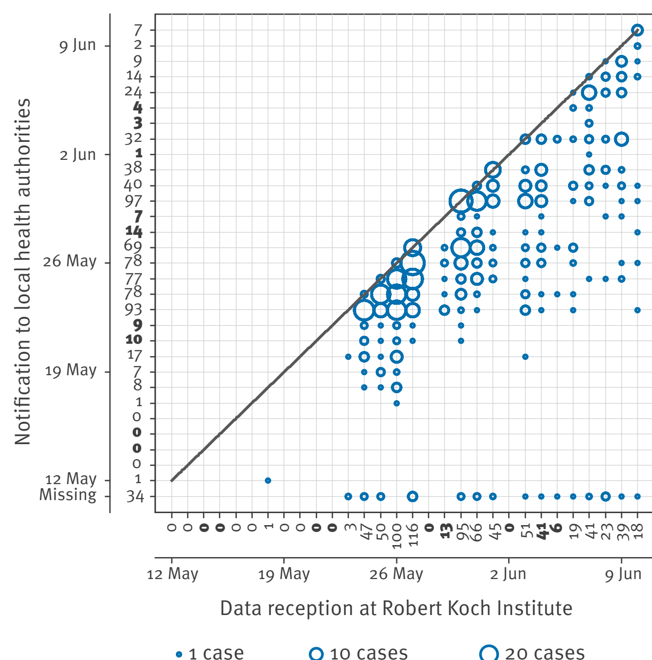


ED: Emergency department; HUS: haemolytic uraemic syndrome; STEC: Shiga toxin-producing *Escherichia coli*.

As of 12 June, a total of 174 ED have participated in the syndromic surveillance system; 27 of which were located within affected areas. The number of ED actively reporting varied from day to day. Thus results may change as further, re¬tro¬spective, reports are received from ED. Between 28 May and 12 June, 4.7% (744/15,884) of all patients presenting to ED in affected regions were reported as having bloody diarrhoea (Figure 5); this proportion was 0.8% (464/55,255) in non-affected regions. Figure 5 shows the sex and age distribution of patients with BD as well as the number of participating ED in affected areas. Women were affected more often than men, with a decreasing proportion of female cases observed after 30 May. Since 6 June, the proportion of all patients with bloody diarrhoea among the patients presenting to emergency departments has remained on an average of 3.6%.

Figure 4

Date of notification of HUS cases to local health authority in relation to date of reception at Robert Koch Institute, Germany, May–June 2011



HUS: haemolytic uraemic syndrome; PH: public holiday; STEC: Shiga toxin-producing *Escherichia coli*; WE: weekend.

Weekends and public holidays in bold; the x- and y-axis additionally show the number of reports received.

The size of the circle is equivalent to the number of cases (examples for 1, 10 and 20 cases shown in the legend).

Assessing the capacities for treatment of haemolytic uraemic syndrome in Germany

From 30 May onwards, the German Society for Nephrology collected data on the HUS treatment capacities in Germany and reported these regularly via e mail to the RKI. During the outbreak period, 79 hospitals, located in 15 of the 16 federal states, provided almost daily information: all but two confirmed having sufficient capacities for treating HUS patients.

Initiating active laboratory surveillance

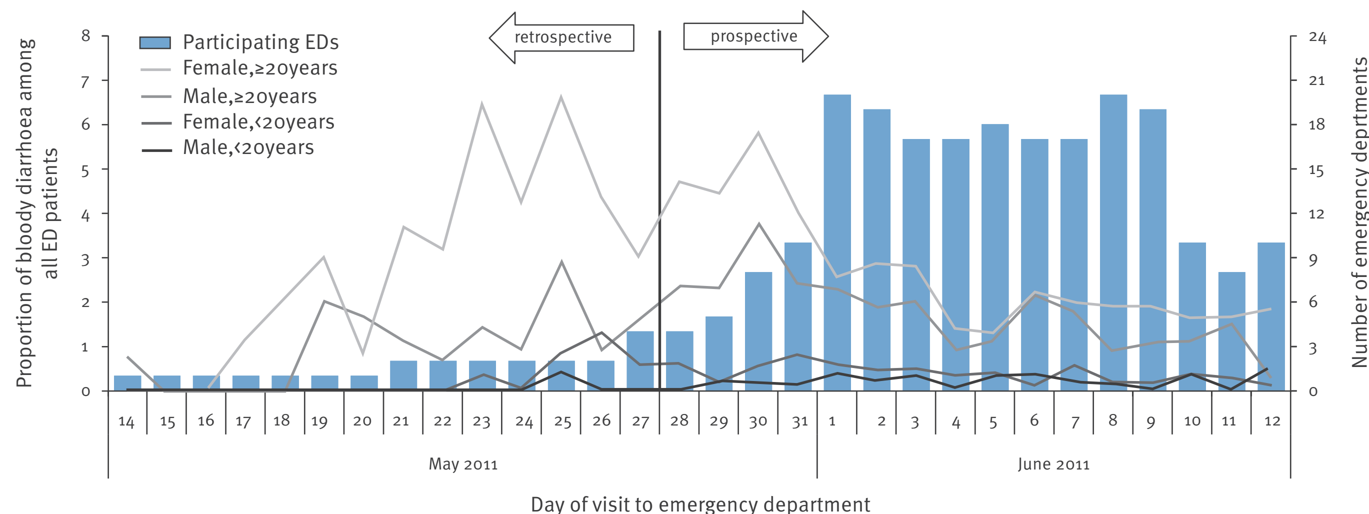
Since 25 May, the RKI has asked four laboratories for daily data transfer per email or telephone. As of 12 June, a total of 195 (6%) of all 3,228 STEC/HUS cases have been confirmed through the routine mandatory system as caused by the outbreak strain STEC/VTEC O104, whereas the active system provided evidence that at least 335 patient samples were related to the outbreak strain.

Reports to the European Union and the World Health Organization

Following international law, Germany informed the European Union (EU) of the STEC/HUS outbreak via

Figure 5

Proportions of patients with bloody diarrhoea among all patients presenting to emergency departments, by age and sex, in areas affected by the STEC/HUS outbreak, Germany, May–June 2011 (n=744)



D: emergency department; HUS: haemolytic uraemic syndrome; STEC: Shiga toxin-producing *Escherichia coli*.

the Early Warning and Response System (EWRS) on 22 May 2011, and notified the event as a potential public health emergency of international concern within the framework of the International Health Regulations (IHR) 2005 on 24 May. The RKI sent updates on the situation to EWRS, the Epidemic Intelligence Information System (EPIS) and the World Health Organization (WHO) on a daily basis.

Both the European Centre for Disease Prevention and Control (ECDC) and the WHO immediately supported the outbreak investigations by staying in close contact with Germany and other countries and reporting imported STEC/HUS cases (in travellers) associated with the outbreak.

Conclusions

Germany has a well established broad statutory surveillance system for infectious diseases. However, the rather long time limits permitted for communicating information on cases from the local to the state/national level led to delayed recognition of this outbreak: The first report at the national level was received on 18 May 2011, while the first outbreak-associated cases fell ill on 1 May, with a sharp increase in case numbers on 9 May. This is a limitation requiring further evaluation. In this specific outbreak situation, the mandatory surveillance system required enhancement that was rapidly and effectively implemented. Physicians, laboratories, local and state health authorities supported the acceleration and extension of the system extraordinarily well. Feedback to the public, the responsible authorities, physicians and laboratories was ensured daily, e.g. by updates on websites, teleconferences and reports.

The additional surveillance instruments were voluntary and allowed for more timely monitoring of this public health emergency. Laboratory surveillance permitted assessment of the actual number of laboratory-confirmed outbreak cases particularly in the early stages. Monitoring capacity for treating HUS patients in German hospitals allowed us to evaluate whether or not international help would be needed. Syndromic surveillance in ED permitted us to follow the temporal trend of bloody diarrhoea patients as a proxy for potentially new STEC/VTEC cases.

We conclude that infectious disease surveillance in Germany can rapidly be adapted to specific outbreak situations. Nevertheless, data flow within the statutory surveillance system should be accelerated, e.g. by use of an electronic notification system by physicians and laboratories, and a common central data base. We recommend continuing syndromic surveillance in ED for at least the next three months to ensure timely detection of possible new trends.

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We gratefully acknowledge the contribution of all physicians and laboratories as well as the local and state health departments, whose investigations and notifications were the data basis of this report. We especially thank the various emergency departments participating in the syndromic surveillance system, the laboratories involved in the laboratory surveillance system and the German Society for Nephrology for their support.

HUS surveillance and laboratory team

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Rapidly controlled outbreak of *Serratia marcescens* infection/colonisations in a neonatal intensive care unit, Pescara General Hospital, Pescara, Italy, April 2011

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In April 2011, an outbreak of *Serratia marcescens* infection/colonisations occurred in the neonatal intensive care unit of Pescara General Hospital. Rapid microbiological investigations lead to identification of five cases of likely cross-transmission from a neonate hospitalised for *S. marcescens* sepsis: four infections and one neonate colonised post-mortem. Two low birth weight neonates died. The environmental investigation detected *S. marcescens* from two soap dispensers. Strict hygiene measures lead to early interruption of the outbreak, without recurrences to date.

Serratia marcescens is an opportunistic pathogen able to rapidly spread in the nosocomial environment, being identified in up to 16% of nosocomial Gram-negative bloodstream infections [1]. Several outbreaks of *S. marcescens* in neonatal intensive care unit (NICU) were documented in recent years [2-6], causing potentially fatal sepsis, meningitis or pneumonitis in very premature or low birth weight neonates with mortality rates as high as 44%, significantly higher compared with those caused by *Enterobacter cloacae*, another well-known cause of disease and death in premature neonates [2,7].

Outbreak description and control measures

In early April 2011, a normal weight neonate was born at 41 weeks of gestational age and was transferred to NICU of Pescara General Hospital because of fever, failure to thrive and increasing C-reactive protein (CRP) values. Blood cultures grew *S. marcescens* fully susceptible to quinolones and carbapenems. The neonate was treated with antibiotics for 11 days and discharged after full recovery on day 11. Three days after, four of 16 neonates in the NICU developed a clinical picture suggestive of sepsis, with rising CRP values. All four were cared for in the same room. Aware of the recent case of *S. marcescens*, clinicians on duty asked for immediate testing and molecular characterisation by Septifast® (Roche) on blood samples which detected *S. marcescens* DNA from all four cases. Antibiotics were administered, based on susceptibility data from the possible source. Two of the four neonates died, after 20 and 22 days of hospitalisation. Both had been premature births (week 26 and 29) with birth weight <1,200 g. Due to respiratory distress at birth, they had been transferred to NICU directly from delivery rooms, 10 and four days before becoming symptomatic. The other two septic neonates were born at 31 and 38 weeks of gestational age, one with low and one with normal birth weight. They had been delivered through Caesarian section and transferred to NICU for respiratory distress, nine and five days before becoming septic. They were discharged in good condition after 49 and 25 days of hospitalisation respectively, after their CRP values were normal.

A fifth very low birth weight (<800 g) neonate, cared for in the same room, had died the day before the four neonates became septic. Post-mortem sampling from his umbilical cord catheter grew *S. marcescens*, suggestive of colonisation.

Environmental sampling

On the day after the four neonates became symptomatic, extensive environmental microbiological investigations were started. Swabs were taken from numerous surfaces, including walls, floors with their edges and corners, doors and door handles, shelves, benches, hoods, sinks, cradles and ventilators, stethoscopes and other personal medical devices, milk

collecting devices, medical records and trolleys. To address the possibility of human carriage, hands and cellular phones of some of the healthcare workers and auxiliary staff who worked in the particular room and of additional staff operating in other rooms in the NICU were checked. Bottles of saline used for dilutions and one phosphate buffer, drug bottles and boxes, soaps and disinfectants were either swabbed or cultured. Only two soap dispensers among all investigated environmental and human surfaces yielded *S. marcescens* isolates.

Control measures

The four infected neonates were cohorted in a single room and new admissions discontinued for 10 days.

Moreover, immediately after the microbiological sampling, a thorough sanitation procedure was started: possible sources were disposed and renewed, including parenteral nutrition, milk collection devices, disinfectants, soaps and soap dispensers, cotton and tissues. Already recommended measures, such as hand washing with alcohol based solutions at all sites in the ward, and using gloves when assisting and caring for neonates, whenever possible, were reinforced. Water and air filters had been routinely renewed a few days in advance and were left in place after negative cultures. After the first sampling, additional swabs were taken from 70 soap dispensers and from bottles of chlorhexidine-based hand disinfectants in stock. Microbiological sampling was extended to other potential sites of environmental persistence, including wards in the obstetrics and gynecology unit, delivery rooms and theatres for Caesarean sections. In these environments, floors, tissues, cradles, door handles and cabinets, sinks, soaps, soap dispensers, several surfaces in the delivery rooms, baby changing tables, scales, air ducts and filters in the nursery, all yielded negative results.

Repeated microbiological controls as of 14 June 2011 failed to identify any further environmental contamination with *S. marcescens*.

Discussion

The present outbreak involved six neonates. Five cases were due to likely cross-transmission from a neonate hospitalised for *S. marcescens* sepsis, four of whom were infected and one colonised post-mortem. Two neonates with clinical signs of sepsis were successfully treated with antibiotics. Two died likely from *S. marcescens* sepsis, leading to a mortality rate of 40%. It is of note, that both deceased neonates had a positive epicutaneus culture for *Klebsiella* spp. after the isolation of *S. marcescens*, one of them having also a minor amplicon for *K. pneumoniae* at his molecular test. Both of them were premature, low weight (≤1,200 g at birth) neonates.

The *S. marcescens* infections occurred at the same time, pointing towards a common exposure to a source(s) present in a narrow period of time in the NICU, after 18 years of negative environmental sampling.

Hand carriage, although not demonstrated in this outbreak, was the most likely way of spread to and from soap dispensers, of which two tested positive. This is in-line with the literature that shows that the organism can survive on the human skin for extended periods of time, and hand carriage is thought to be a mode of transmission when no environmental source is identified [3]. Moreover, other reports document soaps and detergents as potential site for the spread of *S. marcescens* [4].

In outbreaks with *S. marcescens*, extensive environmental microbiological investigations are needed to identify sources and reservoirs [2]. Environmental sources of *S. marcescens* in NICU include breast pumps, breast milk, parenteral nutrition, soap and disinfectant dispensers, laryngoscope blades, ventilators and air conditioning ducts [2,6]. Secondary and even tertiary waves of transmission have been recently reported [8-11]. Under such circumstances, strict measures such as nurse cohorting, closure of wards to new admissions and even temporary total closures of NICU were necessary to contain the spread of the microorganism [10,11]. After the implementation of strict sanitation and control measures in our NICU, there was no evidence of further transmission or environmental persistence of *S. marcescens*.

For this reason, molecular cluster analysis of the eight available isolates (six from the neonates and two from the soap dispensers) was not requested. Fortunately, this outbreak was limited to a small number of cases, with a single epidemic peak of colonisation/infection and so far no recurrence. In recent NICU reports, greater numbers of patients were involved, the fraction of infected/colonised neonates among all neonates present in the wards higher, and environmental colonisation more protracted, with more than one epidemic peak; mortality rates were however comparable.

Prompt clinical suspicion of *S. marcescens* and the immediate use of a molecular assay allowed for early etiological diagnosis, supported by an epidemiological link with a recent case and paved the way to immediate measures for containment of nosocomial infections. These measures adopted before any evidence from traditional microbiological cultures, enabled the rapid interruption of the outbreak. Moreover, this report serves as a reminder of the importance to keep up hygiene precautions at any time specifically in high risk settings such as a NICU.

***Erratum:** At the moment of publication, the names of V Cortesi and V Fortunato were left out in the list of authors. This mistake was corrected on 21 June 2011. We apologise to the authors.

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Intestinal parasites isolated in a large teaching hospital, Italy, 1 May 2006 to 31 December 2008

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Intestinal parasites account for the majority of parasitic diseases, particularly in endemic areas. Most are transmitted via contaminated food. Because of increased immigration and travel, enteric parasitoses are now distributed worldwide. Between May 2006 and December 2008, we examined stool specimens from 5,351 patients (4,695 Italians, 656 non-Italians) for ova and parasites using microscopy, culture techniques, and molecular methods. Stools from 594 patients (11.1%) were contaminated and for all patients samples combined, a total of 700 intestinal parasites were counted. Ninety of the 594 infected patients had more than one parasite in their stools. Parasites causing intestinal disease occurred in 8.8% of patients. The prevalence was over twice as high among non-Italians (26.8% vs 8.9% in Italians, $p<0.001$) and higher in males (13.0% vs 9.5% in females, $p=0.003$). Most isolates were pathogenic protozoa, including in decreasing order of frequency: *Blastocystis hominis, Giardia intestinalis, Entamoeba histolytica,* and *Cyclospora cayetanensis*. The latter two species tended to be more common in Italians, although not at significant level (3.6% (15/418) vs 1.7% (3/176) in non-Italians, OR: 2.15; 95%CI: 0.60–11.70, $p=0.22$). Helminthes were found in 28 patients, mainly non-Italians (5.7% (10/176) vs 4.3% (18/418), OR: 1.34; 95%CI: 0.54–3.13, $p=0.47$). *Ascaris lumbricoides* and *Hymenolepis nana* were the most common. *Strongyloides stercoralis, Enterobius vermicularis, Taenia* spp. and *Trichuris trichiura* were also found. Intestinal parasites are a serious problem in developing countries, but should not be underestimated in industrialised countries.

Introduction

Enteric parasites are the most common cause of parasitic diseases, and they cause significant morbidity and mortality, particularly in endemic areas [1]. Patients with intact or compromised immunity are affected with similar frequencies [2]. Children and young adults are the most affected group, particularly in regions with limited resources and those in which observation of hygienic measures is lax [3]. Intestinal symptoms are frequent and include abdominal pain and acute or chronic diarrhoea and/or constipation, but systemic manifestations (fatigue, anaemia, weight loss, rash) are by no means uncommon. Most intestinal parasites are transmitted by the faecal-oral route as a result of the ingestion of water, vegetables, and/or soil contaminated with ova, cysts, or oocysts; in other cases (i.e. *Ancylostoma duodenale*) transmission occurs via the skin through direct penetration by larvae living in the soil.

The diseases caused by intestinal parasites, once considered rare phenomena confined to the tropics, are now being diagnosed with increased frequency in Europe and other industrialised countries [1]. This trend can be attributed to various factors, including globalisation of the food supply, the increased consumption of fresh foods, increased travel to developing countries, and more intensive immigration originating from these areas. Contact with other cultures has led to increased consumption of raw or undercooked foods, a potential source of parasites that could be eliminated by proper food processing [4]. Seafood is a classic example, but the rapid transport of fresh fruits and produce from developing countries has made such products more available to European consumers, and thus increased their contact with intestinal parasites. In at-risk groups, sexual practices may also represent a contributor to the transmission of parasites such as *Entamoeba histolytica, Hymenolepis nana and/or Enterobius vermicularis* [5].

Physicians in non-endemic areas are often poorly prepared to deal with these 'exotic' diseases. Microscopic examination of stool for ova and parasites is commonly ordered to investigate gastrointestinal complaints, eosinophilia, or liver abscesses or as part of preventive assessments of travellers, immigrants, or patients scheduled for transplantation procedures. However, the physicians who order these tests often have a limited knowledge of what they actually entail and how the results should be interpreted. Paradoxically, laboratory and physician surveys suggest that physicians often test for parasites when the likelihood of infection is low and fail to use essential tests when suspicion is high [6].

There is an abundance of epidemiological data on the diffusion and prevalence of intestinal parasitic diseases in developing areas [2,7,8], but in industrialised countries these infections are rarely reported [9]. The microbiology laboratory of our hospital is receiving an increased number of requests for the analysis of stool specimens for ova and parasites. This trend prompted us to evaluate the distribution of intestinal parasites isolated by our laboratory over a 30-month period.

Patients and methods

Patient population and study design

This retrospective study was conducted at the Catholic University Hospital, a 1,600-bed academic medical centre located in Rome, Italy, that admits approximately 60,000 patients per year. We systematically searched the computerised database of the medical centre's central microbiology laboratory to identify all stool specimens submitted for parasitology investigation from 1 May 2006 through 31 December 2008.

Laboratory methods

All commercial products and devices described below were used in accordance with manufacturers' instructions unless otherwise stated.

Samples were stored in fresh normal saline smears. Formalin ethyl acetate concentration was performed with a Midi PARASEP kit (DiaSys Europe Ltd – Wokingham, UK) with Lugol's iodine. Smears were examined with a 40x phase-contrast objective.

Permanent smears were prepared from unpreserved stool and examined with a 100x oil-immersion objective after specific staining, which included trichrome stain (Scientific Device Laboratory, Inc.; Des Plaines, Illinois) and/or Weigert's iron hematoxylin for the detection of protozoa; modified Ziehl-Neelsen acid-fast stain for *Cryptosporidium, Cyclospora,* and *Isospora* species; and modified trichrome (chromotrope) stain for Microsporida.

We also used DNA-based methods to detect *E. histolytica, E. dispar, Cryptosporidium* spp., *Microsporidia* spp. and *Cyclospora*. In brief, we extracted genomic DNA from samples using the EZ1 DNA Tissue Kit (Qiagen, Valencia, CA - USA). DNA extracts were stored at -20°C prior to PCR analysis, which was done as previously described [10-15]. PCR for Microsporidia was performed only in patients who were immunocompromised or immunosuppressed.

Stool specimens were cultured to detect the presence of certain parasites. For isolation of *E. histolytica*, we used a culture medium based on BOECK & DRBOHLAV formulation (DiaSys Entamoeba kit, DiaSys Europe Ltd; Wokingham, UK). Cultures were incubated at 35–37°C for four days, and each day a drop of culture medium was examined under a 40x phase-contrast objective. Cell cultures were used to detect human-infecting Microsporidia [16]. For detection of *Strongyloides stercoralis* larvae, we streaked stool samples onto nutrient agar plates (1.5% agar, 0.5% meat extract, 1.0% peptone, 0.5% NaCl) and incubated them for at least two days at room temperature. As the larvae crawl over the agar, they carry bacteria with them, creating visible tracks [17]. When requested by clinicians, IgG antibodies to *Strongyloides stercoralis* were searched using a commercial ELISA (Bordier Affinity Products SA, Crissier, Switzerland).

Laboratory quality control

United Kingdom National External Quality Assessment Service (UKNEQAS – Department of Clinical Parasitology – Hospital for Tropical Diseases- London) provides specimens for faecal parasitology quality control since 2003. The quality management system of the laboratory of Microbiology is certified by Det Norske Veritas (DNV Italia S.r.l.) to conform to the quality management standard ISO 9001:2008.

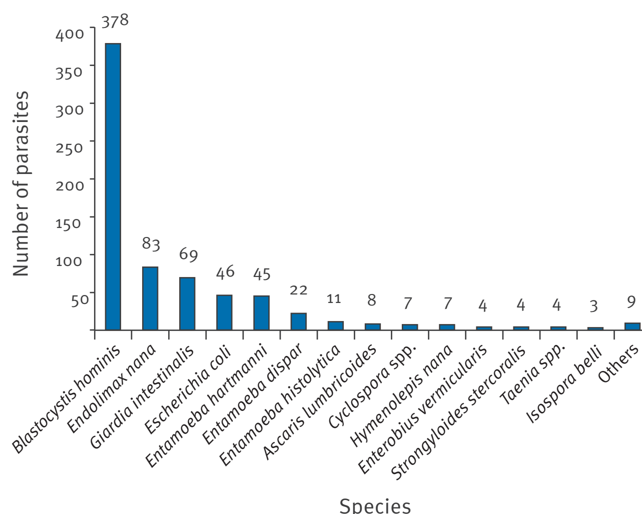
Data analysis

For each case considered, the patient's demographic characteristics were obtained from the hospital and laboratory databases, and these data were used to calculate age-, sex-, and nationality-specific incidence rates for each type of pathogenic parasites.

We also reviewed the results of stool examinations for ova and parasites to determine the incremental value of examining more than one specimen. For categorical variables, results are expressed as absolute numbers or percentages. Statistical significance was determined with the chi-square test. Odds ratios (ORs) and 95%

Figure

Intestinal parasite counts detected in stool samples from 594 patients, by species, Rome, Italy, 1 May 2006–31 December 2008 (n=700)



'Others' include *Cryptosporidium* spp. (n=2), *Encephalitozoon intestinalis* (n=2), *Dientamoeba fragilis* (n=1), *Trichuris trichiuria* (n=1), *Trichomonas hominis* (n=1), *Chilomastix mesnili* (n=1), and *Iodamoeba bütschlii* (n=1).

Taenia spp. include *Taenia saginata* and *T. solium*.

confidence intervals (CIs) were calculated to evaluate the strength of the associations that emerged. Two-tailed tests were used to determine statistical significance; a p value of <0.05 was considered significant.

Results

From May 2006 to December 2008, 9,456 faecal samples from 5,351 patients were examined for ova and parasites. Most patients (87.7%, n=4,695) were of Italian nationality; the remaining (12.3%, n=656) were born in Africa, Asia, Central and South-America. Ages ranged from 0 to 99 years and slightly more than half of the patients (54.43%, n=2,913) were female. Inpatients accounted for 60% (n=3,216) of the patients in the study.

Overall, 11.1% (594/5,351) of patients had stools contaminated with parasites, and for all combined patients' samples, a total of 700 intestinal parasites were counted. Ninety patients (90/594) had more than one parasite count in their stools. Enteric parasites were more commonly detected in non-Italian patients than in Italians (26.8% (176/656) vs 8.9% (418/4,695); OR: 3.01; 95% CI: 2.47–3.68, $p<0.001$), and in males than in females (13.0% (263/2,135) vs 9.5% (276/2,913); OR: 1.30; 95% CI: 1.08–1.56, $p=0.003$). Figure summarises the findings from the parasitological survey. Protozoa predominated and were identified in 96.8% (575/594) of the subjects, while helminths were present in 4.7% (28/594).

Table 1 provides specific quantitative data regarding the association between number of stool samples examined and the successful recovery of parasites. The majority of series (60.5% (3,237/5,351)) consisted of only a single faecal specimen. Intestinal parasites were detected in 7.3% (392/5,351) of patients when either a single specimen (when only one was submitted) or the first of a series of multiple specimens was examined. Examination of a second specimen, when submitted, increased the percentage of positivity by another 1.9% of patients (100/5,351). Examination of three faecal samples allowed the detection of parasites in another 1% of patients (53/5,351) who had not had the first or second specimen test positive for parasites. Examination of more than three specimens, when obtained, uncovered the remaining 0.5% of patients (29/5,351) not diagnosed in the initial specimen of a series.

When only parasites known to cause intestinal disease were considered, an overall prevalence of parasitic infections of 8.8% (469/5,351) was found. The prevalence of pathogenic parasites was lower 7.8% (30/387) in children aged less than five years, increased to 14% among patients aged 5–14 years, and for patients aged more than 14 years the prevalence decreased with age (Table 2).

The distribution of intestinal parasites stratified by nationality and sex is shown in Tables 3 and 4. The prevalence of parasitic infections was about 2.6 times higher among patients in the non-Italian group (19.3% (127/656) vs 7.3% (342/4,695) in the Italian group; OR: 2.66; 95% CI: 2.12–3.32; $p<0.001$) and 1.3 times higher in males than in females (9.9% (241/2,438) vs 7.8% (228/2,913); OR: 1.26; 95% CI: 1.04–1.53; $p=0.02$). Protozoa accounted for the vast majority of the infections, being detected in 97.6% (458/462) of patients harbouring pathogenic parasites. Among those, *Blastocystis hominis* was the most common parasite, detected in 7.1% (378/5,351) of the patients, particularly among non-Italians (14.1% (93/656) vs 6.1% (285/4,695) in Italians; OR: 2.56; 95% CI: 1.97–3.30, $p<0.001$). Although not at significant level, more males also appeared to be infected than females (7.5% (184/2,438) vs 6.6% (194/2,913); OR: 1.14; 95% CI: 0.92–1.42, $p=0.21$). The level of infection by B. hominis was lower among children less than five years of age (2.3% (8/387) vs 7.5% (370/4,964); OR: 0.28; 95% CI: 0.13–0.58, $p<0.001$) and equal among the individuals in all the other age groups. *Giardia intestinalis* was the second most common species. The overall prevalence of infected patients was 1.3% (69/5,351). *Giardia intestinalis* was detected more frequently in non-Italians than in Italians (5.0% (33/656) vs 0.8% (36/4,695); OR: 6.86; 95% CI: 4.11–11.39; $p<0.01$), in males than in females (1.6% (38/2,438) vs 0.7% (21/2,913); OR: 2.18; 95% CI: 1.24–3.92; $p<0.001$). Prevalence of giardiasis

Table 1

Detection of patients infected by intestinal parasites in function of number of stool samples per patient examined, Rome, Italy, 1 May 2006–31 December 2008

Number of patients	Number of samples examined per patient	Patients testing negative for intestinal parasites n (%)	Patients testing positive for intestinal parasites n (%) in function of number of samples examined					
			1	2	3	4	5	≥6
3,237	1	3,020 (93.3)	217 (6.7)					
841	2	730 (86.9)	74 (8.8)	37 (4.3)				
949	3	811 (85.5)	69 (7.2)	32 (3.4)	37 (3.9)			
146	4	122 (83.6)	8 (5.48)	9 (6.2)	4 (2.7)	3 (2.0)		
49	5	35 (71.4)	3 (6.1)	8 (16.3)	2 (4.1)	0 (0.0)	1 (2.0)	
129	≥6	58 (45.0)	21 (16.3)	14 (10.8)	10 (7.7)	12 (9.3)	3 (2.3)	11 (19.0)
5,351	**total**	**4,776 (89.2)**	**392 (7.3)**	**100 (1.9)**	**53 (1.0)**	**15 (0.3)**	**4 (0.05)**	**11 (0.2)**

Table 2

Patients infected with intestinal parasite pathogens, by age group, Rome, Italy, 1 May 2006–31 December 2008 (n=469)

Pathogenic parasites	Number of patients positive for intestinal parasite pathogens					
	Patient age in years	<5	5–14	15–44	45–65	>65
	Number of patients	(n=387)	(n=410)	(n=1,865)	(n=1,259)	(n=1,430)
Ascaris lumbricoides [a]	8		1	3	3	1
Blastocystis hominis [b]	378	8	35	142	89	104
Cryptosporidium spp.	2			1		1
Cyclospora cayetanensis	7	1	1		4	1
Dientamoeba fragilis	1			1		
Entamoeba histolytica [b]	11		3	4	3	1
Enterobius vermicularis [a]	4	1	1	1	1	
Giardia intestinalis [b]	69	17	11	23	10	8
Hymenolepis nana [a]	7	2	4		1	
Isospora belli	3	1	1	1		
Microsporidia	2			1	1	
Strongyloides stercoralis [a]	4		1	1		2
Taenia spp. [a]	4			2	2	
Trichuris trichiura [a]	1					1
Total	**501**	**30 (7.7%)**	**58 (14.1%)**	**180 (9.6%)**	**114 (9.0%)**	**119 (8.3%)**
p value for each group distribution				<0.001		

Total is higher than 469, because a patient could be infected with more than one parasite species.

[a] Helminth.

[b] Pathogenic protozoan.

Table 3

Patients having a single intestinal parasite species, by nationality or sex, Rome, Italy, 1 May 2006–31 December 2008 (n=389)

	Total	Number of positive patients that were:			
		Italians (n=4,695)	Non-Italians (n=656)	Female (n=2,913)	Male (n=2,438)
Ascaris lumbricoides	7	4	3	5	2
Blastocystis hominis	305	250	55	163	142
Cryptosporidium spp	1	0	1	1	0
Cyclospora cayetanensis	2	2	0	1	1
Dientamoeba fragilis	1	0	1	0	1
Encephalitozoon intestinalis	2	2	0	0	2
Entamoeba histolytica	6	6	0	3	3
Enterobius vermicularis	3	3	0	2	1
Giardia intestinalis	52	30	22	15	37
Hymenolepis nana	1	1	0	0	1
Isospora belli	2	0	2	1	1
Strongyloides stercoralis	2	2	0	1	1
Taenia spp.	4	3	1	3	1
Trichuris trichiura	1	0	1	1	0
Total	**389**	**303 (6.4%)**	**86 (13.1%)**	**196 (6.7%)**	**193 (7.9%)**
p value		<0.001		0.11	

Taenia spp. include *Taenia saginata* or *solium*.

was highest in children under five years of age (4.4% (17/387); OR: 6.81; 95% CI: 3.27–14.14; $p<0.001$) and in people aged 5–14 years (2.7% (11/410); OR: 4.09; 95% CI: 1.73–9. 21, $p<0.001$), and lowest in people aged 45 years or more (0.7% (18/2,689); OR: 1, reference group). *E. histolytica* was the third most common species; it was isolated from 11 patients, all but one, Italians. It was detected more commonly in males. The fourth common species was *Cyclospora cayetanensis*, which was detected in seven patients, five were Italians, and three of these denied travel to endemic areas within the previous 12 months. *Encephalitozoon intestinalis* and *Cryptosporidium parvum* were detected in faecal samples of four HIV-infected patients. As regard to helminthes, nematodes such as *Ascaris lumbricoides*, *Strongyloides stercoralis*, *Enterobius vermicularis and Trichiuris trichiuria* were identified in 17 patients, of whom 10 were Italians, while the plathelminthes *Hymenolepis nana* and *Taenia* species were responsible for 11 infections which occurred in seven Italians with no history of travel, and four non-Italians, three of whom were adopted children from endemic areas.

Table 4

Patients with mixed infections, by combinations of pathogenic parasites, by patient nationality or sex, Rome, Italy, 1 May 2006–31 December 2008 (n=80)

Pathogenic parasites	Patients				
	Total	Italians (n=4,695)	Non-Italians (n=656)	Female (n=2,913)	Male (n=2,438)
Blastocystis hominis/Ascaris lumbricoides	**1**	0	1	1	0
B. hominis/Cryptosporidium parvum	**1**	1	0	0	1
B. hominis/Cyclospora cayetanensis	**4**	2	2	2	2
B. hominis/Endolimax nana	**23**	15	8	10	13
B. hominis/E. nana/Escherichia coli	**1**	0	1	1	0
B. hominis/E. nana/E. coli/Entamoeba hartmanni	**1**	0	1	1	0
B. hominis/E. nana/E. hartmanni	**2**	0	2	0	2
B. hominis/E. nana/Entamoeba histolytica	**1**	0	1	0	1
B. hominis/E. nana/Giardia intestinalis	**2**	1	1	2	0
B. hominis/E. nana/Hymenolepis nana	**1**	1	0	0	1
B. hominis/E. nana/E. coli	**1**	0	1	1	0
B. hominis/E. coli	**11**	3	8	5	6
B. hominis/E. coli/G. intestinalis	**2**	0	2	1	1
B. hominis/Entamoeba dispar	**2**	2	0	0	2
B. hominis/E. dispar/G. intestinalis	**1**	1	0	0	1
B. hominis/E. hartmanni	**6**	3	3	4	2
B. hominis/E. histolytica	**2**	2	0	0	2
B. hominis/E. histolytica/G. intestinalis	**1**	1	0	1	0
B. hominis/Enterobius vermicularis	**1**	1	0	0	1
B. hominis/G. intestinalis	**1**	0	1	0	1
B. hominis/G. intestinalis	**4**	2	2	1	3
B. hominis/H. nana/G. intestinalis/Strongyloides stercoralis	**1**	0	1	0	1
B. hominis/H. nana	**1**	0	1	1	0
B. hominis/H. nana/G. intestinalis	**1**	0	1	0	1
B. hominis/S. stercoralis	**1**	0	1	0	1
C. cayetanensis/E. hartmanni	**1**	1	0	0	1
E. histolytica/E. nana	**1**	1	0	0	1
G. intestinalis/E. coli	**1**	0	1	0	1
G. intestinalis/H. nana	**1**	1	0	0	1
G. intestinalis/Iodamoeba Butschlii	**1**	0	1	0	1
G. intestinalis/Isospora belli	**1**	0	1	1	0
H. nana/Chilomastix mesnili/E. hartmanni	**1**	1	0	0	1
Total	**80**	**39 (0.8%)**	**41 (6.2%)**	**32 (1.1%)**	**48 (2.0%)**
p value		0.31		0.51	

Discussion

An estimated 3.5 billion people—roughly half of the world's population—have intestinal parasitoses, which cause almost 450 million deaths every year. The problem is particularly serious in endemic areas with poor sanitation, but parasitic infections are also becoming more common in non-endemic areas as a result of intensified immigration and travel [1].

Very little is known about the prevalence of intestinal protozoa and helminthes in Italy [9]. The present study shows that, over a 30-month period, 8.8% of the patients referred to a large teaching hospital in Rome for ova and parasite analysis of stool samples harbour at least one species of pathogenic parasite.

The infection rate was lowest in children under five years of age and peaked in the 5–14 year-old group, declining progressively thereafter. The frequency of parasitic infections was found to be associated with patient, sex and nationality. The prevalence was substantially higher (almost 20% (127/656)) among the non-Italian patients of our study, and even higher rates have been reported in the immigrant population living in southern Italy (61.9%) [18]. On the whole, intestinal parasites were more common in males, although certain species, such as *A. lumbricoides* and *Taenia* spp., were detected more often in females.

Overall, *Blastocystis hominis* was the most common parasite encountered in this study. It was detected in 7.1% (378/5,351) of the patients we examined (14.1% (93/656) of the non-Italians and 7.5% (184/2,438) of the males). *Blastocystis* is an enteric protozoan that parasitises humans and many animals. It has a worldwide distribution and is often the most commonly isolated organism in parasitological surveys [8-19]. Accumulating evidence (epidemiological, in vivo, and in vitro) strongly suggests that *Blastocystis* is a pathogen [20]. Many genotypes exist in nature, and recent observations indicate numerous zoonotic genotypes also infect humans. Tan et al. have suggested that conflicting observations on the pathogenesis of *Blastocystis* stem from genetic diversity within the species, which includes both pathogenic and non-pathogenic genotypes [19]. The controversial pathogenicity of *Blastocystis* has been attributed to subtype variations in virulence, and while more recent studies seem to support this view, other factors also appear to contribute to the clinical outcome of the infection [19].

The second most common species in our study population was *Giardia intestinalis*. This common, ubiquitous flagellated protozoan has a significant impact on public health. Giardiasis is characterised by high prevalence, and it frequently causes major outbreaks and substantial effects on the growth and cognitive functions of infected children [21]. *Giardia* generally causes a self-limited illness characterised by diarrhoea, abdominal cramps, bloating, weight loss, and malabsorption. However, asymptomatic infections are frequent, especially in developing countries [22]. Infection rates are generally lower in industrialised countries, and figures ranging from 0.4% to 6.2% have been reported in Italy [23]. The overall prevalence observed in the present study was 1.3% (69/5351), but the rate in children less than 15 years of age was three times higher (4.8% (38/797)). Rates were also higher in the non-Italian subgroup (5% (33/656) vs 0.8% (36/4,695) in Italians), and 78.6% (22/28) of infected patients aged less than 15 years were non-Italians. High infection rates have been reported for certain relatively poor regions in developed countries, and Giangaspero et al. reported a rate of 42.9% in a small, socially deprived Roma community in Italy [24]. A lower prevalence (4.5%) was found, however, in immigrants living in southern Italy [18].

The third most common species, although only detected in 11 patients, was *Entamoeba histolytica*. *Entamoeba* spp. can cause harmless colonisation of the intestine or severe infections characterised by invasion of the colon wall and damage to other host tissues, such as the liver, lung, and brain (amoebiasis). In most cases, a clinical diagnosis of amoebiasis can be confirmed microbiologically, generally by light microscopic visualisation of parasites in a wet smear or stained specimens. This procedure is simple and inexpensive, but it has several limitations. The most important is that it cannot distinguish between the cysts and trophozoites of *E. histolytica* (the disease-causing species), *E. dispar* (which is non-pathogenic), and E. moshkovskii, the amphizoic amoeba that occasionally infects humans. In addition, multiple samples often have to be examined, and the diagnosis can be even more difficult when there are cysts of different species of Entamoeba, Iodamoeba, or *Endolimax* [25]. Differentiating between these three species has become increasingly important (for diagnosis and for epidemiological studies) since sporadic cases of human infection with *E. moshkovskii* were reported [26]. Recent findings indicate that this species is highly prevalent in young children in Bangladesh, where it is often associated with *E. histolytica* and *E. dispar* [27]. In our study, *E. histolytica* and/or *E. dispar* were detected by microscopy in 33 patients. However, when these cases were analysed with molecular tools, the presence of *E. histolytica* was confirmed in only 11 patients (10 of whom were Italians): the other 22 (18 Italians) were infected with *E. dispar*, *Isospora belli*, E. intestinalis, and *Cryptosporidium parvum* were detected only in immunocompromised patients, all but one of who were HIV-infected. Helminthes were found in only 0.5% (27/5,351) of our patients.

Our findings confirm that certain intestinal parasites, such as *E. histolytica, C. cayetanensis, S. stercoralis,* and *H. nana*, are not restricted to endemic areas in the tropics. However, our study has certain limitations that must be acknowledged. Our analysis was retrospective, and it was performed at a single hospital, so the results are not necessarily applicable to other settings. However, this shortcoming is to some extent

outweighed by the large size of the sample population and the fairly large variety of the parasites identified. In addition, the relationship between parasitic infection rates and nationality has been consistently demonstrated in other studies [18-24].

In conclusion, knowledge of the organisms responsible for intestinal parasite infections in non-endemic areas is an essential step toward effective patient care and the implementation of appropriate control measures. Our findings demonstrate that intestinal parasitoses must be considered in the differential diagnosis of gastrointestinal diseases even in industrialised areas. Examination of multiple stool specimens per patient is still the recommended approach for excluding parasitic infection [28]. We agree that multiple stool analyses for ova and parasites can be clinically useful in populations with increased risk of infection, but it also is important to identify populations at low risk for infection, such as that in our study. Stool examinations are costly and labour-intensive, and routine requests should be avoided unless they are supported by epidemiological (e.g. international travel, unsafe drinking water) and clinical features (e.g. diarrhoea for more than seven days, especially if the patient is immunocompromised). In this context, close collaboration between physicians and clinical microbiologists should produce significant positive effects.

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