We investigated a Q fever outbreak with human patients showing high fever, respiratory tract symptoms, headache and retrosternal pain in southern Hungary in the spring and summer of 2013. Seventy human cases were confirmed by analysing their serum and blood samples with micro-immunofluorescence test and real-time PCR. The source of infection was a merino sheep flock of 450 ewes, in which 44.6% (25/56) seropositivity was detected by enzyme-linked immunosorbent assay. *Coxiella burnetii* DNA was detected by real-time PCR in the milk of four of 20 individuals and in two thirds (41/65) of the manure samples. The multispacer sequence typing examination of *C. burnetii* DNA revealed sequence type 18 in one human sample and two manure samples from the sheep flock. The multilocus variable-number tandem repeat analysis pattern of the sheep and human strains were also almost identical, 4/5-9-3-0-5 (Ms23-Ms24-Ms27-Ms28-Ms33-Ms34). It is hypothesised that dried manure and maternal fluid contaminated with *C. burnetii* was dispersed by the wind from the sheep farm towards the local inhabitants. The manure was eliminated in June and the farm was disinfected in July. The outbreak ended at the end of July 2013.

**Introduction**

*Coxiella burnetii* is the causative agent of Q fever, a worldwide zoonotic disease [1,2]. Domestic ruminants are the most important recognised reservoirs of *C. burnetii*; they are often asymptomatic carriers, but the agent may also cause abortion in these animals [1,3]. Ticks may also act as reservoirs of *C. burnetii* in nature [1,4]. Domestic ruminants are considered the most important source of human Q fever infection. Outbreaks in human populations have been linked to slaughterhouses or dispersion of *C. burnetii* by wind from farms where infected ruminants were kept [1,5]. Q fever is typically an acute febrile illness with non-specific clinical signs such as atypical pneumonia and hepatitis in roughly 40% of cases, while 60% remain asymptomatic after infection [6]. A small percentage (ca 5%) of infected people may develop chronic infection with life-threatening valvular endocarditis [7,8].

Q fever is a notifiable disease in Hungary. Antibodies against *C. burnetii* were first detected in the sera of abattoir workers in 1950 [9], and infections were first diagnosed in 1956 in dairy and sheep farms [10]. The last major outbreaks were registered in the period of 1976–80. According to a recent survey (2010–11), seroprevalence among cattle and sheep in Hungary were 38.0% and 6.0%, respectively, by enzyme-linked immunosorbent assay (ELISA), which is in agreement with the European averages [3,11]. The number of yearly reported acute human infections in Hungary ranged between 36 and 68 between 2008 and 2012.

The aim of this study was the epidemiological, diagnostic and genetic investigation of the Q fever outbreak that occurred in Hungary during the spring and summer of 2013.

**Methods**

During the epidemic investigation a suspected human case was defined by high fever and radiological evidence of pneumonia occurring after 17 April 2013, and by geographic proximity to the outbreak area after 4 April. The human serum samples were tested with micro-immunofluorescence test (MIF) (Focus Diagnostics, United States). Based on MIF results of the first serum samples, the suspected human cases were evaluated as seronegative or possibly infected (showing seropositivity, i. e. any phase of specific IgM/IgG antibodies). Cases were considered confirmed if there was seroconversion and/or an elevated level (greater than 1:252 dilution) of IgG-II/IgG-I antibodies when paired sera were available. DNA was extracted from EDTA anticoagulated human blood and serum samples (collected in the one to three weeks after
onset of clinical symptoms) using the MagNA Pure LC total nucleic acid isolation kit (Roche Diagnostics, Switzerland).

Animal serum samples were tested with complement fixation test (CFT) (Virion/Serion GmbH, Germany) and ELISA (IDEXX Laboratories Inc., Switzerland). Questing ticks were collected using the dragging/flagging method. DNA was extracted from milk, manure and individual tick samples with the ReliaPrep gDNA Tissue Miniprep System (Promega Inc., United States).

All extracted DNA samples were screened with a real-time PCR assay targeting the IS1111 element of *C. burnetii* [12]. Strong positive samples (with Ct values below 30) were further genotyped with multispace sequence typing (MST) based on 10 loci and multilocus variable-number tandem repeat analysis (MLVA) based on six loci [13-15].

**Results**

**Description of the epidemic area**

The centre of the outbreak region was a hilly area of approximately 10 km² encompassing primarily the small rural towns of Vokány (851 population) and Kistótfalu (321 population) of Baranya county in southern Hungary (45.90 °N, 18.33 °E) (Figure 1). Although the area has a continental climate, an unusually cold and snowy March was followed by a warm, dry and occasionally windy April in 2013. A non-dairy merino sheep flock (450 ewes) near Vokány, a small cattle herd (40 animals), a small mixed flock of sheep and goats (20 animals) and some individually kept animals were the only livestock in the region. The lambing season occurred from January to April in 2013 (Figure 2).

**Investigation of human cases**

During systematic screening for respiratory pathogens, six patients of the initial cluster of eight febrile cases (since 17 April) showed *C. burnetii*-specific phase II IgM
positivity from serum samples received on 8 May, while other respiratory agents could be ruled out. After that laboratory finding, all suspected cases were tested for *C. burnetii* antibodies and an epidemiological investigation was initiated by the local authorities of Baranya County. The serological sampling was done primarily by general practitioners and extended to hospitalised patients living in the outbreak area. A centralised laboratory investigation was performed on all samples from suspected cases.

Starting on 17 April 2013 until 31 July, 176 possible human cases with high fever, respiratory tract symptoms, headache and retrosternal pain were reported. Of those, 103 were male and 73 were female. The age of the cases ranged from 14 to 84 years. Most of them were local residents of Vokány and Kistótfalu, although some had only transient connections with the region. Twenty-six patients needed hospital admission but no fatalities were recorded.

Based on MIF results of the first serum samples taken from the 176 suspected cases, 100 patients were evaluated as seronegative and 76 as possibly infected. Based on serological results, the infection was confirmed in 70 (60%) of 117 cases from whom paired sera were available. There was a predominance of men (45 male vs 25 female) in confirmed cases. Fifteen of the 26 hospitalised suspected cases were confirmed. *C. burnetii* real-time PCR was positive in three of the 26 anticoagulated blood samples and in one third (17/51, Ct: 29.4–36.1) of the tested serum samples collected in the one to three weeks after onset of clinical symptoms. The rate of PCR positivity decreased over time, as the samples taken in the first week of illness yielded the highest rate of PCR positivity (48%), with only 23% in the second and 0% in the third week. In the 20 PCR-positive human cases, seroconversion was detected in 19. Other important respiratory agents (*Mycoplasma pneumoniae, Chlamydia pneumoniae, Chlamydia psittaci, Legionella spp.*) were ruled out by laboratory examination (data not shown).

A three-week course of doxycycline therapy (200 mg daily) was administered to all laboratory-confirmed cases and also to the majority (no exact data are available) of the suspected cases living in the outbreak area. Serological follow-up was advised for all patients diagnosed with acute Q fever to control their IgG-I level because persisting high titres (≥800) may indicate chronic infection. As of July 2014, third serum samples have been sent from 21 of the 70 confirmed cases, and high titres of IgG2/IgG1 (≥512) were detected in all of them.

**Animal and environmental investigations**

Fifty-six serum and 20 milk samples were collected from the Vokány merino sheep flock and from all animals on other farms. Manure samples were also collected from the breeding stable of each farm within the outbreak region.
The CFT and ELISA examinations of the serum samples collected in the merino sheep flock revealed 23.2% (13/56) and 44.6% (25/56) seropositivity, respectively, while C. burnetii was detected in four of 20 (Ct: 30.1–33.5) of individual’s milk and two thirds (41/65, Ct: 28.9–36.82) of manure samples collected from the merino sheep flock. Of the four sheep with PCR-positive milk, two were also seropositive by ELISA. The ELISA showed 11 of 40 and two of 20 seropositive animals in the cow herd and the mixed sheep and goat flock, respectively, and only one of the milk samples from a cow contained C. burnetii DNA (Ct: 33.7). The manure samples collected from these small farms as well as all samples (serum, milk, manure) collected from the individually kept animals in the epidemic region were negative for C. burnetii infection.

Seven questing ticks (five Ixodes ricinus and two Haemaphysalis concinna) were collected from the vegetation at the merino sheep farm pasture in July and September 2013. Another 115 archived ticks collected from two dogs (four Dermacentor marginatus) and 23 goats (91 I. ricinus and 20 H. concinna) residing with the merino sheep flock in 2011 and 2012 were also included in this study. C. burnetii DNA was detected in five archived I. ricinus (three larvae, one nymph, one female, Ct: 35.0–36.58) collected from goats kept in the merino sheep flock in 2011.

Genetic characterisation of C. burnetii strains
The MST examination of C. burnetii DNA detected in one human sample and two manure samples from the merino sheep flock revealed sequence type (ST)18. The MLVA pattern of the sheep and human strains were also almost identical, 4/5-9-3-3-0-5 (Ms23-Ms24-Ms27-Ms28-Ms33-Ms34). Genotyping of samples from the ticks collected in 2011 and from the cow milk failed because of their low C. burnetii DNA content.

Discussion
Seventy human cases were confirmed during the Q fever outbreak in Hungary in 2013. The laboratory diagnosis of the first patients on serology and real-time PCR identified the aetiologic agent within a few days and enabled targeted screening and adequate therapy of further infected individuals. Combining these two methods was also an effective diagnostic strategy in the first two weeks after the onset of clinical symptoms. The merino sheep flock in Vokány village was identified as the source of the outbreak. Interestingly, the farmer had not observed an elevated abortion rate during the lambing season from January to April. The 44.6% seropositivity rate at this farm was far higher than the 6.0% recorded in other Hungarian sheep flocks and represents strong evidence of a localised outbreak [11].

The causative agent of the outbreak described here was an ST18 C. burnetii strain, which argues against a direct connection with the 2007–09 outbreak in the Netherlands which was caused primarily by ST33 [16,17]. ST18 has previously been detected in France, Germany, Italy, Romania and Slovakia [13]. A recent human serum sample from Belgium was also likely to be (or be closely related to) ST18 [17]. Our subtyping data confirmed that the source of the human Q fever infections was the merino sheep farm. The one VNTR difference between the ovine and human isolates at Ms23 is likely to be due to the rapid mutation capacity of this locus [15]. We hypothesise that dried manure and birth fluids contaminated with C. burnetii were dispersed by the wind from the sheep farm towards the towns and their inhabitants. This hypothesis is supported by the genotyped human sample. This patient had not had any direct contact with the merino sheep flock in Vokány. He was an inhabitant of a neighbouring town but was working on a hillside close to Vokány at the time of his supposed exposure. Based on the available epidemic information, laboratory data and the large number of human cases it is highly unlikely that this epidemic was caused either by tick-bites or raw milk consumptions. The origin of the ST18 strain at the merino sheep farm remains unknown. C. burnetii isolates originating from other parts of Hungary have been either ST20 (cattle) or ST28 and ST37 (sheep) genotypes [18].

Public health countermeasures included elimination of manure in June 2013, followed by disinfection of the merino farm (using VIROCID, Cid Lines Inc, Belgium and Disinflex, Hexachem Kft, Hungary) in July. In this region, no further acute human Q fever cases have been confirmed since July 2013. Manure and milk samples collected in May 2014 from the merino sheep flock in Vokány and other farms within a 30 km diameter, tested negative by real-time PCR. As a precaution, the merino farm in Vokány was disinfected in June 2014 as well.

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Conflict of interest
None declared.

Authors’ contributions
MG, KS, and ÁD performed the PCR screening of livestock and tick samples, genotyped the positive samples, analysed the data and wrote the manuscript. EB, TM and AB performed the serological and PCR analysis of human samples and edited the manuscript. ZS and PB collected the livestock samples in the field. BD performed the serological examination of the livestock samples. SH collected and identified the ticks. HMH, TP and PK helped in genotyping and in the writing of the manuscript.
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