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From SARS to Ebola – 10 years of disease prevention and control at ECDC

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A decade ago, the European Centre for Disease Prevention and Control (ECDC) appeared as a new player among international health organisations, with the mandate 'to identify, assess and communicate current and emerging threats to human health from communicable diseases' in the European Union (EU) [1]. As part of the ECDC 10-year anniversary celebrations, *Eurosurveillance* compiled a print issue with a selection of articles published over this period in the journal. The 10 articles, representing a year each, mark the organisation's evolution and show its leadership and influence in the areas of its mandate.

The first five years

During 2005 to 2010, the focus was on developing the Centre's core functions. ECDC officially started its operations on 20 May 2005 and in the autumn of that year, wild birds were found positive for influenza A(H5N1) virus in Croatia, Romania, and Turkey. The then newly established ECDC was asked to answer questions from public health experts and policymakers in EU Member States and the European Commission. Without having the current systems and processes, ECDC experts had to 'build the plane while flying'. An editorial by Nicoll in the first year shows that ECDC was, from the very start, able to strategically shape the activities needed to improve the level of preparedness – for influenza and in general – in Europe [2]. Even retrospectively and in the light of the 2009 influenza pandemic, the answers given to the questions posed in the editorial published in 2005 still hold. Some of the issues raised have been addressed in the meantime by the Commission Decision 1082/2013 [3].

One of ECDC's key tasks is to identify threats from current or emerging infectious diseases. In its second year of operations, ECDC presented a proposal to complement the traditional indicator-based surveillance, using epidemic intelligence as an early detection and warning system [4]. Such epidemic intelligence would take into account changes in the information sector, and pick up relevant information from sources such as traditional and social media and others, and analyse it. The proposed framework became the basis for rapid risk assessments, one of the cornerstones of the Centre's work today and one of its most appreciated outputs.

Another ECDC core function is capacity building. The European Programme for Intervention Epidemiology Training (EPIET) was transferred to ECDC in 2007 and the article by Varela and Coulombier describes the efforts to define and agree on standards for core competencies required for epidemiologists, which still serve as foundation for this important ongoing task [5]. A short-term vision for surveillance of infectious diseases in the EU was presented in October 2005 to ECDC's governing bodies and in 2008, the single EU surveillance database, The European Surveillance System (TESSy), was successfully established. EU-wide supranational surveillance is at the core of ECDC's mandate and the start of TESSy was accompanied by a long-term strategy with challenging goals, with the aim of adding value, on top of national surveillance systems [6]. Even if not all goals have been achieved today, it is of note that TESSy data are increasingly used, also by non-ECDC scientists as basis for their analyses indicated by the increasing numbers of request to access TESSy data. This demonstrates the added value and that TESSy has become a point of reference for EU data on infectious diseases.

The emergence of a new disease in 2003, severe acute respiratory syndrome (SARS), together with a perceived pandemic threat, sparked the establishment of ECDC. The 2009 influenza pandemic could thus be considered its first 'real' test. In June 2009, early in the pandemic, an article was published with contributions from a large group of collaborators from all EU countries, demonstrating the capability of ECDC to rapidly collate and disseminate information necessary for public health action during a public health event [7]. The article specifically pointed out two important features of the pandemic that were confirmed in several publications thereafter: the relatively mild clinical course and children and adolescents as the main groups affected by and involved in indigenous transmission.

After the pandemic: 2010–14

A new era began in 2010, with a focus on further developing disease-specific functions. Antimicrobial resistance (AMR) is one of the most important infectious disease threats today and most likely also in the future. It has increasingly become a crucial aspect of ECDC's work. The article by the ECDC Antimicrobial Resistance and Healthcare-Associated Infections Programme (ARHAI) provides an overview of the initiatives that ECDC undertook from an early stage to improve the understanding of the risks associated with AMR and to support the response [8]. It also demonstrates the priority given to raising awareness about the relevant health threats.

Another example of how ECDC fulfils its mandate to strengthen prevention and control of cross-border threats in Europe is the guidance described in an article by Leitmeyer [9]. Since its finalisation, the European risk assessment guidance for infectious diseases transmitted on aircraft (RAGIDA) has become a de facto reference for many public health authorities in Europe.

Aside from AMR, healthcare-associated infections are a health threat posing a major burden on individual patients and health systems alike. Prior to a pilot point prevalence survey (PPS) of healthcare-associated infections and antimicrobial use survey, ECDC and a large group of experts from all EU countries developed a standardised methodology, training materials, a train-the-trainer course for national PPS coordinating staff, free-of-charge hospital software for data collection and a validation methodology. The article by Zarb et al. describes one of the most complex epidemiological activities ECDC has coordinated: the pilot survey included nearly 20,000 patients from 66 hospitals in 23 European countries [10]. National PPS coordinating staff trained an estimated 2,800 healthcare workers from 1,200 hospitals across Europe to implement the standardised PPS methodology. Besides being impressive on a technical level, it also provided the first (relatively) comparable picture of prevalence of a fast-growing public health concern. The initiative is another important marker of ECDC's role in identifying and communicating serious threats to health.

Evidence-based approaches aim at improving the quality of scientific findings as a basis for decision-making. The article selected for 2013 reflects the growing demand for evidence-based methods (EBM) in some of the Centre's core functions, where the currently available tools do not provide good-enough answers. Rapid risk assessments are usually developed under time constraints and yet need to form the basis of public health decisions. ECDC and an interdisciplinary group of experts developed a conceptual framework of how to address the current gaps [11] and support public health experts in the future to produce rapid assessments using the best available evidence, even when the evidence may still be limited.

Launched as an ECDC initiative in 2008, the European Antibiotic Awareness Day, marked on 18 November each year, is another example of ECDC activities in the area of communication. It has grown to become a European-wide coordinated health campaign, joined by many countries beyond the EU. Several public health organisations (in the United States and Canada, and the World Health Organization) have aligned their respective campaigns on the same day [12].

In addition to the disease programmes on influenza and the ARHAI, programmes on other disease groups such as emerging and vector-borne diseases, food- and waterborne diseases, HIV, sexually transmitted infections and viral hepatitis, tuberculosis, vaccine-preventable diseases and the microbiology team were established; some of them took up their work already in the early days of ECDC. A list with scientific peer-reviewed publications from 2005 onwards is available on the ECDC website and illustrates the work done by the programmes and ECDC experts and expert groups [13].

The future

The selected articles show that ECDC tackled from its very first year cross-border health threats in close collaboration with a network of experts across the EU and beyond. The expertise of these networks is one pillar of ECDC as most, if not all, of ECDC's work is based on the collaboration of numerous colleagues in the countries' national public health institutes, research and other institutions. I would like to express, on behalf of ECDC, my sincere gratitude for their dedication and constructive input during all these years, which have contributed to shaping ECDC.

As the emergence of the Middle East respiratory syndrome coronavirus (MERS-CoV) and the Ebola outbreak in West Africa have recently demonstrated, some of the issues described in the articles are still relevant today, others might emerge in the future, indicating both the complexity and dynamics of infectious diseases. ECDC will continue to deliver independent outputs of high scientific quality and will endeavour to further increase their usefulness and value for decision makers. In this and in line with the recently published recommendations from the second external ECDC evaluation, ECDC will work closely with the countries and the European Commission to support them in facing threats to human health from current or emerging infectious diseases.

Conflict of interest

None declared.

Authors' contributions

Andrea Ammon wrote the editorial and approved the final version before publication.

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Research article

Meticillin-resistant *Staphylococcus aureus* CC398 is an increasing cause of disease in people with no livestock contact in Denmark, 1999 to 2011

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Livestock constitutes a potential reservoir of meticillin-resistant *Staphylococcus aureus* isolates belonging to a recently derived lineage within clonal complex 398 (MRSA CC398-IIa). Since its discovery in the early 2000s, this lineage has become a major cause of human disease in Europe, posing a serious public health challenge in countries with intensive livestock production. To retrace the history of human colonisation and infection with MRSA CC398-IIa in Denmark, we conducted a nationwide, retrospective study of MRSA isolates collected from 1999 to 2011. Among 7,429 MRSA isolates screened, we identified 416 MRSA CC398-IIa isolates. Of these, 148 were from people with infections, including 51 from patients reporting no livestock exposure. The first cases of MRSA CC398-IIa infection in Denmark occurred in 2004. Subsequently, the incidence of MRSA CC398-IIa infection showed a linear annual increase of 66% from 2004 to 2011 (from 0.09 to 1.1 per 100,000 person-years). There were clear temporal and spatial relationships between MRSA CC398-IIa-infected patients with and without livestock exposure. These findings suggest substantial dissemination of MRSA CC398-IIa from livestock or livestock workers into the Danish community and underscore the need for strategies to control its spread both on and off the farm.

Introduction

In 2005, two studies, from France and the Netherlands, provided the first evidence of a reservoir of meticillin-resistant *Staphylococcusaureus* (MRSA) in livestock, with transmission to humans [1,2]. The MRSA isolates from these initial cases belonged to clonal complex 398 (CC398), which was very uncommon in humans at the time. Since its discovery, MRSA CC398 has been isolated from cattle, horses, chickens and turkeys, but currently pigs appear to be its primary host [3]. While several other MRSA strain types have been identified in a variety of livestock species worldwide, CC398 is the dominant MRSA strain type in European livestock today [3].

MRSA CC398 has unique genetic characteristics compared with other MRSA strain types: it is nontypeable by *Sma*I-pulsed-field gel electrophoresis (PFGE) [4], it comprises a distinct set of *spa* types [5], and it contains a novel Sau1 type I restriction-modification system [6]. These features challenged early genotyping efforts, which have been aided more recently by whole-genome sequencing.

Whole-genome phylogenetic analyses show that there are multiple *S. aureus* CC398 lineages in circulation, including one recently derived lineage primarily found in livestock, termed CC398-IIa, and several other more basal lineages primarily found in humans, collectively referred to as CC398-I/II-GOI [7]. The CC398-IIa isolates can be distinguished from the CC398-I/II-GOI isolates by lineage-specific canonical single nucleotide polymorphisms (canSNPs) [7,8]. Furthermore, CC398-IIa isolates are typically positive for the tetracycline resistance gene *tet*(M) and negative for the staphylococcal complement inhibitor gene *scn*, whereas CC398-I/II-GOI isolates are typically negative for *tet*(M) and positive for *scn* [7,8]. Finally, CC398-IIa isolates generally lack the *lukS-PV* and *lukF-PV* genes encoding Panton-Valentine

Table 1

Genotypic characterisation of meticillin-resistant *Staphylococcus aureus* (MRSA) CC398 isolates, one isolate per person, Denmark, 1999–2011 (n = 420)

Strain type	tet(M)	scn	canSNP			Number (%)
			748	1002	3737	
MRSA CC398-IIa						
MRSA CC398-IIa	+	–	ND	ND	ND	389 (94)
MRSA CC398-IIa	+	+	T	A	A	25 (6)
MRSA CC398-IIa	–	–	T	A	A	2 (0.5)
Total						**416 (100)**
MRSA CC398-I/II-GOI						
MRSA CC398-I/II-GOI	–	+	ND	ND	ND	4 (100)
Total						**4 (100)**

canSNP: canonical single nucleotide polymorphism; ND: not determined.

+ : positive; – : negative.

leukocidin (PVL), whereas these genes are frequently found in CC398-I/II-GOI isolates [8]. The existence of two epidemiologically and evolutionary distinct *S. aureus* CC398 lineages underscores the importance of strain typing when undertaking epidemiological investigations and source tracking of *S. aureus* CC398.

Despite its recent emergence as a zoonotic pathogen [1-3], MRSA CC398 has become a frequent cause of human colonisation and disease in Europe, especially in countries with intensive livestock production [9]. For example, MRSA CC398 accounts for up to 40% of new cases of MRSA in Denmark, the Netherlands and some areas of Germany [10-12]. MRSA CC398 primarily colonises and infects people with direct livestock contact (primary exposure) and their household members through intra-household transmission (secondary exposure). However, surveillance data from Denmark and the Netherlands show that MRSA CC398 is also found in people with no connection to livestock [10,13]. In addition, MRSA CC398 has been implicated in sporadic outbreaks in Dutch hospitals and nursing homes [14-16]. Unfortunately, these studies did not differentiate MRSA CC398-IIa isolates from MRSA CC398-I/II-GOI isolates, and it is therefore unclear how and to what extent MRSA CC398-IIa is spreading to people with no livestock exposure. *S. aureus* normally spreads through human-to-human contact [17] and this mode of transmission is likely to play a major role in the dissemination of MRSA CC398-IIa. Various studies suggest that environmental contamination of air and soil surfaces may also contribute to MRSA CC398 transmission [18-21]. Moreover, MRSA CC398 is a relatively common contaminant of retail meat in Europe [22], and food-borne transmission has been hypothesised as a possible source of infections in people with no livestock contact. However, epidemiological data suggest that food-borne transmission is rare [22].

The aim of our study was to investigate the epidemiology of MRSA CC398 in humans in Denmark from 1999 to 2011, especially in relation to the presence of MRSA CC-398-IIa in people with no livestock contact.

Methods

National MRSA registry and strain repository, 1999–2011

This study is based on data from the national MRSA registry and strain repository at Statens Serum Institut in Copenhagen. It should be noted that the registry contains data available only at the time of the first diagnosis and does not hold information on subsequent episodes of infection or asymptomatic carriage. The use of data from the national MRSA registry was approved by the Danish Data Protection Agency (protocol no. 2001-14-0021).

Since 1988, one MRSA isolate from each newly identified MRSA-positive person (including patients with infection and people with asymptomatic carriage) has been forwarded to Statens Serum Institut from Danish clinical microbiology laboratories. From 1988 to 2006, this was as part of a voluntary surveillance programme and since 2007, has been part of a national mandatory programme for the management of MRSA [23]. Compliance with both programmes was about 100% during the study period, as assessed by annual feedback between the clinical microbiology laboratories and Statens Serum Institut. From 1999 to 2006, all MRSA isolates were genotyped using *Sma*I-PFGE [24]. In 2007, *Sma*I-PFGE was replaced by PCR-based detection of *mecA* and *lukF-PV* (an indicator of PVL production) and *spa* typing [25].

All MRSA isolates were tested for antimicrobial susceptibility (erythromycin, clindamycin, tetracycline, fusidic acid, rifampicin, norfloxacin, kanamycin, linezolid and mupirocin) by use of the disk diffusion method, in accordance with the European Committee on Antimicrobial Susceptibility Testing guidelines [26]. Screening for reduced susceptibility to glycopeptides was performed on brain-heart infusion agar supplemented with 5 µg/mL teicoplanin. MRSA isolates growing on the screening agar were further tested using Etest glycopeptide-resistance detection strips (0.5–32 µg/mL vancomycin, 0.5–32 µg/mL teicoplanin) (bioMérieux, Marcy l'Etoil, France), as described by Fitzgibbon et al. [27]. Multidrug resistance was defined as resistance to three or more non-β-lactam antibiotics.

Identification and characterisation of MRSA CC398 isolates

MRSA isolates were tentatively designated as CC398 if they were nontypeable by *Sma*I-PFGE (1999–2006) or if they displayed *spa* types previously associated with *S. aureus* CC398 (2007–2011). Putative MRSA CC398 isolates were confirmed by PCR detection of the *sau1-hsdS1* variant in *S. aureus* CC398 [6]. All MRSA CC398 isolates were assessed for the presence of *tet*(M) and

Table 2

Characteristics of meticillin-resistant *Staphylococcus aureus* (MRSA) CC398-IIa isolates and infected patients, Denmark, 1999–2011 (n = 148)

Characteristic	Number (%)[a] of MRSA CC398-IIa-infected patients,		P value[b]
	Livestock-exposed (n = 97)	Livestock-unexposed (n = 51)	
spa type			
t011	10 (10)	6 (12)	0.79
t034	83 (86)	39 (76)	0.18
t108	0 (0)	2 (4)	0.12
t571	1 (1)	0 (0)	1.00
t899	0 (0)	1 (2)	0.34
t1255	1 (1)	0 (0)	1.00
t1446	0 (0)	0 (0)	NA
t1793	0 (0)	0 (0)	NA
t5095	0 (0)	2 (4)	0.12
t5706	1 (1)	0 (0)	1.00
t9345	1 (1)	0 (0)	1.00
t9517	0 (0)	1 (2)	0.34
Presence of genes			
tet(M)	97 (100)	51 (100)	NA
scn	3 (3)	3 (6)	0.42
lukF-PV	0 (0)	0 (0)	NA
Antimicrobial resistance			
Erythromycin	40 (41)	16 (31)	0.29
Clindamycin	74 (76)	34 (67)	0.24
Tetracycline	97 (100)	51 (100)	NA
Fusidic acid	1 (1)	3 (6)	0.12
Rifampicin	1 (1)	0 (0)	1.00
Norfloxacin	21 (22)	10 (20)	0.83
Kanamycin	7 (7)	6 (12)	0.37
Linezolid	0 (0)	0 (0)	NA
Mupirocin	0 (0)	0 (0)	NA
Glycopeptides	0 (0)	0 (0)	NA
Multidrug resistance	51 (53)	23 (45)	0.49
Age and sex			
Female-to-male ratio	0.5	1.3	0.0081*
Median age in years (range)	30 (0–89)	49 (1–84)	0.0007*
Type of infection			
SSTIs	82 (85)	44 (86)	1.00
Ear	8 (8)	3 (6)	0.75
Eye	2 (2)	0 (0)	0.55
Respiratory sites	3 (3)	4 (8)	0.23
Bone and joint	2 (2)	0 (0)	0.55
Blood and CSF	0 (0)	0 (0)	NA
Other	0 (0)	0 (0)	NA

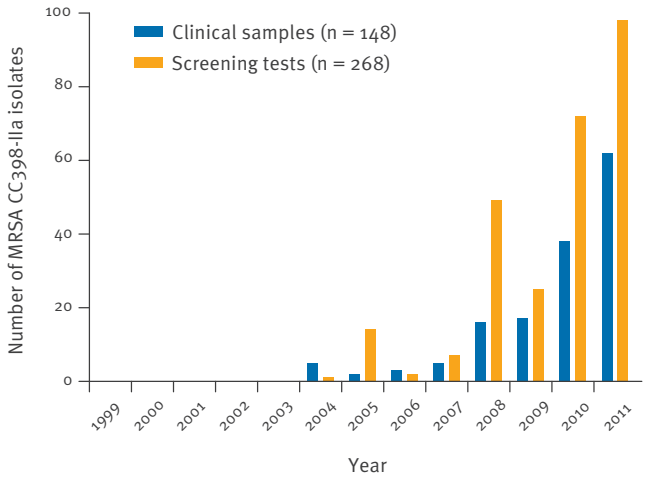
CSF: cerebrospinal fluid; NA: not applicable; SSTIs: skin and soft tissue infections.

[a] Unless otherwise specified.

* P value < 0.05 (significance level set at α < 0.05).

Figure 1

Annual number of meticillin-resistant *Staphylococcus aureus* (MRSA) CC398-IIa isolates, from each newly identified MRSA-positive person, Denmark, 1999–2011 (n = 416)



scn using a multiplex PCR assay [8]. For comparative purposes, MRSA CC398 isolates from 1999 to 2006 were subjected to PCR-based detection of *mecA* and *lukF-PV* and *spa* typing [25]. MRSA CC398-IIa isolates were differentiated from other CC398 lineages using a dual-probe real-time PCR assay targeting CC398-IIa-specific canSNPs [7,8].

Clinical and epidemiological investigations

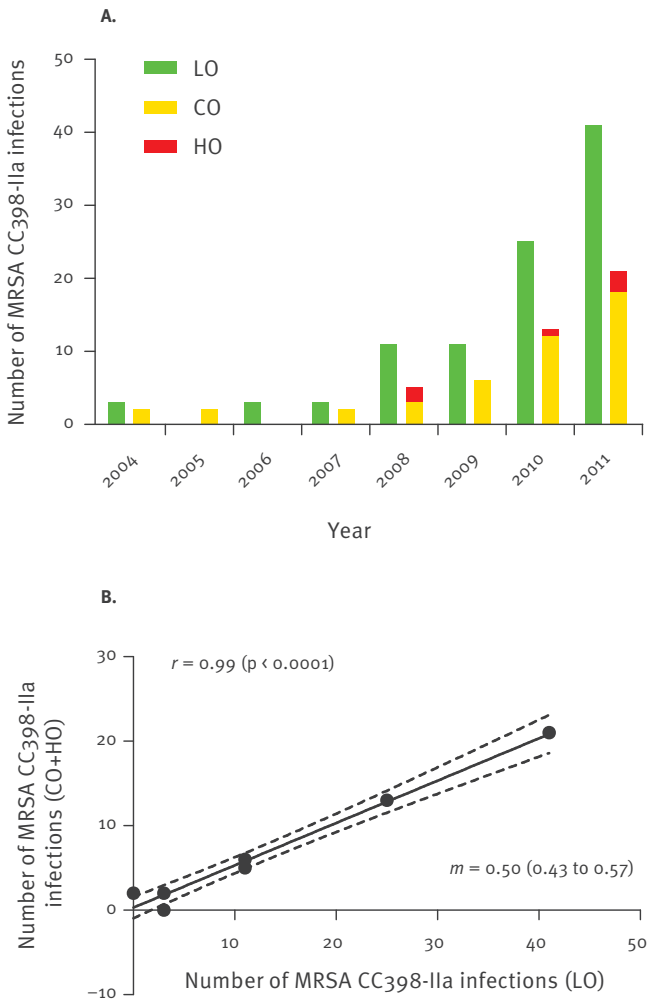
Patient information (i.e. age, sex, residential address, medical history and known livestock contact) was obtained from hospital or general practice records from 1999 to 2006 and from notification forms since 2007, when MRSA became a notifiable organism in Denmark. Patient information, including livestock contact or not, that was insufficiently described in the written records was obtained retrospectively through interviews with the corresponding patients, the relevant hospital or their general practitioner.

MRSA CC398 infections in patients who reported direct livestock contact (primary exposure) or were living together with a person with direct livestock contact (secondary exposure) were classified as livestock-onset. MRSA CC398 infections in patients with no livestock contact were classified either as community-onset when the positive culture was obtained from an outpatient or within the first 48 hours of hospital admission, or as healthcare-onset when the positive culture was obtained from an inpatient after 48 hours of hospital admission.

Infections were grouped as: (i) skin and soft tissue infections (SSTIs), including nose, skin, wound, and abscess; (ii) ear; (iii) eye; (iv) respiratory sites, including tracheal aspirates, sputum and induced sputum; (v) bone and joint; (vi) blood and cerebrospinal fluid; and (vii) others, including all other clinical sites, such as indwelling devices and fluid of unspecified origin.

Figure 2

Annual number (A) and scatter plot (B) of meticillin-resistant *Staphylococcus aureus* (MRSA) CC398-IIa infections, Denmark, 2004–2011 (n = 148)



CO: community-onset disease; HO: healthcare-onset disease; LO: livestock-onset disease.

Pearson's correlation (*r*) with p values in parentheses, linear regression line (solid) and 95% confidence intervals (dashed lines) and slope (*m*) with 95% confidence intervals in parentheses are shown.

Temporal and spatial analyses of MRSA CC398-IIa infections

We assessed the temporal and spatial relationships between MRSA CC398-IIa-infected patients with and without livestock exposure (healthy carriers identified through screening tests were excluded from epidemiological analyses, to eliminate any bias or confounding due to inconsistent screening practices). Discrete and continuous variables were compared between the two exposure groups by use of Fisher's exact test and Student's t-test, respectively (GraphPad Prism software, version 5, GraphPad, La Jolla, California, United States). Poisson regression modelling was used to compare incidence over time and between the two exposure groups (Stata software, version 12, StataCorp, College Station, Texas, United States). Pearson correlation and linear regression were used to describe the strength of the linear relationship between annual numbers of infections among patients with and without livestock exposure (GraphPad Prism software, version 5, GraphPad, La Jolla, California, United States). The significance level was set at $\alpha = 0.05$.

To characterise the spatial distribution of patients, we plotted their georeferenced residential addresses as point data on digital maps along with the population density per km² for each municipality (ArcGIS software, version 10.1, ESRI, Redlands, California, United States). Each data point was placed randomly within a 5-km radius of the exact residential address within a given municipality of residence to protect anonymity of the patient. Data on the number of person-years and the population density per km² for each municipality were obtained from Statistics Denmark.

Results

Identification of MRSA CC398 isolates, spa typing and antimicrobial susceptibilities

Statens Serum Institut received 7,429 MRSA isolates, one isolate per person, over the 13-year study period from 1999 to 2011. A total of 420 isolates were identified as MRSA CC398, of which 416 putatively belonged to CC398-IIa based on the presence/absence of *tet*(M) and *scn* (n = 389) or detection of canSNPs (n = 27) (Table 1). The remaining four isolates belonged to CC398-I/II-GOI.

The 416 MRSA CC398-IIa isolates displayed 12 different *spa* types, including t034 (n = 343; 82%), t011 (n = 45; 11%), t5706 (n = 8; 1.9%), t571 (n = 6; 1.4%), t108 (n = 3; 0.7%), t5095 (n = 3; 0.7%), t1255 (n = 2; 0.5%), t9517 (n = 2; 0.5%), t899 (n = 1; 0.2%), t1446 (n = 1; 0.2%), t1793 (n = 1; 0.2%) and t9345 (n = 1; 0.2%), were negative for *lukF-PV* and demonstrated variable levels of antimicrobial resistance: tetracycline (n = 416; 100%), clindamycin (n = 309; 74%), erythromycin (n = 188; 45%), norfloxacin (n = 91; 22%), kanamycin (n = 26; 6.3%), fusidic acid (n = 7; 1.7%), rifampicin (n = 2; 0.5%), linezolid (n = 1; 0.2%). None were resistant to mupirocin or glycopeptides. Most of the MRSA CC398-IIa isolates (n = 237; 57%) were multidrug resistant.

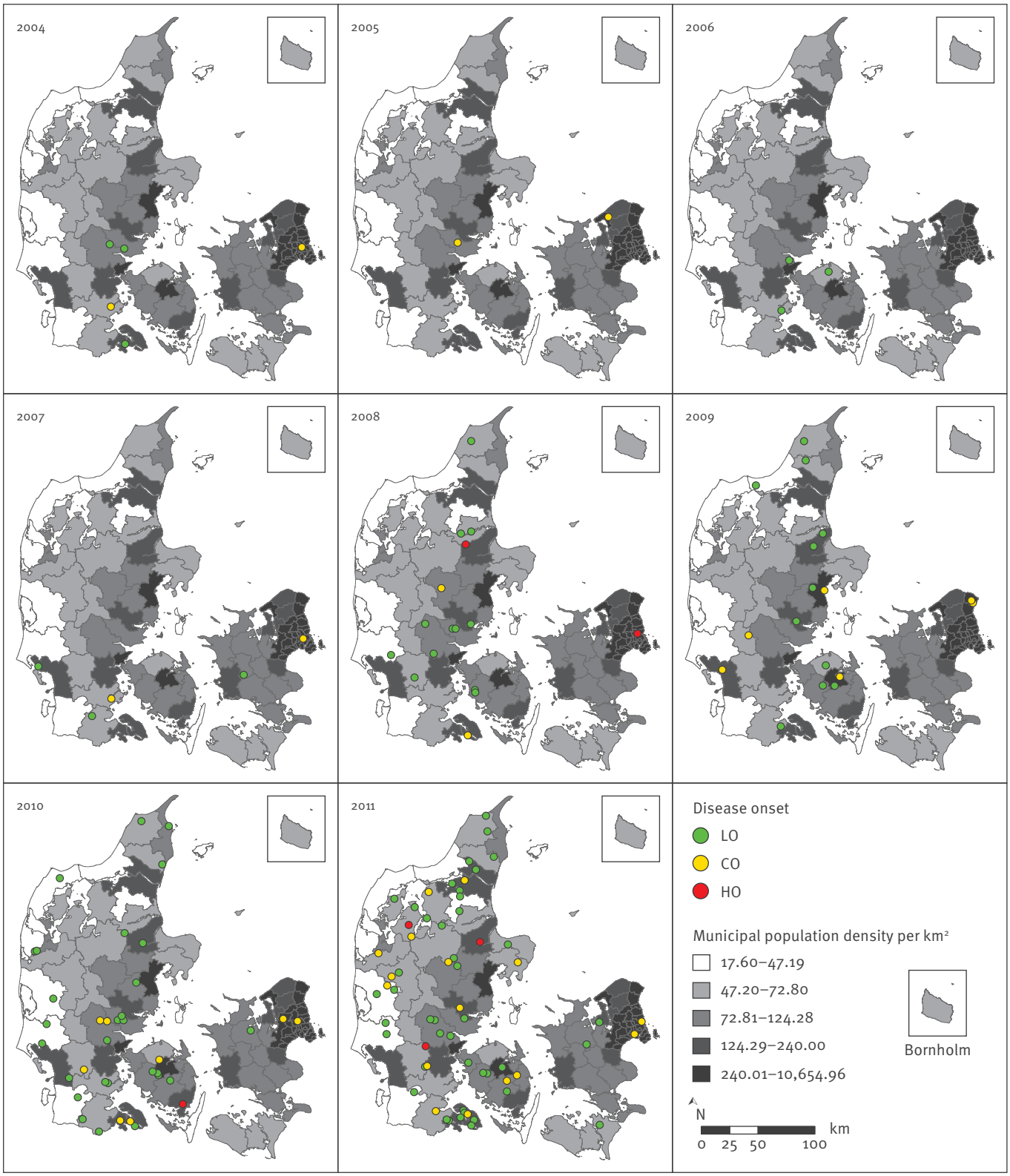
All four MRSA CC398-I/II-GOI isolates displayed *spa* type t034, were positive for *lukF-PV* and were resistant to erythromycin, clindamycin and norfloxacin.

Clinical epidemiology

A total of 151 MRSA CC398 isolates were obtained from clinical cases: the remaining 269 MRSA CC398 isolates originated from screening tests and were excluded from further analysis. Among the 151 cases of MRSA CC398 infection, 97 had primary or secondary exposure to livestock and 54 had no livestock exposure. MRSA CC398-IIa accounted for all 97 infections in patients with primary or secondary exposure to livestock and for 51 of the 54 infections in patients with

Figure 3

Geographical distribution of patients with meticillin-resistant *Staphylococcus aureus* (MRSA) CC398-IIa infection, Denmark, 2004–2011 (n = 148)



CO: community-onset disease; HO, healthcare-onset disease; LO: livestock-onset disease.

Each dot is placed randomly within a 5-km radius of the exact residential address within a given municipality of residence to protect anonymity of the patient. The municipal population density per km² is shown.

no livestock exposure, including 45 cases with community-onset infection and six cases with healthcare-onset infections. MRSA CC398-I/II-GOI was identified in the remaining three patients with no livestock contact, including two adoptees from Asia and a close family member of one of the adoptees, all of whom had SSTIs.

MRSA CC398-IIa isolates from patients unexposed to livestock were highly similar to MRSA CC398-IIa isolates from livestock-exposed patients both in terms of molecular and phenotypic characteristics as well as type of infection (Table 2). Unexposed patients were significantly older than livestock-exposed patients (median age: 49 vs 30 years; p = 0.0007) and were more likely to be female (female-to-male ratio: 1.3 vs 0.5; p = 0.0081) (Table 2).

Temporal and spatial trends of MRSA CC398-IIa infections

The annual numbers of MRSA CC398-IIa isolates, from each newly identified MRSA-positive person, over the 13-year study period from 1999 to 2011 are shown in Figure 1. MRSA CC398-IIa was first identified in January 2004 in a patient with an SSTI. Subsequently, the incidence of MRSA CC398-IIa infections increased from 0.09 per 100,000 person-years in 2004 to 1.1 per 100,000 person-years in 2011, corresponding to a linear annual increase of 66% (incidence rate ratio (IRR): 1.7; 95% confidence interval (CI): 1.5–1.9; p < 0.00001). Pearson correlation and linear regression demonstrated a clear temporal relationship between annual number of infections among livestock-exposed and unexposed patients (Figure 2). Furthermore, most unexposed patients appeared to live in close proximity to livestock-exposed patients (Figure 3).

Incidence of MRSA CC398-IIa infection among people with no livestock contact

In 2011, a total of 62 cases of MRSA CC398-IIa infection were identified in Denmark (5,475,791 inhabitants), of which 66% (41/62) were livestock-exposed and 34% (21/62) were unexposed (see also Figure 2 and Figure 3). The 41 livestock-exposed patients lived in 25 of the 99 Danish municipalities. In these 25 municipalities (1,676,186 inhabitants), the incidence of MRSA CC398-IIa infections among unexposed people was 0.7 per 100,000 person-years, whereas the overall incidence of MRSA infections was 10.9 per 100,000 person-years. In the remaining 74 municipalities (3,799,605 inhabitants), the incidence of MRSA CC398-IIa infections among unexposed people was 0.3 per 100,000 person-years, whereas the overall incidence of MRSA infections was 12.8 per 100,000 person-years. Poisson regression modelling showed that the risk of MRSA CC398-IIa infection among unexposed people was significantly higher in the 25 municipalities in which livestock-exposed patients lived than in the rest of Denmark (74 municipalities) (IRR: 2.5; 95% CI: 1.1 to 5.7; p = 0.041). Nonetheless, MRSA CC398-IIa accounted for only 6% (11/183) and 2% (10/487) of the total number of MRSA infections among unexposed people in the 25 and 74 municipalities, respectively, and the overall risk of MRSA infection was not significantly different between the two groups of municipalities (IRR: 0.9; 95% CI: 0.7 to 1.0; p = 0.062).

In the Capital Region (30 municipalities, 1,645,825 inhabitants), we observed only two cases of MRSA CC398-IIa infection among unexposed people in 2011, corresponding to 0.6% (2/309) of the total number of MRSA infections among unexposed people.

Discussion

The results presented here show that MRSA CC398-IIa was an increasing cause of infection among people with and without livestock exposure in Denmark from 2004 to 2011. During this time, there was a more than fourfold increase in the prevalence of MRSA CC398 among Danish pigs [5,28,29]. Most of the unexposed patients were spatially clustered around livestock-exposed patients. Moreover, MRSA CC398-IIa isolates from livestock-exposed and unexposed patients had similar molecular and phenotypic characteristics. Together, these findings suggest that the expanding livestock reservoir of MRSA CC398 may have led to increased spillover into the surrounding community during the study period.

The MRSA CC398-IIa isolates analysed demonstrated high levels of resistance to tetracyclines, lincosamides (clindamycin), macrolides (erythromycin) and quinolones (norfloxacin), which, with the exception of quinolones, represent some of the most commonly used antibiotics in Danish pig production [29]. Interestingly, the isolates were more often resistant to clindamycin than to erythromycin. This rather unusual resistance pattern has been described in human and porcine MRSA CC398 isolates from Spain, where it was associated with the presence of either the *lnu*(A) or *lnu*(B) gene [30]. Most of the MRSA CC398-IIa isolates were multidrug resistant, thus further limiting the options for clinical therapy beyond β-lactam antibiotics. In Denmark, use of antibiotics for growth promotion and prophylaxis is not permitted in livestock production; therefore, the data presented here suggest that the public health risks of antibiotic use in agriculture may also include therapeutic applications.

While the MRSA CC398-IIa isolates and types of infection did not differ among the two exposure groups, the patients comprising the two populations varied significantly. Livestock-exposed patients were younger than unexposed patients and were more likely to be male. These findings probably reflect the demographics of livestock workers, who are more likely to be working-age men compared with the general population.

MRSA CC398-IIa was primarily associated with SSTIs and other non-invasive infections during the study period; however, there have been four fatal bloodstream infections (BSIs) with MRSA CC398-IIa in Denmark since 2012 [31]. These patients had no livestock exposure

but presented with known predisposing risk factors of MRSA BSI (e.g. severe underlying diseases or haemodialysis). These observations confirm that MRSA CC398-IIa is capable of causing life-threatening disease in at-risk patients, and it is expected that there will be an increasing number of invasive infections in the near future if the prevalence of MRSA CC398-IIa continues to increase in the general population. Thus, there is an urgent need to control this organism in the healthcare setting. In 2012, the Danish Health and Medicines Authority released updated guidelines for the management of MRSA, which recommend that all hospitals perform targeted screening of patients and hospital staff at high risk of MRSA CC398-IIa carriage (i.e. all people with primary or secondary exposure to livestock) [23]. However, spread into the hospital may increase if the prevalence of MRSA CC398-IIa in the unexposed population continues to grow.

Our study does not address how MRSA CC398-IIa spreads from the livestock reservoir into the local community. Other *S. aureus* strain types predominantly spread through human-to-human contact [17]. The same is likely to be true for MRSA CC398-IIa, where livestock workers may be the primary source for the delivery of MRSA CC398-IIa to unexposed people. In addition, some unique features of livestock production may make it more conducive to environmental transmission. For example, powerful tunnel ventilation systems that carry air though livestock production facilities may mediate airborne transmission of MRSA CC398-IIa into nearby communities. Previous studies have shown that antibiotic-resistant *S. aureus* can be isolated from air samples up to 300 m downwind of pig farms [18,19]. Likewise, land application of pig manure as fertiliser, by spray or injection, may also mediate environmental transmission. Two studies from the United States showed significant associations between the cropland application of pig manure and MRSA carriage [20,21]. However, in contrast to our study, these two American studies did not distinguish between people with and without livestock contact and did not report MRSA strain types. More research is needed to quantify the relative roles of human contact and environmental media in the transmission of MRSA CC398-IIa to people with no livestock exposure.

MRSA CC398-IIa is frequently isolated from retail meat in Denmark [28,29,30,32], which has raised concerns about food-borne acquisition of this pathogen in both community and healthcare settings through consumption or handling of contaminated products. These concerns were substantiated by a case–control study from the Netherlands, which found that consumption of chicken meat was a significant risk factor for MRSA carriage [33]. In Denmark, most livestock is slaughtered in a small number of centralised abattoirs, from which meat is distributed to widely dispersed retail stores. Despite this, most cases of MRSA CC398-IIa infection in our study were among unexposed people who lived in close proximity to livestock-exposed patients in rural areas; we observed only a few cases of MRSA CC398-IIa infection among unexposed people in the Capital Region. Taken together, these findings strongly suggest that food-borne transmission does not play a major role in the MRSA CC398-IIa epidemiology.

The low prevalence of MRSA CC398-I/II-GOI in our study suggests that this strain type is not endemic in Denmark. In contrast, MRSA CC398-I/II-GOI is a frequent cause of both community-onset and healthcare-onset infections in China [34] and has been described in Asian adoptees in the Netherlands and Sweden [35,36]. Consistent with these previous studies, the patients with MRSA CC398-I/II-GOI infection identified in our study were linked to adoptees from Asia.

Our study has several strengths. First, we used a nationwide registry-based case-finding strategy in combination with individual-level information on both primary and secondary exposure to livestock. Second, we eliminated any bias or confounding due to inconsistent screening practices by excluding healthy carriers identified through screening tests from the analysis. Third, we used a set of newly developed genotyping tools to differentiate between MRSA CC398-IIa and MRSA CC398-I/II-GOI isolates.

Our study also has limitations that should be considered. First, the national MRSA registry contains data only at the time of the first diagnosis and does not hold information on subsequent episodes of infection. By discounting people with asymptomatic carriage who later develop MRSA infections, the number of MRSA infections may be substantially underestimated. Second, it is unclear if our findings are generalisable to other countries. Nonetheless, our findings are consistent with those from the Netherlands, where MRSA CC398-positive people with no livestock exposure were found to be concentrated in rural, livestock-production areas [37].

Our findings suggest that MRSA CC398-IIa may be spreading from the livestock reservoir into the local community with increasing frequency. However, even in these local communities, MRSA CC398-IIa accounted for a relatively small proportion of the total number of MRSA infections among unexposed people, and there was no apparent association between living inside or outside such an area and the overall risk of acquiring an MRSA infection. The spatial distribution and rate of spillover of MRSA CC398-IIa into the community are likely to increase in the near future as the livestock reservoir expands to MRSA-naive farms. The epidemiology of MRSA CC398-IIa may also change over time as new variant subclones emerge with increased or decreased capacity for human-to-human transmission. Therefore, it is important to continue monitoring MRSA CC398-IIa colonisation and infection rates among livestock and livestock workers, community dwellers and patients and staff in healthcare facilities. Efforts must also be

made to further resolve transmission routes and stem the continued dissemination of MRSA CC398-IIa.

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Conflict of interest

None declared.

Authors' contributions

JL, AP, MS, MS, AL and RS were members of the MRSA surveillance team. JL, LP, PA, AL and RL designed the study. JL, LP and RS prepared the initial manuscript. AP and AL contributed to the subsequent editorial revisions. LvA, PVB, LK, LL and BF performed epidemiological investigations.

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Research article

Prevalence, genotyping and macrolide resistance of *Mycoplasma pneumoniae* among isolates of patients with respiratory tract infections, Central Slovenia, 2006 to 2014

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In this retrospective study we employed real-time polymerase chain reaction (PCR) to analyse the occurrence of *Mycoplasma pneumoniae* among upper and lower respiratory tract infections (RTI) in the Central Region of Slovenia between January 2006 and December 2014. We also used a culture and pyrosequencing approach to genotype strains and infer their potential macrolide resistance. Of a total 9,431 tested samples from in- and out-patient with RTI, 1,255 (13%) were found to be positive by *M. pneumoniae* PCR. The proportion of positive samples was 19% (947/5,092) among children (≤16 years-old) and 7% (308/4,339) among adults (>16 years-old). Overall, among those PCR tested, the highest proportions of *M. pneumoniae* infections during the study period were observed in 2010 and 2014. In these two years, 18% (218/1,237) and 25% (721/2,844) of samples were positive respectively, indicating epidemic periods. From the 1,255 *M. pneumoniae* PCR-positive samples, 783 (614 from paediatric and 169 from adult patients) were successfully cultured. Of these, 40% (312/783) were constituted of strains belonging to the P1 type II genomic group, while 60% (469/783) contained strains of the P1 type I group. Two isolates comprised both P1 type I and II strains. Results of a genotype analysis by year, showed that the dominant *M. pneumoniae* P1 type during the 2010 epidemic was P1 type II (82% of isolates; 81/99), which was replaced by P1 type I in the 2014 epidemic (75%; 384/510). This observation could indicate that the two epidemics may have been driven by a type shift phenomenon, although both types remained present in the studied population during the assessed period of time. Only 1% of strains (7/783) were found to harbour an A2063G mutation in the 23S rRNA gene, which confers macrolide resistance, suggesting that the occurrence of *M. pneumoniae* macrolide resistance still seems to be sporadic in our geographic area.

Introduction

Mycoplasma pneumoniae is a fastidious, slow growing, cell wall-lacking bacteria that can be the causative agent of up to 40% cases of community-acquired respiratory tract infections (RTI) [1-6]. Although most cases present with mild or moderate symptoms, serious disease requiring hospitalisation occasionally occurs [7].

M. pneumoniae incidence usually increases during epidemics, which occur at intervals of four to seven years [8-10]. This pattern is presumably linked to the alternation of P1 adhesin types, which tend to change dominance over the course of time and, consequently, allow *M. pneumoniae* to elude the human host immune response [11]. According to nucleotide (nt) differences in two repetitive regions (RepMP2/3 and RepMP4) in the MPN141 gene, which codes for P1 adhesin, *M. pneumoniae* strains can be divided into two genomic groups, called P1 type I and P1 type II [12], with several subtypes [13].

Macrolide-resistant strains among *M. pneumoniae* isolates are increasingly described, with reported resistance rates among studies from European Union Member States [11,14-17], Israel [18] and the United States [19] of up to 30%. In contrast, rates of over 90% have been observed in some parts of Asia [20-25]. Low to high grade resistance against many if not all macrolides in *M. pneumoniae* has been shown to be related to nt substitutions in two regions of the V domain in the 23S rRNA gene, namely at positions 2,063, 2,064, 2,067 and 2,617 (*M. pneumoniae* numbering). Among

Table 1

Samples of patients with upper and lower respiratory tract infections tested by *Mycoplasma pneumoniae* real-time polymerase chain reaction and annual positivity rate, Central Slovenian region, 2006–2014 (n=9,431 samples)

Samples	Year									Period 2006–2014
	2006	2007	2008	2009	2010	2011	2012	2013	2014	
Total tested N	391	470	393	659	1,237	1,185	1,005	1,247	2,844	9,431
Positive N (%)	9 (2)	19 (4)	16 (4)	48 (7)	218 (18)	117 (10)	46 (5)	61 (5)	721 (25)	1,255 (13)

these mutations, the A2063G mutation appears to be the most common, followed by A2064G [26]. All other mutations A2063C [27], A2063T [28], A2067G, C2617G and C2617A are found much less frequently [26].

In Slovenia, *M. pneumoniae* infections are not notifiable and *M. pneumoniae* genotyping as well as antibiotic resistance testing are not yet provided as a routine service. Moreover, limited data are so far available for longitudinal studies looking into changes in the prevalence of *M. pneumoniae* infections, genetic diversity and antibiotic resistance.

In the only two published serology-based studies available from Slovenia, one from April 1996 to March 1997 [29] and the other from November 1999 to April 2001 [30], *M. pneumoniae* was frequently found as the causative agent of community-acquired pneumonia in hospitalised patients (5.7% [29] and 24.8% [30]). Based on these reports and the observation of an increased number of *M. pneumoniae* cases from routine laboratory testing all over Slovenia in 2010 and 2014 (data not shown), we decided to analyse the occurrence of *M. pneumoniae* infections in in- and out-patients with RTIs. At the end of 2005, real-time polymerase chain reaction (PCR) was introduced as the main diagnostic tool for *M. pneumoniae* infections in the Institute of Microbiology and Immunology at the University of Ljubljana, which covers the entire Central Slovenian Region. This allowed us to analyse tested samples from January 2006 to December 2014. We also used culture and molecular assays to further characterise the genotype of isolated strains during this period, and infer their potential macrolide resistance.

Methods

Patient samples and *Mycoplasma pneumoniae* testing

Our study was conducted between January 2006 and December 2014. Patients considered, were inhabitants of the Central Region of Slovenia, which on average represents 41% (843,528/2,038,281) [31] of the whole Slovenian population during the designated period of time. The distribution of patients did not have any apparent specific clustering in space and time. Thirty-two per cent (2,996/9,431) of patients were from the capital Ljubljana and 68% (6,435/9,431) from other parts of the Central Slovenian region. Depending on the severity of the symptoms, patients were treated as in- or out-patients. During the study period, all consecutive (n=9,431) upper or lower respiratory tract specimens obtained from 5,092 (54%) paediatric (≤16 years of age) and 4,339 (46%) adult (>16 years of age) patients with RTI were enrolled. Together with each sample, we received data of the patient's sex, age, address, attending physician and his/her institution, sample type, date and place of collection and basic diagnosis (RTI). An aliquot of each sample was subjected to routine laboratory testing by *M. pneumoniae* real-time PCR (Argene biosoft, France). This protocol was not changed during the time of the study. The remainder of each PCR positive sample was cultivated in order to obtain pure *M. pneumoniae* isolates.

Culture of *Mycoplasma pneumoniae* positive samples

Culture was performed by using *Mycoplasma* selective broth and agar plates (OXOID, United Kingdom (UK)) enriched with *Mycoplasma* supplement G or P (OXOID, UK) according to standard methods described elsewhere [32]. The obtained isolates were stored at -80 °C until further testing.

Mycoplasma pneumoniae genotyping and macrolide resistance detection

Neither mutations in the 23S rRNA gene domain II, ribosomal proteins L4 and L22 [26], nor genomic or plasmid *erm* genes have been shown to be implicated in macrolide resistance in *M. pneumoniae* [33]. Moreover as *M. pneumoniae* harbours only one copy of the 23S rRNA gene [34], molecular tests targeting the 23S rRNA gene domain V offer valuable tools for quick identification of resistant strains. Such tests have been developed, as well as some allowing rapid and reliable genotyping, and these can even be applied directly to clinical samples [11,15,35-39]. Recent publications by Spuesens et al. [40,41] describe a pyrosequencing approach for genotyping *M. pneumoniae* and determination of macrolide resistance. Based on single nt polymorphisms (SNPs) in the MPN141 gene's constant region, and on additional information from the constant region of the MPN528a gene, the method enables quick classification of strains into P1 type I and P1 type II. Macrolide resistance implicated mutations in the two regions of the 23S rRNA gene V domain can also be detected. This

pyrosequencing approach was used for our study with some modifications which are further described.

Mycoplasma pneumoniae DNA was purified from 200 µL of liquid culture (OXOID, UK) using the MagNA Pure Compact automated DNA extraction system (Roche, Germany). A total of 5 µL of the 200 µL eluate was used in all PCR reactions.

All primers, reaction conditions and master mix compositions used for the amplification of *M. pneumoniae* MPN141, MPN528a and two parts of the 23S rRNA domain V (designated as Assay 1 – region encompassing macrolide resistance important positions 2,063, 2,064, 2,067 and Assay 2 – region encompassing macrolide resistance important position 2,617) were as described by Spuesens et al. [40], with several modifications. Briefly, for all PCR assays the number of cycles was increased to 50, with the denaturation step prolonged to 15 s and the extension step shortened to 15 s. The annealing step was 30 s with the temperature set to 55 °C for all PCR assays. The master mix was as previously published [40]. The resulting biotinylated amplification products were checked for expected sizes and suitable amounts by 1.5% agarose gel electrophoresis stained with 1X SYBR Safe (Invitrogen, Germany).

All remaining amplified fragments of MPN141, MPN528a, 23S rRNA Assay 1 and Assay 2 were pyrosequenced on a PyroMarkID instrument (Biotage, Sweden) with PyroMark Gold Q96 SQA Reagents (Qiagen, Germany) using previously designed sequencing primers (TIB MOLBIOL, Germany) [40]. For the detection of P1 type specific SNP, we adopted the dispensation order developed earlier [40], but we constructed our own specific nt dispensation orders that cover all so far detected mutations linked with macrolide resistance in respective portions of the *M. pneumoniae* 23S rRNA gene domain V. Moreover, the dispensation order for the *M. pneumoniae* 23S rRNA pyrosequencing Assay 1 was extended to produce 79 bases long pyrosequencing products, which were later used to additionally check the specificity of the PCR assay, by National Center for Biotechnology Information (NCBI) basic local alignment tool (BLAST) search.

Genotype and possible macrolide resistance designations were attributed using IdentyFire software (Biotage, Sweden) loaded with custom built libraries.

Mycoplasma pneumoniae 23S rRNA polymerase chain reaction Assay 1 and Assay 2 specificity

NCBI BLAST search of the primers [40] used in the 23S rRNA domain V Assay 1 and Assay 2 showed a possible nonspecific annealing with *Mycoplasma genitalium*. Although *M. genitalium* is not a commonly recognised pneumonia agent and despite the fact that RTI isolates were used as starting material, we decided to additionally check the specificity of the primers. Both *M. pneumoniae* 23S rRNA assays were therefore performed with DNA from *Chlamydia pneumoniae, C. psittaci, C. trachomatis, Corynebacterium spp., Enterococcus fecalis, Escherichia coli, Klebsiella pneumoniae, Moraxella cattarhalis, Mycoplasma genitalium, Mycoplasma hominis, Neisseria gonorrhoeae, Proteus mirabilis, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus pyogenes, Streptococcus agalactiae* and *Ureaplasma* spp.

Results

Patient samples and *Mycoplasma pneumoniae* testing

From 9,431 patient samples, 1,255 (13%) were found to be *M. pneumoniae* positive by PCR. Of the latter, 51% (n=640) were from males and 49% (n=615) from females. The median age of patients infected by *M. pneumoniae* was 8 years (range: 43 days to 85 years).

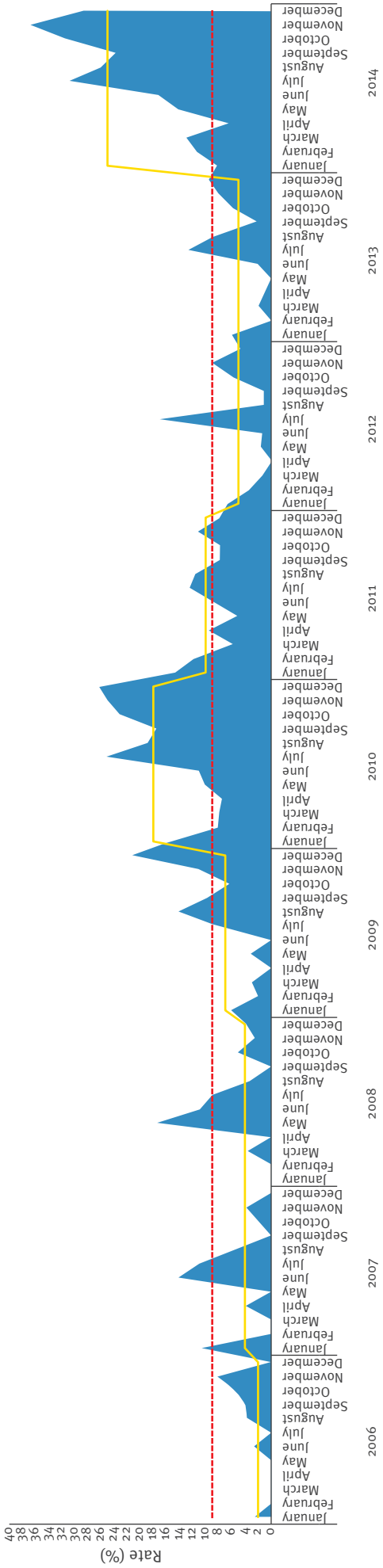
While *M. pneumoniae* was found among 19% (947/5,092) of children samples, 7% (308/4,339) of adult samples tested positive. The highest proportion of positive samples were found in the age group comprising individuals between six and 16 years-old (26%; 565/2,162), followed by preschool children ≤5 years-old (13%; 382/2,930), adults between 17 and 65 years-old (8%; 288/3,253) and elderly persons ›65 years-old (2%; 20/1,086).

A closer look at the distribution over time of positive patient samples revealed that the majority were found in 2010, 2011 and 2014, when 218/1,237 (18%), 117/1,185 (10%) and 721/2,844 (25%) of patient samples contained detectable amounts of *M. pneumoniae* DNA, respectively (Table 1).

An even more detailed month to month analysis shows that the rate of detected *M. pneumoniae* infections was overall low from 2006 to 2009 (between 2% and 7%), with seasonal peaks between June and August. However, in December 2009 the rate of *M. pneumoniae* infections quickly rose to 21% (15/70) and remained high through to November 2011, when 11% (13/115) of patients with RTIs tested *M. pneumoniae* positive. Only later did the rate of *M. pneumoniae* infections return below the 2006 to 2014 average (9%) and remained low, with seasonal peaks during the summer, similarly to the time before 2010. Another unusual increase in the rate of *M. pneumoniae* positive samples occurred after the typical summer peak in 2013, during winter 2013 and spring 2014, until November 2014, when 37% (214/580) of patients were positive by *M. pneumoniae* PCR (Figure). It was also found that 15% (436/2,996) of patients in Ljubljana and 13% (819/6,435) in other parts of the Central Slovenian region had *M. pneumoniae* acute infections, which seems to indicate that *M. pneumoniae* was overall similarly present in the geographical area included in the study. Additionally, the rise in the number of *M. pneumoniae* positive patients during 2010 and 2014 occurred almost evenly throughout the whole studied area. We also did not observe

Figure

Rates of samples testing positive for *Mycoplasma pneumoniae* among samples from patients with upper and lower respiratory tract infections, Central Slovenian region, January 2006–December 2014 (n=9,431 tested samples)



any specific clustering that could indicate a merely localised outbreak during the time of the study.

Of the 1,255 PCR positive samples, *M. pneumoniae* was successfully cultured from 62% (n=783). The distribution of the obtained isolates according to sex was 50% (390/783) men, 50% (393/783) women, and overall 78% (614/783) of cultured samples were from children and 22% (169/783) from adults.

Mycoplasma pneumoniae genotyping and macrolide resistance detection

For all 783 *M. pneumoniae* cultured isolates, PCR products were successfully obtained for all targets, namely MPN141, MPN528a and both parts of 23S rRNA gene domain V (Assay 1 and Assay 2). All assays produced good quality pyrosequencing products of 7, 10, 79 and 16 nt for MPN141, MPN528a, 23S rRNA domain V Assay 1 and Assay 2, respectively. During the assessed time period, *M. pneumoniae* P1 type I and P1 type II strains were found in 60% (469/783) and 40% (312/783) of cultured isolates, respectively. We also detected two isolates with both P1 type I and P1 type II *M. pneumoniae* DNA.

A more detailed analysis on a year-to-year basis showed that although both P1 types remained present during the whole study period, the most observed *M. pneumoniae* P1 type during the 2010 epidemic was P1 type II (82%; 81/99), while in the 2014 epidemic P1 type I dominated (75%; 384/510) (Table 2). Moreover it seems that between these two epidemic years, the proportion of isolates with P1 type II decreased in favour of P1 type I (Table 2).

Seven samples (1%; 7/783), all from inpatients, were found to contain an A2063G mutation (Table 2). Six mutated strains were cultured from respiratory samples of paediatric patients and one from an adult patient. Three of the presumed macrolide resistant strains belonged to the P1 type I and four to the P1 type II. All other isolated *M. pneumoniae* strains had no mutation in the two sequenced areas of the 23S rRNA gene and were therefore presumed to be macrolide sensitive.

Mycoplasma pneumoniae 23S rRNA polymerase chain reaction Assay 1 and Assay 2 specificity

As predicted by the NCBI BLAST search, no tested organism except *M. genitalium* produced a positive PCR result in either *M. pneumoniae* 23S rRNA Assay 1 or Assay 2. However, after pyrosequencing the PCR product from Assay 1, the sequence of *M. pneumoniae* had six differences (73/79; 92% identity) distinguishing it from *M. genitalium*.

Discussion

From our data, we observed that in 2006, 2007, 2008, 2009, 2012 and 2013 the number of *M. pneumoniae* positive cases usually rose during the summer but quickly returned to low values during the autumn,

Table 2

Annual number of *Mycoplasma pneumoniae* isolates, P1 type distribution, percentage of inferred macrolide-resistant strains and detected 23S rRNA mutations, Central Slovenian region, 2006–2014

Year	Number of isolates	P1 type I N (%)	P1 type II N (%)	P1 type I+II N (%)	Mutated strains N (%)	23S rRNA mutation
2006	4	2 (50)	2 (50)	0 (0)	0 (0)	None
2007	19	9 (47)	10 (53)	0 (0)	0 (0)	None
2008	7	2 (29)	5 (71)	0 (0)	0 (0)	None
2009	33	12 (36)	21 (64)	0 (0)	1 (3)	A2063G
2010	99	17 (17)	81 (82)	1 (1)	2 (2)	A2063G
2011	46	12 (26)	33 (72)	1 (2)	0 (0)	None
2012	29	8 (28)	21 (72)	0 (0)	0 (0)	None
2013	36	23 (64)	13 (36)	0 (0)	1 (3)	A2063G
2014	510	384 (75)	126 (25)	0 (0)	3 (1)	A2063G
Total	783	469 (60)	312 (40)	2 (0)	7 (1)	–

winter and spring, suggesting that in these years the occurrence of *M. pneumoniae* infections was most likely endemic. On the other hand, a different pattern with unusual increases in *M. pneumoniae* infections was seen from November 2009 to December 2011 and from May 2014 to December 2014. Based on these results, we suggest that a *M. pneumoniae* epidemic took place in Slovenia from November 2009 to December 2011 similarly to other European countries [42]. Interestingly, our results also seem to show that another, even more extensive, outbreak of *M. pneumoniae* infections began in May 2014, reached its peak in November 2014, when 37% (214/580) of patients were positive for *M. pneumoniae* DNA and persisted at least until December 2014, when 29% (171/598) of patients were infected. Comparable results from other countries are not yet available, so we cannot say whether such a scenario is limited to Slovenia or correlates with a new European-wide epidemic.

We do not believe that the rises in the number of *M. pneumoniae* positive patients were due to increased awareness or testing availability, since no changes were made in the healthcare system in Slovenia during the time of the study. Additionally, the same test was used throughout and increased numbers of positives cannot therefore be ascribed to methodological changes in sensitivity. Moreover, a similar increase in the number of *M. pneumoniae* cases was also observed by other laboratories in Slovenia (data not shown). Finally, the number of tested patients did increase from 2006 to 2014 but most likely as a result of more patients seeking help at healthcare facilities during epidemics.

Analysis of *M. pneumoniae* P1 type distribution in Central Slovenia from 2006 to 2014 shows that both P1 types were present in the studied population during the whole period. No complete type shift phenomenon, as described in a Japanese study [43], could be observed in our population but it would seem that the 2010/11 epidemic was caused mostly by *M. pneumoniae* P1 type II and the 2014 epidemic by *M. pneumoniae* P1 type I. Although we observed a large increase in *M. pneumoniae* cases in 2014, we cannot be sure that the reason for the new outbreak was an increase in the number of P1 type I strains. Further studies using multilocus variable-number tandem repeat analysis (MLVA) [44] are needed to assess whether the 2010/11 and 2014 epidemics were caused by a specific MLVA type or multiple types, as seen in a recent study from France and Israel [45]. On the basis of P1 typing, however, it might be concluded that the prevalence of the two major *M. pneumoniae* genetic types in Slovenia does oscillate during time.

The results of our study suggest that macrolide-resistant *M. pneumoniae* strains, although represented by small numbers of isolates, are already present in Slovenian patients, which seems to be in concordance with most European studies, which also show low level of macrolide resistance in *M. pneumoniae* [11,14,15,17]. An exception is a study from 2011, in which macrolide resistance was detected in 26% of children presenting with *M. pneumoniae* pneumonia or bronchitis in an Italian city [16].

Our low prevalence of *M. pneumoniae* macrolide resistant strains, however, is not unexpected, since a national antibiotic usage survey shows that macrolides are being carefully used in Slovenia with Daily Doses (DD) per 1,000 inhabitants of 2.14, 2.43, 2.22, 2.11, 1.85, 1.75, 1.84, 1.67 and 1.65 in 2006, 2007, 2008, 2009, 2010, 2011, 2012, 2013 and 2014, respectively [46,47]. Interestingly, *M. pneumoniae* macrolide-resistant strains were first detected in 2009, and then in 2010, 2013 and 2014, when macrolide antibiotic DDs per 1,000 inhabitants were constantly declining.

We noted that all mutated strains harbour an A2063G substitution, which is recognised as the most prevalent and high level macrolide resistance mediating mutation [27]. Although all strains harboured the same type of mutation, we did not find any connection among the patients from whom these were derived. The patients were from different geographical parts of Central Slovenia and the dates of sample collection were several months or even years apart. Moreover, basic molecular typing studies showed that three presumed macrolide-resistant strains belonged to P1 type I and four to P1 type II subgroup, which additionally supports a lack of connection between the strains. Studies using more accurate genetic methods (MLVA) could shed further light on the phylogeny of these strains.

To summarise, we observed two epidemics of *M. pneumoniae* infections. The first was, similarly to some other European countries, in 2010/11. The second epidemic seems to have started in Slovenia in 2014. We also found that *M. pneumoniae* P1 type II may have been the main cause of the 2010 epidemic while P1 type I may have been responsible for the new 2014

outbreak. Additionally, in concordance with the situation in Europe, we documented the presence of macrolide-resistant *M. pneumoniae* strains in children and in adults at very low level.

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Conflict of interest

None declared.

Authors' contributions

RK: execution of experiments, analysis and interpretation of results, manuscript writing; DK: project leader, analysis and interpretation of results, manuscript writing, TM and MP: sample collection, patient data, pre-submission manuscript reviewing.

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Research article

Surveillance of endemic foci of tick-borne encephalitis in Finland 1995–2013: evidence of emergence of new foci

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The geographical risk areas for tick-borne encephalitis (TBE) in Finland remained the same until the beginning of the 21st century, but a considerable geographical expansion has been observed in the past 10 years. In order to support public health measures, the present study describes the number of laboratory-confirmed TBE cases and laboratory tests conducted and the associated trends by hospital district, with a particular emphasis on the suspected geographical risk areas. An additional investigation was conducted on 1,957 clinical serum samples throughout the country taken from patients with neurological symptoms to screen for undiagnosed TBE cases. This study identified new TBE foci in Finland, reflecting the spread of the disease into new areas. Even in the most endemic municipalities, transmission of TBE to humans occurred in very specific and often small foci. The number of antibody tests for TBE virus more than doubled (an increase by 105%) between 2007 and 2013. Analysis of the number of tests also revealed areas in which the awareness of clinicians may be suboptimal at present. However, it appears that underdiagnosis of neuroinvasive TBE is not common.

Introduction

Tick-borne encephalitis virus (TBEV) is a clinically important flavivirus causing encephalitic disease with thousands of cases annually in Europe [1]. There are three known subtypes of TBEV: European (Eur), Siberian (Sib) and Far-Eastern (FE). The European subtype is mainly carried by *Ixodes ricinus* ticks in central and north-eastern Europe, whereas the other two are mainly found in *I. persulcatus* ticks in an area reaching from north-eastern Europe to the Russian Far East, China and Japan [2].

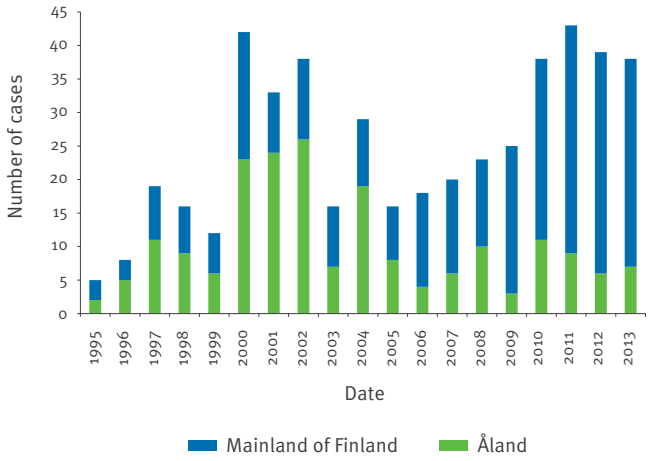
TBEV is maintained in nature by ticks and their hosts. Ticks serve both as a reservoir and as vector for the virus [3]. Persistence in small mammals may also contribute to maintenance [4,5]. *Ixodes* ticks are widely spread generalist ectoparasites [6]. However, circulation of TBEV is dependent on the local vegetation, dynamics of the vertebrate and tick host populations and microclimatic conditions, leading to focal occurrence of TBEV within the distribution area of the host ticks [3].

Humans do not contribute to sustain the circulation of TBEV but can get infected as accidental hosts by a tick bite or through consumption of unpasteurised milk from infected ruminants, particularly goats [7]. Besides genetic host factors, the course and severity of the infection may depend on the virus subtype and strain. Of the three subtypes, TBEV-FE is the one that most frequently induces severe tick-borne encephalitis (TBE), and the reported fatality rate reaches 35% [7]. TBEV-Sib causes a mortality of 2–3% and has been associated with chronic forms of TBE [1]. A biphasic course of infection is considered characteristic for TBEV-Eur: After an average incubation period of eight days, the viraemic phase, lasting one to eight days, manifests with unspecific influenza-like symptoms or remains subclinical [7,8]. After an asymptomatic period, 20–30% of the patients present with a neuroinvasive disease [7]. Although mortality is low for TBEV-Eur, less than 2% [1,9], neuronal degeneration and post-encephalitis syndrome occur in up to one third of the patients and can have sequelae that reduce the quality of life [8,9]. Effective vaccines are available to prevent TBE caused by any of the three subtypes [2,7].

In Europe, the laboratory diagnosis of acute TBE is based on specific IgG and IgM antibody detection from serum and/or cerebrospinal fluid (CSF) [10,11]. Vaccination and natural infection with other flaviviruses produce cross-reactive antibodies, which should be taken into consideration in the interpretation of IgG test results.

Figure 1

All tick-borne encephalitis cases reported in the National Infectious Diseases Register, by place of diagnosis, Finland, 1995–2013 (n = 478)



Cases as reported by hospital district of the treating unit [41].

There is no official definition of a TBEV-endemic area in Finland. However, foci with repeated human cases are considered risk areas. Most of these areas have been visited by our research group for collection of ticks and rodents to confirm the presence of TBEV in ticks and/or host animals and to analyse the TBEV strains and tick species present in the area. Evidence from the past decade suggests a TBEV prevalence of up to 2% in *I. ricinus* and *I. persulcatus* ticks in TBEV foci throughout Finland [12-15].

Finland, together with the Baltic countries and western Russia, lies in the zone where the two main host tick species mix [2]. The overall distribution of tick species in Finland has only been investigated in restricted areas, most of which are endemic TBEV foci. *I. ricinus* is the main tick species found in southern and south-western Finland, while *I. persulcatus* is present in the north-west coast and at the eastern border of the country [12-16]. The TBEV subtypes endemic in Finland are TBEV-Eur and TBEV-Sib [13,15,16]. Interestingly, TBEV-Eur was recently found in *I. persulcatus* ticks in the northernmost known TBE focus, in Simo, Finland [14]. All three subtypes are found in Russia and have been reported in Estonia and Latvia [17-19].

The first cluster of encephalitis cases in Finland occurred in Kumlinge, Åland in the 1940s; TBE, then known locally as Kumlinge disease, was described in 1956 [20] and TBEV was isolated in Kumlinge in 1959 (isolate A52) [8]. The number and geographical distribution of human TBE cases in Finland remained stable for many decades, but in the past 10 years, the disease has become more common and spread to new foci.

In the present study we analysed the development of the geographical distribution and the number of TBE cases in Finland notified to the National Infectious Diseases Register (NIDR) in the period from 1995 to 2013. In order to further support public health measures, we also describe the number of laboratory-confirmed TBE cases and of conducted laboratory tests as well as the associated trends for each of the 21 Finnish hospital districts, with a particular emphasis on the suspected risk areas. In addition, we used clinical serum samples from patients with neurological infection to investigate to which extent TBE cases and foci may have occurred unnoticed in Finland.

Methods

Tick-borne encephalitis diagnostics, case definition, and reporting

TBE is a notifiable disease in Finland according to the Communicable Disease Act and Decree. Two diagnostic laboratories located in Turku (University of Turku) and Helsinki (HUSLAB, Helsinki University Hospital), perform TBE diagnostics and report acute cases to the Finnish NIDR at the National Institute of Health and Welfare (NIHW). Laboratory-confirmed cases are registered by the hospital district of the treating hospital and dated by the first TBE IgM-positive clinical sample. Cases have been registered since 1995 with information on the place of residence and since 2007, detailed information on place and date of infection, date of first symptoms and clinical features have been collected from medical records and, when possible, in interviews.

The results submitted by the diagnostic laboratories to the Finnish NIDR are reviewed annually by specialists representing NIHW and the reporting laboratories using medical reports of the patients. During the study period (since 2007), this evaluation usually took place during the first months of the following year. Only cases that fulfilled the criteria for acute TBE (described below) are considered as cases and left in the register. In this study, we compared the data in the NIDR before and after the annual evaluation.

In Finland, the case definition of acute TBE is based on disease-specific medical history and clinical findings, absence of previous exposure to other flaviviruses, and detection of TBEV-specific IgG and IgM antibodies in serum and/or CSF. The criteria are consistent with the case definition from the European Centre for Disease Prevention and Control (ECDC) [21], but in addition, also cases with a mild clinical picture, without inflammation in the central nervous system (CNS), are registered. Only the classification 'confirmed' is used in Finland.

The TBEV IgM and IgG tests used In Turku are enzyme immunoassays (EIA) (Virion/Serion FSME virus/TBE, Wuerzburg, Germany). In HUSLAB, TBEV IgM is tested by an in-house μ-capture IgM EIA and confirmed by a haemagglutination inhibition (HI) test (described below). In the present study, we used the laboratory-confirmed

data, the re-evaluated data from the NIDR and the data from the interviews conducted by the NIHW.

Screening for antibodies against tick-borne encephalitis virus in sera from patients with central nervous system infection

Clinical serum samples, collected throughout Finland from patients with a CNS infection with suspected viral origin, were screened to identify previously undiagnosed TBE cases (Research permit §32 HUSLAB, 2013). Convenience sampling representing the season of tick activity (i.e. daily mean temperature ≥ 5°C, in southern Finland this is late April to end of October, in northern areas a shorter period) was conducted in a sampling frame of serum specimens from patients with a suspected neurological infection, retrieved in 1997, 2005–07 and 2011–12 and representing different geographical areas in Finland.

The samples had at the time of collection been sent to HUSLAB for screening of IgG and IgM antibodies against a panel of agents potentially causing CNS infection, including human herpesvirus 6, herpes simplex virus 1 and 2, varicella zoster virus and *Mycoplasma pneumoniae*, but not TBEV. We excluded samples without diagnostic finding for the agents listed above as well as samples from patients who already had a diagnosis of TBE following an independent request to study anti-TBEV antibodies. Altogether 1,957 specimens were selected for this investigation.

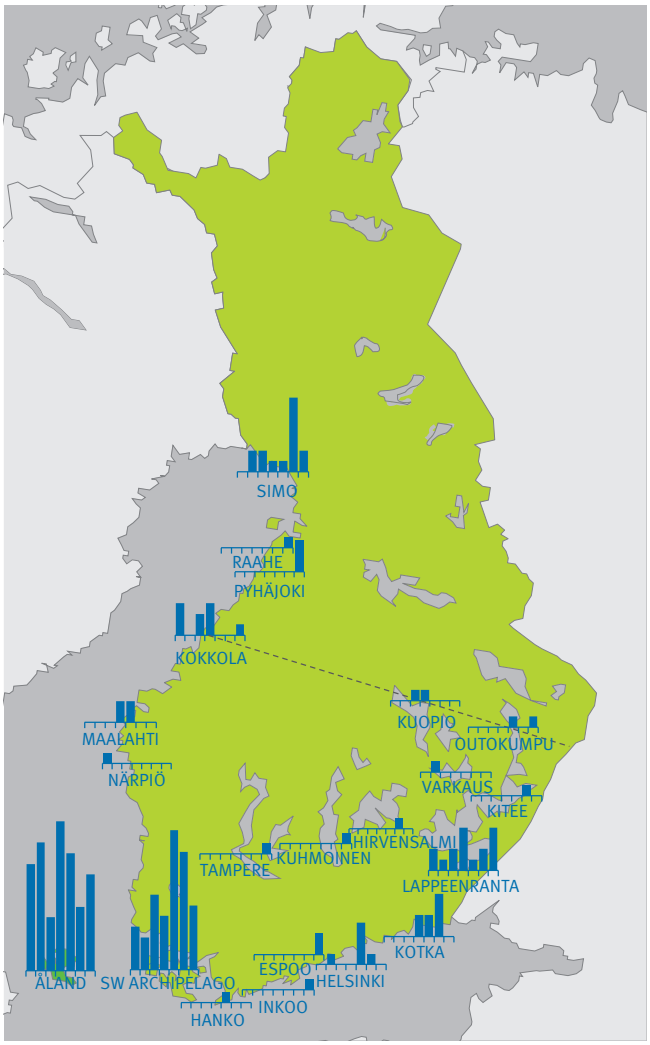
The samples from 2005 to 2007 and 2011 to 2012 were screened with a modified µ-capture IgM EIA decribed earlier [22], except than an anti-FSME monoclonal antibody (Mab) [23] and a peroxidase-conjugated donkey anti-mouse IgG antibody (Jackson immunoresearch, West Grove, United States) were used in place of a peroxidase-conjugated anti-TBEV-Mab. The specificity of positive IgM reactions was further confirmed with an in-house HI test [24]. These methods are in routine use in the diagnostic laboratory at present. Samples from 1997 were screened with an IgM-EIA (Progen Biotechnik GmbH, Heidelberg, Germany) and with an in-house HI test [24], the methods in routine use in the diagnostic laboratory at that time.

Data from diagnostic laboratories conducting tick-borne encephalitis virus serology in Finland 2007 to 2013

We surveyed the databases of the two diagnostic laboratories conducting TBEV serology in Finland, the University of Turku and HUSLAB, including the referral information and TBE diagnostic test results (ethical permit THL/402/5.05.00/2014). Each patient was included only once in the data analysis. The patients were grouped by hospital district. Information on sex and age was only available for patients tested in HUSLAB, which represented 57% of all diagnostic data. Each municipality or private healthcare provider in a hospital district can choose the laboratory to which they send the specimens for TBEV antibody testing.

Figure 2

Cases of tick-borne encephalitis, by geographical place of infection, Finland, 2007–13 (n = 192)



All 192 cases with adequate information of the place of infection in Finland are included in the figure. Data of some geographically continuous areas were combined as follows: Simo: Simo and Kemi; Helsinki: Helsinki and Sipoo archipelago; Lappeenranta: Lappeenranta, Imatra, Lemi and Joutseno; Kotka: Kotka and Hamina archipelagos; Kokkola: Kokkola and Kannus. The south-western (SW) archipelago extends to Kemiö in the south-east, includes the archipelago of Uusikaupunki in the north and excludes Åland in the west. The x axis of the graphs represents the date in years from 2007 to 2013. The y axis represents the cases (min=1 case, e.g. Tampere, Kuhmoinen and Inkoo 2013; max=14 cases, e.g. Åland 2010)

The northern limit of distribution of Ixodes ricinus in the 1960s is marked with a dashed line.

The proportion of specimens sent to HUSLAB or to the University of Turku by each hospital district varied (the proportion sent to HUSLAB varied between 2.4% and 100%).

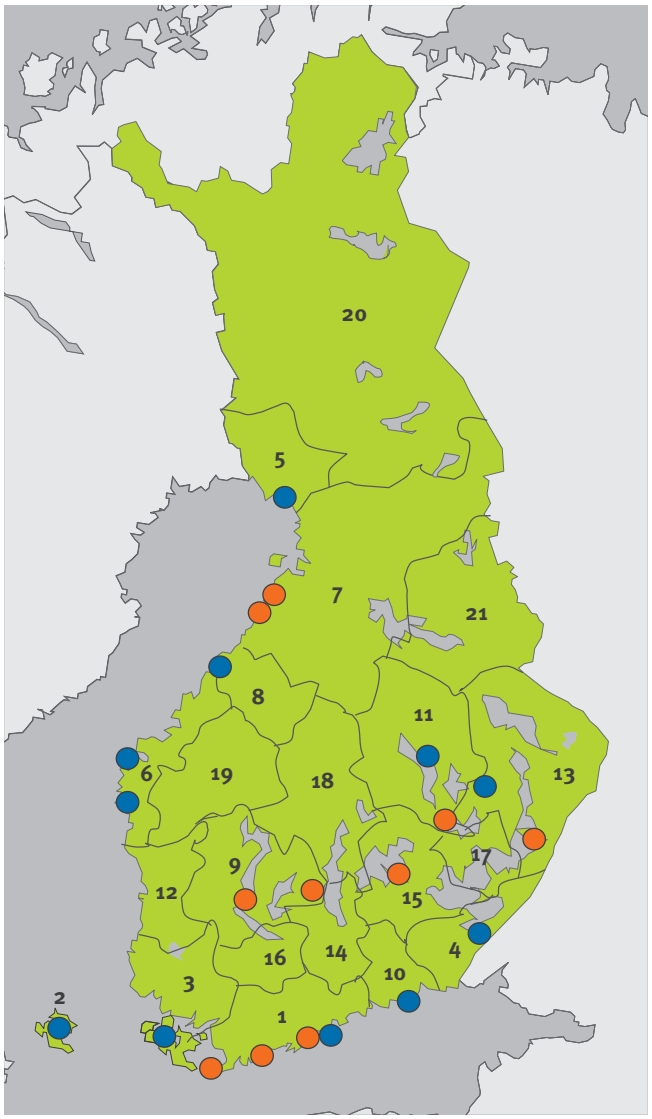
Results

Descriptive analysis of notified tick-borne encephalitis cases in Finland in 1995 to 2013

Altogether 478 acute TBE cases were reported to the NIDR in the period from 1995 to 2013 (annual average: 25 cases; range: 5–43) (Figure 1). Of these, 196 were

Figure 3

Hospital districts with continuous or sporadic occurrence of tick-borne encephalitis, Finland, 2007–13 (n = 192)



Blue circles: districts with continuous occurrence of human cases; red circles: places with sporadic cases. Sporadic was defined as only one case registered in a single year or as occurrence of the case/cases during the last year of the study. Cases are shown by geographical site of infection.

Numbers represent hospital districts as listed in Table 1.

reported from the Åland islands, a highly endemic area in south-western Finland. After 2006, when the national immunisation programme started providing vaccination for the population of Åland (n = 28,666 in 2013 [25]), the majority of infections occurred on the Finnish mainland (i.e. areas other than Åland) (Figure 1), however, the incidence still remained highest on Åland.

Geographical distribution

Adequate data on the place of infection was available for 208 (92%) of the 226 cases reported between 2007 and 2013 (Figure 2). Of the total 226 cases, 74 (33%) were female; the median age was 48 years for men (range: 1–85) and 50 years for women (range: 4–81). In comparison, the median age for men in 2013 in the whole population was 39 years and for women 43 years according to Statistics Finland (personal communication Niina Haataja, June 2014). The median age of 3,190 patients tested for suspected acute TBEV infection was 43 years (range: 0–91), of whom 50% were female.

Of the 208 TBEV cases for whom detailed information was available, the infection site of 192 cases was in Finland (Figure 2).

Figure 2 shows recent, earlier unreported areas with human TBE cases in Finland, namely Raahe, Pyhäjoki, Maalahti, Kuopio, Outokumpu, Varkaus, Kitee, Hirvensalmi, Kuhmoinen, Kotka, Tampere and Espoo. In many of these areas, infections have remained sporadic (Figure 3), however, some of these areas emerged only in the last year of the study and the development cannot be assessed yet.

Of the cases with the infection site in Finland, 129 (67%) acquired the infection in their place of residence or in a neighbouring municipality. In the highly endemic areas of Åland and the south-western archipelago, respectively two thirds (46/67) and half of the cases (25/49) were local residents. In the Simo-Kemi region, 13 of 16 infections were acquired by local residents, whereas the proportion was four of eight in Kotka, archipelago seven of nine in Kokkola and 15 of 16 in Lappeenranta. Infections among local residents predominated also in the rest of the endemic areas. Altogether 16 cases were acquired abroad, mainly in countries neighbouring Finland: 10 persons acquired their infection in Estonia, three in Sweden, and one each in Russia, Austria and Switzerland.

Seasonality

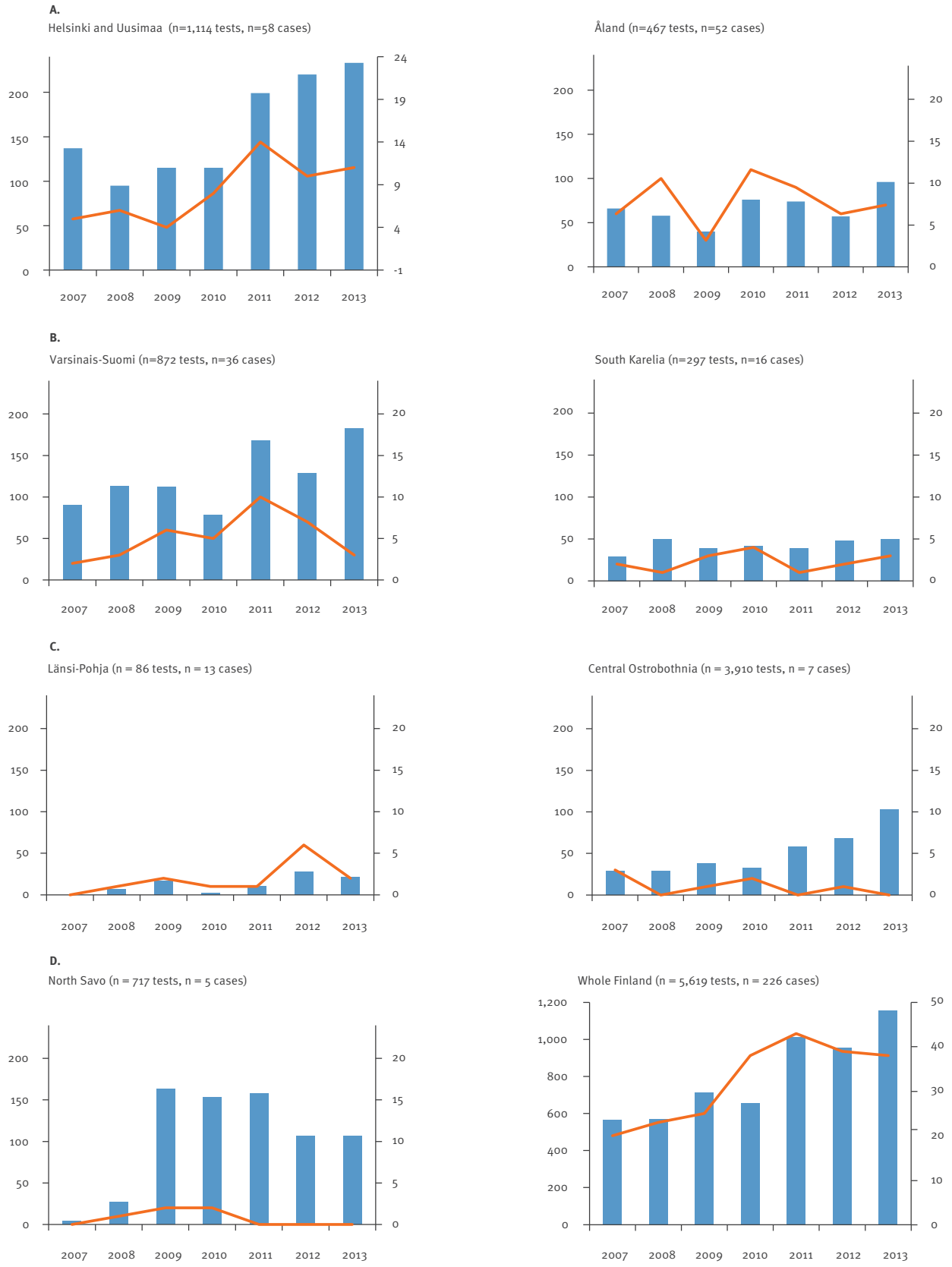
Acute TBE cases were reported from 20 April through 19 November. At both the start and the ends of this period, cases were detected in the south-western region. In Åland, 37 of 65 cases were reported evenly distributed from June to August, while 17 of the 65 cases were observed in September. In the south-western archipelago, there was a small peak in the number of cases in July and August, corresponding to the holiday season. Note that cases among residents of Åland were registered by the date of the first TBEV-positive sample, while the cases from other parts of Finland were reported by the first date of symptoms. In Åland and the south-western archipelago, infections in late autumn were particularly common in 2010, 2011 and 2013. In the south-eastern endemic area of Lappeenranta, 16 cases were reported from May to October, nine of them in August and September, while in the northern parts of the country, most cases were reported in June, with only one case in August and none later.

Diagnosis

The average time from first symptoms to test-positive laboratory specimen was 13.4 days (range: 0–68,

Figure 4

Diagnostic laboratory tests for the detection of acute tick-borne encephalitis (n = 5,619) vs diagnosed cases (n = 226), Finland 2007–13



Columns: number of tests performed are presented in columns (scale on left). Line graphs: cases reported in the National Infectious Diseases Register by the hospital district of the treating unit (scale on right).

The areas in Finland with highest case numbers are shown. North-Savo is included as the area with the largest ratio of tests conducted vs cases detected.

Table 1

Diagnostic tests for the detection of acute tick-borne encephalitis, by hospital districts, Finland, 2007–13 (n = 5,619)

Hospital district		Patients tested	Tests/100,000 inhabitants [a]	Cases[b]	Positive tests %	Cases by site of infection[c]	Incidence/100,000 average 1995–2014
1	Helsinki and Uusimaa	1,114	70	58	5.2	10	0.55
2	Åland	467	1,629	52	11.1	67	39.47
3	Varsinais-Suomi	872	184	36	4.1	49	0.85
4	South Karelia	297	225	16	5.4	16	1.46
5	Länsi-Pohja	86	134	13	15.1	15	1.24
6	Vaasa	471	279	10	2.1	5	0.38
7	Northern Ostrobothnia	139	34	10	7.2	4	0.17
8	Central Ostrobothnia	357	456	7	2.0	9	1.22
9	Pirkanmaa	175	34	4	2.3	2	0.14
10	Kymenlaakso	72	41	4	5.6	8	0.20
11	North Savo	721	290	4	0.6	3	0.23
12	Satakunta	167	74	2	1.2	0	0.18
13	North Karelia	84	50	3	3.6	3	0.12
14	Päijät-Häme	96	45	1	1.0	0	0.03
15	South Savo	36	34	1	2.8	1	0.15
16	Kanta-Häme	42	24	1	2.4	0	0.09
17	East Savo	37	83	1	2.7	0	0.17
18	Central Finland	42	17	1	2.4	0	0.04
19	Southern Ostrobothnia	17	9	1	5.9	0	0.03
20	Lapland	59	45	0	0.0	0	0.04
21	Kainuu	4	5	0	0.0	0	0
Private laboratories [d]		264	NA	NA	NA	NA	NA
All		**5,619**	**102**	**255**	**4.5**	**192**	**0.53**

NA: not applicable; NIDR: National Infectious Diseases Register; NIHW: National Institute of Health and Welfare; TBE: tick-borne encephalitis.

Incidence data and the cases were provided by the NIHW in the NIDR [41]. Hospital districts are listed by case numbers reported in the NIDR in descending order. For a map of the hospital districts see Figure 3.

[a] Number of inhabitants in the hospital district in 2013.

[b] TBE cases reported by the NIHW in the NIDR in 2007 to 2013, by hospital district of the treating unit.

[c] TBE infections in 2007 to 2013 that occurred in the geographical area of the hospital district.

[d] The sending unit is not known for the private laboratories, however, the positive cases are included in the register of NIHW by area of the laboratory. In our analysis, we only analysed the frequency of the positive samples in this set of samples.

median: 13). From 2007 to 2013, 106 TBE patients (68%) had noticed a tick bite before developing clinical TBE (75% of the 208 interviewed patients provided this information). The number of laboratory tests conducted in each hospital district varied between five and 1,629 tests conducted per 100,000 inhabitants in total in the period from 2007 to 2013 (Figure 4, Table 1). Åland had the largest number of cases (n = 67, Åland as geographical site of infection) and the largest number of tests conducted per 100,000 inhabitants (1,629/100,000). Of the five hospital districts with most cases (Åland, Varsinais-Suomi, South Karelia, Länsi-Pohja, and Helsinki and Uusimaa), the smallest ratio of tests conducted vs cases diagnosed 2007 to 2013 was seen in Varsinais-Suomi and Länsi-Pohja (Figure 4, Table 1). Of the areas with sporadic cases (between one and 10 cases altogether in the study period), the largest ratio of tests conducted vs cases detected was seen in North Savo, Vaasa, and Central Ostrobothnia (Table 1).

During the study period, three patients died with acute TBE (1.3% of all reported cases): a person in their 60s with a neuroinvasive TBE, a person in their 70s with TBEV encephalitis and a person in their 80s with confirmed TBEV encephalitis. The latter patient had received only one dose of TBE vaccine three weeks before the infection.

Screening of clinical samples for antibodies to tick-borne encephalitis virus

Of the 1,957 clinical serum samples from patients who had an infection with CNS symptoms in summer or autumn of the studied years, but had not previously

Table 2

Patients with central nervous system infections with unknown aetiology, Finland, 1997–2012 (n = 1,957)

Year	Period of samples collection	Number of samples	New cases [a]	Hospital district [b]	Proportion %	All cases registered in that year (NIHW)
1997	1 August–31 October	383	1	Helsinki and Uusimaa	0.26	19
2005	15 May–31 August	409	0	No cases	0.00	16
2006	16 May–31 August	250	1	Åland	0.40	18
2007	20 June–23 August	221	0	No cases	0.00	20
2011	1 June–31 October	374	3	Helsinki and Uusimaa (2 cases), Kymenlaakso (1 case)	0.80	43
2012	1 June–31 October	320	0	No cases	0.00	39

NIHW: National Institute of Health and Welfare; TBEV: tick-borne encephalitis virus.

[a] Patients with no previous TBEV diagnosis.

[b] Hospital districts as presented in Figure 3.

been tested positive for anti-TBEV antibodies, five (0.3%) were positive in both serological tests and fulfilled the diagnostic criteria of acute TBE (Table 2). Of these five cases, three were from the hospital district of Helsinki and Uusimaa, one from Åland and one from Kymenlaakso.

Tick-borne encephalitis surveillance system in Finland

Positive TBE test results, mainly positive TBEV IgM EIA results, are reported automatically to the NIDR as diagnostic TBE findings by the clinical laboratories. For the period 2007 to 2013, the case re-evaluation procedure at the NIHW resulted in a 12.4% decrease (range: 0–30.7 per year) in the annual number of TBE cases (data not shown). In most cases, this was because no IgG or HI seroconversion could be shown and the positive IgM test result was most probably unspecific.

Discussion

This study demonstrates that in the past eight years, human TBE cases have emerged in new areas in Finland. An increasing number of TBE cases have been reported particularly along the north-western coast and in the south-western archipelago. Case numbers in Finland peaked in the early 2000s, mainly because of a significant increase in TBE cases reported in Åland. The TBE incidence on Åland reached a peak in 2002 with 100 per 100,000 population. Awareness of the disease led to an increase in TBE immunisations and since 2006, the national vaccination programme has provided the vaccine for residents of Åland, where the vaccination coverage in 2012 was estimated at 70.7% [26,27]. While the number of TBE cases on Åland has been steadily decreasing, the overall incidence between 2007 and 2013 was still higher than in other parts of Finland. Furthermore, there have been indications of vaccine failure infections in Finland during the recent years (data not shown), as also reported in Sweden [28]. Vaccine failures have so far been studied systematically only on Åland. Age and number of vaccination doses have been reported as the most important factors influencing the immunological response to vaccination [29].

Case numbers have decreased considerably also in some of the smaller TBE foci in Finland that have been known for decades. These include Kokkola and the island of Isosaari in Helsinki, where our research group detected the Siberian and European TBEV subtypes, respectively, in rodents in 2008 and 2009, indicating that TBEV is still endemic, even if human cases are rare or absent [4]. We can assume that the observed decrease in case numbers was mainly due to increased awareness and subsequent immunisation. However, fluctuation of TBEV prevalence in ticks, annual fluctuations in host rodent and tick populations, virulence of the circulating virus strains [30], and the probability for tick co-feeding may also regulate the risk of acquiring TBEV infection.

TBEV is, at least currently, found in very local foci in Finland. Even if a municipality is recognised as an endemic area, the true risk for a TBEV infection is restricted to areas smaller than the municipality. Studied foci and the patient cases reported are concentrated, in addition to Åland, along the coastline of the Baltic Sea and of Lake Saimaa, the largest inland waterway in Finland. Typically the foci are surrounded by water and appear on islands or headlands. This may be due to the favourable microclimatic conditions, vegetation and a suitable structure of host mammal populations for TBEV and ticks. Also human exposure to ticks by the water is emphasised. Åland, the south-western archipelago and the Kotka archipelago are common holiday destinations and summerhouses, characteristic of the Finnish people, are usually located by water. In 2013, there were 498,700 summerhouses in Finland (population 5,451,270) [31]. In the south-western parts of the country, the highest density of summer houses overlaps with the areas where the TBE incidence peaks. The majority of the cases in Finland were recorded in the municipality of residence or in the neighbouring municipality. Still, even in those cases, the infections were often acquired at a summerhouse

located by the sea or a lake and only rarely at the permanent residence. The focal nature and the infection risk at the summerhouse should be considered when the immunisation policies are planned and the budget for the provided immunisations are allocated.

During the last two decades, TBE case numbers have been increasing in many European countries, especially in northern Europe and northern parts of Russia [1-3,33,34]. Climate change is one, not completely understood, factor in this development [2,35]. In Finland and the neighbouring countries Sweden and Russia, host tick species and TBEV endemic foci have moved to more northern latitudes. In addition, *I. ricinus* has become more abundant in Sweden [14,34,36], and *I. ricinus* is found at higher altitudes in the central European mountain area [37]. At the same time, it has been predicted that TBEV may become rare or that the seasonality of cases may change in the traditionally highly endemic areas in central Europe [3,38]. Besides climate, human behavioural factors such as land use, socioeconomic changes, increased awareness of the disease and changes in the healthcare system have an influence on reported TBE case numbers [3,39].

No recent systematic countrywide survey on the distribution of TBEV and the host *Ixodes* ticks is available in Finland, although areas with known human infections have been investigated. The geographical distribution of TBEV in Finland was last surveyed in the 1960s by screening of anti-TBEV antibodies in cattle sera collected throughout the country [40]. The northern limit of *I. ricinus* ticks was found at the latitude of Kokkola-Joensuu [40] (indicated in Figure 2). In a survey of 106 TBE patients between 1959 and 1987 the identified geographical sites of infection were Åland, the south-western region of Finland, Kokkola, the Helsinki region and north-eastern Finland [8]. In the present study, TBE cases were detected beyond these regions, particularly more to the north at the west coast (i.e. in the Raahe, Pyhäjoki and Simo-Kemi regions, 64°5' to 65°7' N). However, according to earlier unpublished results (personal communication: Markus Brummer-Korvenkontio, March 2015), anti-TBEV antibodies were already detected (by HI test and neutralisation) twice, two years apart, in a single cow in the 1960s in Tervola, a municipality next to Simo, suggesting that TBEV may have been endemic in the area already 50 years ago, even if no human cases were noted. The foci of Raahe and Pyhäjoki, as well as those in the inland lake region described here have not been reported previously.

I. persulcatus ticks are found on the north-west coast, overlapping, at least in Simo and in North Karelia, with emerging foci; at least in the Simo-Kemi region the ticks are carrying TBEV [13-16]. In south-western endemic areas, where *I. ricinus* is carrying TBEV, human cases were reported in the period between 20 April and 19 November. The course of TBEV infection suggests that tick exposure occurred a few days to three weeks earlier. In the northern regions, where *I. persulcatus* ticks are present, no autumnal cases were recorded. This may be due to different behaviour of the tick species or because the vegetation period is shorter in the northern parts of the country.

The number of samples sent to diagnostic laboratories for TBE testing in Finland increased by 105% during the study period. However, the number of samples sent for TBE diagnostic tests did not in all hospital districts correlate with the TBE incidence. Awareness of TBE in northern parts of the country has increased since the first cases reported in Simo in 2008, but the number of tests conducted is still moderate. The district of Helsinki and Uusimaa stood out with a high number of tested samples, even if only a few infections were acquired in this area. TBE cases connected to travel in Finland or abroad or to a summerhouse in Finland were characteristic for the capital region. The observed increase in the number of infections with Parainen (south-western archipelago) as the geographical place of infection is reflected in the number of cases registered in Helsinki and Uusimaa. The third highest number of diagnostic samples was from North Savo, although only three sporadic human cases are known from this region (in Kuopio and Varkaus). Comparing the number of tests conducted with the number of cases diagnosed, the ratio was particularly low for Varsinais-Suomi and Länsi-Pohja, suggesting potential lack of awareness among clinicians for TBE in these known endemic areas.

We screened 1,957 patients for anti-TBEV antibodies. Patients had CNS infection of suspected viral origin but no previous TBEV suspicion or diagnosis. Five of them (0.25%) were found positive. We conclude that undiagnosed TBE forms only a minor fraction of this particular patient group. In addition, it is evident that the TBEV surveillance system in Finland, i.e. reporting the cases by laboratory results even if the clinical case definition is not fulfilled, is not sufficiently specific to avoid false positive reporting. As the Finnish NIDR is publicly available online, registering TBE cases without full laboratory support (i.e. unspecific results) may raise unnecessary public concern in certain areas.

Conclusion

The overall TBE incidence in Finland is increasing and the virus is emerging in new geographical locations. Based on the present study, we have the following recommendations on TBE surveillance and prevention: (i) surveillance should be based on cases that fulfil the case definition at the time when the data are deposited and the data should be evaluated frequently; (ii) irrespective of the funding arrangements, vaccination programmes should be targeted to all people staying in TBE-endemic regions for several weeks between May and October, not only permanent residents; (iii) in certain areas with a higher risk of transmission, awareness among clinicians should be increased; (iv) geographical information of TBE foci should be more precise and up-to-date, and used to estimate the population at risk.

A comprehensive study of TBEV prevalence (TBEV RNA in ticks and antibodies in host mammals in Finland) and a survey of the distribution of the two tick species should be conducted.

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Conflict of interest

None declared.

Authors' contributions

ET, OV and TM designed and initiated the study. ET and TM collected the serum samples and performed and analysed the laboratory work. ET, SK, ST, and MK collected and analysed the data on laboratory confirmed TBE cases and ET, SK and TV the data on laboratory tests conducted. ET, SK, ST, TV, MK and OV wrote the manuscript.

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Research article

Presence of antibodies but no evidence for circulation of MERS-CoV in dromedaries on the Canary Islands, 2015

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In 2012, a new betacoronavirus, Middle East respiratory syndrome coronavirus (MERS-CoV), was identified in humans. Several studies confirmed dromedary camels to be a potential reservoir and a source for human infection. Camels located on the Canary Islands were included in those studies and ca 10% of them were positive for MERS-CoV-specific antibodies. However, these findings could not be correctly interpreted because epidemiological information was not provided. Thus, further investigations were necessary to clarify these results. A total of 170 camels were investigated in this survey, of which seven (4.1%) were seropositive by ELISA. Epidemiological information revealed that all seropositive camels had been imported from Africa 20 or more years prior. We conclude that seropositive camels had contact with MERS-CoV in Africa and that there is no shedding of the virus among camels or people around the farms on the Canary Islands. However, the presence of antibodies in the camel herds should be monitored.

Introduction

In 2012, a new betacoronavirus, Middle East respiratory syndrome coronavirus (MERS-CoV), was identified in patients suffering from severe respiratory disease in the Middle East. As of 11 September 2015, 1,545 cases of laboratory-confirmed MERS have been reported to the World Health Organization (WHO) [1].

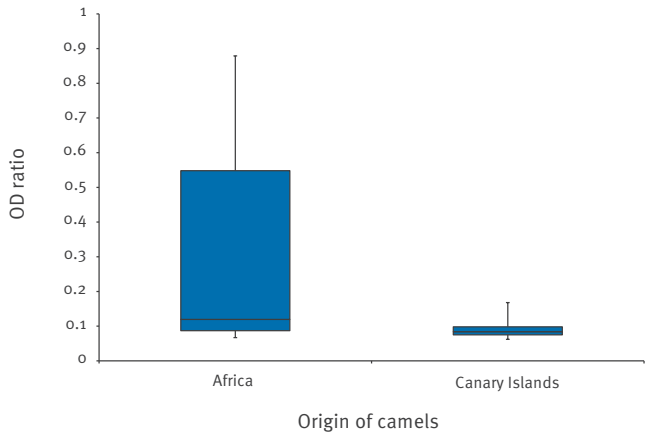
MERS-CoV has been shown to be phylogenetically related to bat coronaviruses [2,3] but the origin of MERS is still unclear. Dromedary camels were found to harbour specific antibodies against MERS-CoV, identifying them as the main potential reservoir, at least among the animal species investigated [4]. Comparison of genome sequences between viruses isolated from infected humans and camels indicated that there is transmission of MERS-CoV from camels to humans [5,6]. Moreover, in a nationwide serological survey evaluating 10,009 patients in Saudi Arabia, 15 were seropositive. Compared with the general population, seroprevalence was 15-fold higher in shepherds and 23-fold in slaughterhouse workers [7]. However, a recent study failed to find a correlation between exposure to infected camels and serology in humans, reporting that zoonotic transmission of this virus from dromedaries is rare [8]. MERS-CoV and related genetic material were identified in camels in countries in the Middle East and North Africa. The implications of these findings for management and control recommendations would need further investigation [9].

A serological survey investing camels as the likely source of zoonotic transmission of MERS-CoV included sera from dromedary camels from Oman as well as the Canary Islands to compare regions affected and not affected by MERS [4]. All of the Omani camels had antibodies to MERS-CoV. Surprisingly, also ca 10% of the Canary camels were seropositive, which confirmed that they must have had previous contact with MERS-CoV. However, from an epidemiological viewpoint, the significance of these findings was limited because the animals were not well identified. The sampled camels came from the same farm and epidemiological information was not provided. Nevertheless, the media impact was enormous and drastically affected the camel trade in the Archipelago. These findings could potentially have a serious impact on human and animal health on the Canaries and, given the continuous movement of people from and to the Islands (ca 14 million tourists in 2014), also by extension, in Spain and the rest of Europe. Thus, further investigations seemed to be needed to clarify the presence of antibodies in camels on the Canaries.

The purpose of this study was to investigate the presence of MERS-CoV in camels on the Canary Islands, evaluating the prevalence and the epidemiology of the disease in order to implement sanitary and/or control measures if necessary.

Figure

Anti-MERS-CoV ELISA (IgG) in camels, by their origin, on the Canary Islands, 2015 (n = 170)



OD: optical density.

Photometric measurements at 450 nm, Camels born in Africa (n = 17): min: 0.067; median: 0.1195; max: 0.879. Camels born on the Canaries (n = 153): min: 0.062; median: 0.084; max: 0.168.

Methods

Animals

We carried out a transversal study between January and February 2015 including 170 dromedary camels from the four islands (Gran Canaria, Lanzarote, Fuerteventura and Tenerife) on which camels are kept. Per island, we selected 40 to 45 camels from at least two different farms. Sampling size was calculated as a minimum of 138 individuals, based on the 95% confidence level, 5% of desired absolute precision and 10% of expected prevalence [10]. With a current census of ca 1,500 camels, the sampling size of 170 individuals was ca 11% of the total camel population of the Islands.

Information taken at farm level was the following: island, farm location, owner, animal identification, place of birth (Canary Islands vs Africa), age and sex. We selected camels with a placid temperament in order to avoid unnecessary suffering of the animals or possible accidents of the handling personnel. Camels showing clinical signs of any disease or without official identification were excluded from this study.

Serological analysis

Blood sera were analysed using the anti-MERS-CoV ELISA Camel (IgG) manufactured by EUROIMMUN AG (Lübeck, Germany) to detect specific antibodies. This test is based on the S1 antigen of MERS-CoV and has successfully been used by other authors evaluating MERS-CoV in camels [11].

Statistical analysis

Data were entered in a database and statistically processed using Epi info version 3.03l, an open source epidemiological statistics software for public health (http://www.openepi.com/Menu/OE_Menu.htm). Two-by-two tables were used to evaluate the association between a possible risk factor (exposure to the virus) and the presence of circulating antibodies. Chi-square and exact measures of association tests were also used.

Results

Of 170 camels analysed, 17 originated from Africa and 153 were born on the Canaries; 101 were male and 69 female, and the mean age was 14 years, ranging between 2 and 26 years. Seven camels (4.1%) were seropositive, with photometric measurements ranging from 0.378 to 0.879 (Figure). These camels belonged to three different farms located on three different islands. Positive samples were re-assessed using the same ELISA and all showed positive reactions again. Epidemiological information revealed that all seropositive camels originated from Africa, all were female and between 20 and 26 years-old. In contrast, 10 other camels born in Africa and all camels born on the Canaries (n = 153) were negative. The likelihood to have had contact with the virus was 0.41 for camels born in North-West Africa, while it was 0 for those born on the Canaries. Chi-square and other measures of association between camels born in Africa or on the Canary Islands and presence or absence of antibodies were statistically significant ($p<0.0000001$).

Farmers and the veterinarians in charge were interviewed about the medical records of the seropositive camels. They informed that clinical signs related to MERS such as respiratory distress or nasal or ocular discharges had not been observed in these animals.

Discussion

This study intended to further assess the presence of specific antibodies against MERS-CoV in camels on the Canary Islands, as previously reported [4]. The main purpose of this work was to assess the whole camel population using a significant sampling size and to interpret the results according to the epidemiological information.

Firstly, the efficacy of the serological test used in this survey needs to be considered. The manufacturer's information indicates that sensitivity and specificity are almost 100%, although there may be cross-reaction with some coronaviruses affecting ruminants and camels and therefore possible false positive results. Indeed, many coronaviruses affect livestock, particularly ruminants [12] and camels [13] worldwide. Thus, immunogenic proteins of closely related coronaviruses that share common structural or linear epitopes can elicit cross-reactive and cross-neutralising antibodies in the host [14]. In fact, in the previous study about MERS on the Canaries, camel sera reacted with human coronavirus OC43 and bovine coronavirus antigen [4]. Nevertheless, none of the camels born on the Canaries, including some young animals, showed circulating anti-MERS-CoV antibodies. Other CoV are common in young animals, causing infections and peaks of

antibodies: Therefore the fact that we did not find antibodies in these animals would indicate high sensitivity and specificity of the kit detecting specific anti-MERS-CoV antibodies based on S1 antigen.

All seven seropositive camels had been introduced from the near North-West African coast. None of them had shown any clinical signs related to MERS infection, but we know that infected camels do not usually show any signs of infection [1]. Serological evidence of the circulation of MERS-CoV among dromedary camels was found in African countries like Egypt (81.4%), Sudan (86.7%), Somalia (83.7%) [11], Kenya (9.8% in farm animals, 57.9% in nomadic animals) [15], Nigeria (94%), Ethiopia (97%) or Tunisia (54%) [16], the latter in the north of the continent and closer to the Canary Islands. All 17 camels imported to the Canaries came from the Western Sahara, a region not previously studied for MERS-CoV; 10 of them did not have antibodies in our study. Mean values obtained for the camels born in Africa vs those born on the Canaries showed statistically significant differences (ANOVA, $p<0.005$). The photometric values in the positive camels from North-West Africa (mean: 0.302) can be considered low compared with those obtained from Middle East countries in which MERS-CoV is actively circulating (ca 2.500–3.000, EUROIMMUN database (data not shown). These findings indicate that MERS-CoV is not circulating among camels on the Canary Islands. The fact that no camel born on the Canaries was seropositive indicates that there is no shedding of the virus among the camels nor, by extension, among the people working in close contact with these animals (personnel, farmers, veterinarians) or tourists who have occasional contact riding camels. No human cases of MERS have occurred on the Canaries.

Given that the last import of live camels from Africa was authorised in 1995, we can assume that the animals originating from Africa have not had any contact with the virus for more than 20 years. This could be indicative of MERS-CoV being a highly immunogenic virus in camels, generating and maintaining a specific immune response (IgG) for at least 20 years. Studies carried out in camels in Somalia and Sudan using archived serum samples accumulated during the past 30 years detected seropositive animals by neutralisation assay, suggesting long-term virus circulation in these animals [11], which was also confirmed in Kenya [15]. MERS-CoV seems to have been present in North-West Africa before 1995 and it MERS-CoV-specific antibodies may not be detectable in Canary camels in the future.

In conclusion, we propose that MERS-CoV does not present a threat for humans or animals on the Canary Islands. However, surveillance measures should be taken to monitor the presence of antibodies in the camel herds. Further studies including more animals and herds not only on the Canary Islands would be needed to evaluate the whole camel population, which is not large, and the focus should be on the imported camels.

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Conflict of interest

EL and SR are employees of EUROIMMUN AG, the company producing the anti-MERS ELISA used in this paper.

Authors´ contributions

CG: Designing the survey, providing samples, processing samples, drafting the manuscript; MT T-J: Designing the survey, processing samples, performing ELISA tests, drafting the manuscript; MG: Designing the survey, performing ELISA tests; EL: Designing the survey, drafting the manuscript; SR: Designing the survey, critical review of the manuscript.

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