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Targeted vaccination of teenagers following continued rapid endemic expansion of a single meningococcal group W clone (sequence type 11 clonal complex), United Kingdom 2015

H Campbell (helen.campbell@phe.gov.uk)[1], V Saliba[1], R Borrow[2], M Ramsay[1], S N Ladhani[1]
1. Public Health England, Immunisation Department, London, United Kingdom
2. Public Health England, Meningococcal Reference Unit, Manchester Royal Infirmary, Manchester, United Kingdom

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Since the epidemiological year 2009/10, the United Kingdom has experienced a year-on-year increase in meningococcal group W (MenW) disease due to rapid expansion of a single endemic hyper-virulent strain belonging to sequence type 11 clonal complex (cc). This strain was identified among cases diagnosed across all regions and was not linked to travel abroad. Consequently, an adolescent MenACWY conjugate vaccination programme for 13-18 year-olds will be introduced in August 2015, with priority given to 17-18 year-olds (school leavers).

Here we describe the epidemiology of invasive meningococcal disease and the emergence of a single meningococcal group W (MenW) clone in England in the last six years and the resulting public health measures.

Background

Neisseria meningitidis remains a major cause of meningitis and septicaemia worldwide [1]. While incidence varies by individual country and by age group, meningococcal group B (MenB) has been responsible for the majority of cases of invasive meningococcal disease (IMD) over the past decade in England (84%) like in most European countries, where 74% of all IMD is due to MenB [2]. In England, meningococcal group C (MenC) disease has been virtually eliminated following the introduction of the MenC conjugate vaccine into the national immunisation programme in 1999 [3].

Historically, MenW has been a rare cause of IMD in England, accounting for<5% of all laboratory-confirmed cases [4,5]. An outbreak of invasive MenW disease following the Hajj (pilgrimage to Mecca) in the early 2000s was soon controlled following mandatory MenACWY vaccination for all pilgrims [6]. In the 2008/09 epidemiological year (running from 1 July to 30 June the following year), MenW was responsible for only 19 of 1,109 (1.7%) cases in England. Since then, however, MenW cases started to increase, with cases nearly doubling annually in recent years (Figure 1).

By end May 2015 170 cases have already been confirmed for the epidemiological year 2014/15, compared with 88 and 46 cases for the same period in 2013/14 and 2012/13, respectively. MenW is responsible for 25% of all IMD cases in England in 2014/15 to date, compared with 15% in 2013/14 and 7% in 2012/13. This increase has been accentuated by the concomitant decrease in MenB disease, which has been declining since 2000/01 [4,5].

In England, the initial increase in MenW cases was seen in older adults, but soon extended across all age groups, especially adolescents (15–19 year-olds) and infants (<1 year-olds) (Figure 2).

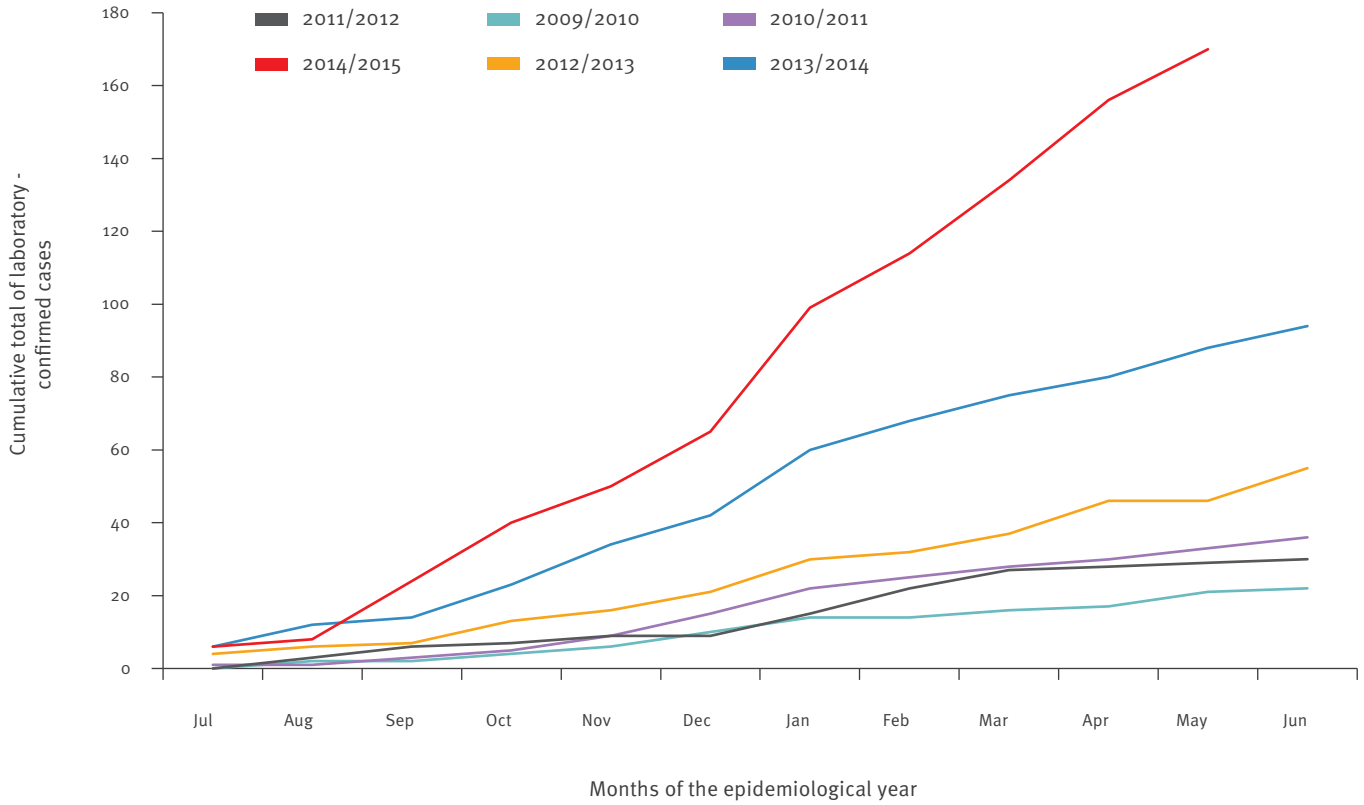
Of the 26 MenW cases among 18–24 year-olds in 2014/15, 16 were attending higher education settings at the time. More recently, there has been a notable rise in cases among pre-school children (1–4 year-olds) from three to seven cases annually during 2009/10–2013/14 to 18 by the end of May in 2014/15. The contribution of MenW to total IMD cases varied by age group, ranging from 49% in ≥65 year-olds, to 32% in 15–19 year-olds and 15% in<5 year-olds. This compares with 11%, 2% and 1% for the same age groups in England and Wales during 2007–11 [4].

Clinical follow up of laboratory-confirmed meningococcal group W cases

Clinical follow-up of 129 MenW cases diagnosed during 2010/11 to 2012/13 revealed that most MenW cases, especially children and young adults, were previously healthy (n=105; 81%), and had not travelled abroad before illness, indicating that this strain is endemic and already established in carriage [5]. Half the MenW cases presented with septicaemia (49%), followed by

Figure 1

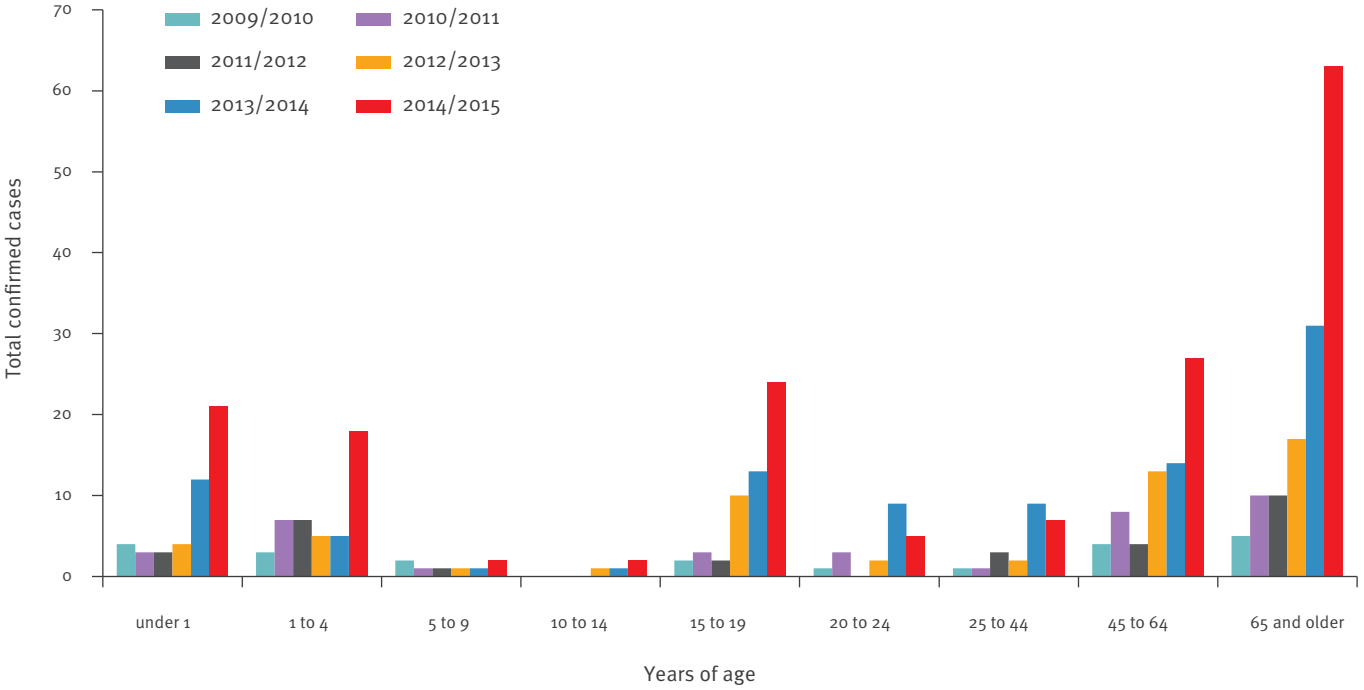
Cumulative number of laboratory-confirmed cases of invasive meningococcal group W (MenW) disease by epidemiological year, England, 2009/10–2014/15 (n=407)



Data for the most recent epidemiological year (2014/15) are complete until end May 2015.

Figure 2

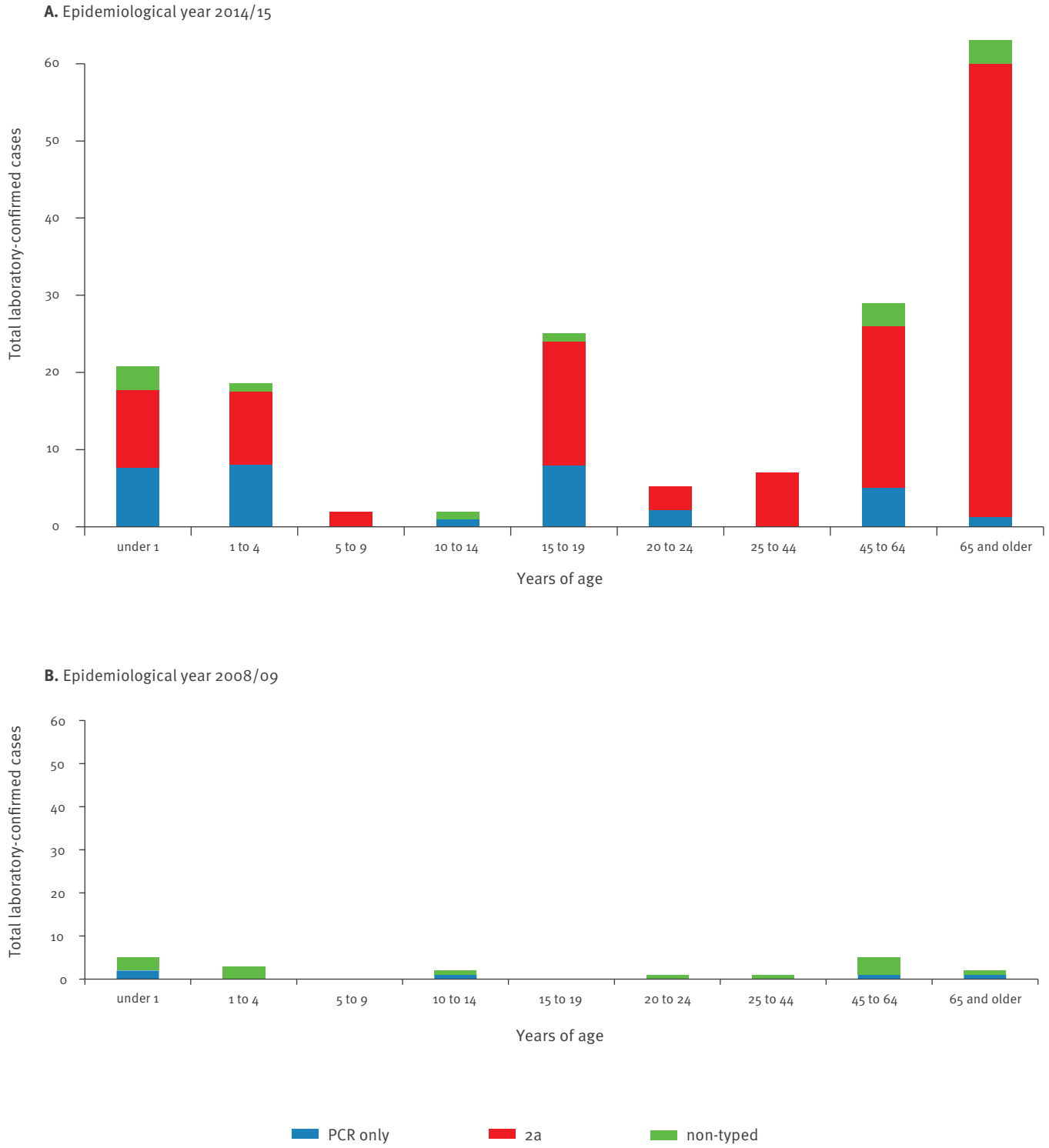
Age distribution of laboratory-confirmed cases of invasive meningococcal group W (MenW) disease by epidemiological year, England, 2009/10–2014/15 (n=407)



Data for the most recent epidemiological year (2014/15) are complete until end May 2015.

Figure 3

Phenotypic characterisation of invasive clinical isolates of meningococcal group W (MenW) during A. Epidemiological year 2014/15 (n=170) and B. Epidemiological year 2008/09 (n=19) in England



Data for the most recent epidemiological year (2014/15) are complete until end May 2015.
PCR: polymerase-chain reaction.

meningitis (12%) or both (16%), while a quarter had atypical presentations such as pneumonia (12%), septic arthritis (7%) and epiglottitis/supraglottitis (4%). In 2014/15, the MenW case fatality ratio was 12% (21/170 cases), consistent with previous years, and significantly higher than that reported for MenB [4]. Half the documented deaths were among ≥ 65 year-olds and older (11 deaths), with six among 45–64 year-olds and two each among 18–30 years and < 5 year-olds.

Characterisation of clinical meningococcal group W isolates

MenW isolates from IMD cases have been phenotypically characterised as expressing PorB serotype 2a, a surrogate marker for MenW:cc11 (Figure 3), and confirmed as belonging to cc11 by whole genome sequencing [5].

The increase in PCR-confirmed MenW cases is also most likely due to emergence of MenW: cc11. Comparison of whole genome sequences with a range of other strains belonging to cc11 has revealed the emerging MenW strain to be very similar to the one responsible for current South American outbreaks, [7,8] which have been associated with very high case fatality rates [9].

Public health action

Because of the continuing rapid increase in MenW disease, the UK Joint Committee on Vaccination and Immunisation (JCVI) recommended vaccinating adolescents against MenACWY as a national emergency outbreak response to provide both direct and indirect (herd) protection [10]. This recommendation was accepted by the UK Departments of Health which, on 21 June 2015, announced the rapid introduction of an adolescent MenACWY conjugate vaccine programme to begin in August 2015. The programme will target 13–18 year-olds, the age group at increased risk of IMD and with the highest meningococcal carriage rates [11,12]. Adolescents aged 17–18 years (current school year 13) will be the first group to be offered the vaccine, from August 2015, in order to prioritise those leaving school and entering higher education in the next academic year. A time-limited Freshers' programme offering the MenACWY conjugate vaccine to unvaccinated university entrants up to 25 years of age will also be in place. The adolescent MenC conjugate vaccine currently recommended for 13–14 year-olds will be replaced with the MenACWY conjugate vaccine. Over the next two years, all remaining adolescents in the 13–18 year age groups will be offered MenACWY conjugate vaccine. In addition to providing adolescents with direct protection against these capsular groups, it is expected that, by reducing carriage in individuals with high meningococcal carriage rates [12], all other age groups will be protected indirectly over the coming years. Full details of the adolescent MenACWY vaccination programme can be found on the PHE website [13].

Implications for other countries

The rapid introduction of a MenACWY conjugate vaccination programme in the UK will provide direct protection for adolescents at the time when they are most vulnerable to IMD and, is expected consequently, to contribute to indirect (herd) protection by interrupting transmission through carriage prevention. So far, no other European country has reported an endemic increase in invasive MenW disease. In France, six epidemiologically- and geographically-unlinked MenW cases were diagnosed in the first three months of 2012 [14]. Unlike the situation in the UK, all cases were associated with recent travel by the patient or patient contacts to sub-Saharan Africa, where large multinational outbreaks of MenW:cc11 were occurring at the time. Given how quickly this hypervirulent MenW:cc11 clone has established itself in populations across different continents [8], European countries should remain highly vigilant and be prepared to control this aggressive but vaccine-preventable infection.

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Conflict of interest

The Public Health England (PHE) Immunisation Department has provided vaccine manufactures with post-marketing surveillance reports which the marketing authorisation holders are required to submit to the UK licensing authority in compliance with their risk management strategy. A cost recovery charge is made for these reports. RB performs contract research on behalf of PHE for GSK, Novartis, Pfizer, Sanofi Pasteur and Sanofi Pasteur MSD.

Authors' contributions

The Immunisation Department (HC, VS, MR, SL) and the Meningococcal Reference Unit (RB) at Public Health England conduct enhanced national surveillance of meningococcal disease in England. All authors have contributed equally to writing and commenting on the draft manuscript. All authors have seen and approved the final manuscript.

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Evaluation of immunochromatography tests for detection of novel GII.17 norovirus in stool samples

P Khamrin1,2,3, A Thongprachum2,3, S Takanashi2,3, S Okitsu2,3, N Maneekarn1, S Hayakawa3, H Ushijima (ushijima-hiroshi@jcom.home.ne.jp)2,3

1. Department of Microbiology, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand
2. Department of Developmental Medical Sciences, School of International Health, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan
3. Division of Microbiology, Department of Pathology and Microbiology, Nihon University School of Medicine, Tokyo, Japan

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A novel GII.17 norovirus has emerged as a major cause of epidemic and endemic acute gastroenteritis in several countries in Asia. We used a small panel of stool samples in which GII.17 virus had been quantified by real-time RT-PCR to evaluate four commercially available norovirus immunochromatography (IC) kits. At least 10^8 copies/mL of GII.17 virus were required by each IC kit for a positive result, which is 1,000-fold more than that reported for these assays for GII.4 viruses.

In the winter of 2014–15, a novel GII.17 norovirus variant emerged in several countries in Asia [1]. From September 2014 to March 2015, 70% of all outbreaks in Guangdong and Jiangsu provinces in China were caused by a novel GII.17 virus [2,3]. A similar increase in the number of infections with this novel GII.17 virus has been reported in Japan [4] and Thailand since December 2014. In this study, we assessed whether current immunochromatography (IC) tests can detect these novel GII.17 noroviruses.

Laboratory investigation

For the rapid detection of norovirus, an IC test is one of the most convenient and accessible diagnostic tools commonly used in primary care units and private clinics in Japan [5]. However, these IC tests were developed mainly for the detection of genotypes such as GII.3 and GII.4. To evaluate if these IC tests are able to detect these novel GII.17 noroviruses, we tested four commercial IC kits available in Japan: GE test Noro Nissui, Nissui Pharmaceutical Co., Ltd.; ImmunoCatch-Noro, Eiken Chemical Co., Ltd.; Quick Navi-Noro 2, Denka Seiken Co., Ltd.; Quick Chaser-Noro, Mizuho Medy Co., Ltd.

A panel of six GII.17-positive stool samples from patients in Japan (n = 5) and Thailand (n = 1), in which the virus copy numbers had been quantified by real-time RT-PCR (reference values), were randomly selected from stool samples for which there was a large quantity available. Two of the six GII.17 stool samples tested positive by all four IC kits (Table).

Testing of the six specimens by real-time RT-PCR demonstrated that the two samples that were positive by IC test contained high virus titres (1.90 x 10^9 and 8.06 x 10^9 virus copies/mL). In contrast, the other four specimens that were negative in the IC test had virus titres ranging from 4.91 x 10^3 to 2.50 x 10^8 virus copies/mL. One of the two specimens positive in the IC tests (HU-2015) was re-tested using 1:10 and 1:100 stool dilutions, using three of the four kits (one was not available at the time of re-testing). The results demonstrated that at 1:10 dilution (virus titre 1.90 x 10^8 copies/mL) two of three tests still showed the positive results, with a weak positive band, while at 1:100 dilution (virus titre 1.90 x 10^7 copies/mL), all three IC tests were negative. These data demonstrated that the sensitivity of the IC kits for the detection of this novel GII.17 virus was about 10^8 copies/mL.

Discussion

Norovirus is one of the most common etiological agents of acute gastroenteritis in people of all ages in developing and developed countries [6]. The virus is transmitted mainly via food and water and by person-to-person. On the basis of sequence differences in the virus VP1 region, noroviruses can be divided into seven genogroups (GI to GVII): viruses from GI, GII and GIV cause disease in humans. GI is further divided into nine genotypes (GI.1 to GI.9) while GII contains at least 22 genotypes (GII.1 to GII.22) [7]. Of all genotypes,

Table

Virus copy numbers of six GII.17 noroviruses tested and results of four norovirus immunochromatography tests

Norovirus GII.17 sample ID	Date sample taken	Virus titre (copies/mL)[a]	Norovirus immunochromatography tests			
			ImmunoCatch-Noro	Quick Navi-Noro 2	GE test Noro Nissui	Quick Chaser-Noro
12860	2 Feb 2015	2.50×10^8	Negative	Negative	Negative	Negative
12868	2 Mar 2015	8.06×10^9	Positive	Positive	Positive	Positive
12870	4 Mar 2015	4.91×10^3	Negative	Negative	Negative	Negative
12880	17 Mar 2015	6.46×10^3	Negative	Negative	Negative	Negative
HU-2015	31 Jan 2015	1.90×10^9	Positive	Positive	Positive	Positive
R1-Thai	19 Dec 2014	1.82×10^6	Negative	Negative	Negative	Negative

[a] Quantified by real-time RT-PCR (reference values).

GII.4 is the most common infection worldwide and new GII.4 variants emerge every two to three years [8]. Although based on a small sample size, our findings suggest that the commercial IC kits for the detection norovirus available on the market in Japan are able to detect the novel GII.17 norovirus, but with relatively low sensitivity. Only samples that contained more than 10^9 copies/mL were positive in all four IC tests. Previous data have shown that the minimal detection limit of an IC test for GII.4 norovirus was about 10^6 virus copies/mL, which is a 1,000-fold more sensitive [9]. Therefore, redesign of the currently available norovirus IC tests may be required to detect the novel GII.17 noroviruses with the same sensitivity as for the more commonly circulating norovirus genotypes. Laboratories and physicians should be aware of these findings, in particular where the novel GII.17 norovirus has been shown to be circulating.

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Conflict of interest

None declared.

Authors' contributions

PK: conducted the laboratory characterisation of norovirus and drafted the manuscript; AT: conducted the laboratory characterisation of norovirus; ST: involved in laboratory investigation; SO: involved in the data interpretation and revised the manuscript; NM: revised the manuscript; SH: revised the manuscript; HU: conceptualised the study and revised the manuscript.

* Authors' correction

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Design and application of a core genome multilocus sequence typing scheme for investigation of Legionnaires' disease incidents

J Moran-Gilad (giladko@post.bgu.ac.il)[1,2,3], K Prior[4], E Yakunin[5], T G Harrison[6], A Underwood[6], T Lazarovitch[7], L Valinsky[5], C Lück[8], F Krux[4], V Agmon[5], I Grotto[1,3], D Harmsen[4]

1. Public Health Services, Ministry of Health, Jerusalem, Israel
2. Surveillance and Pathogenomics Israeli Centre of Excellence, National Institute for Biotechnology in the Negev, Ben-Gurion University of the Negev, Beer-Sheva, Israel
3. Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer-Sheva, Israel
4. Department of Periodontology, University of Münster, Münster, Germany
5. Central Laboratories, Public Health Services, Ministry of Health, Jerusalem, Israel
6. Reference Microbiology Services, Public Health England, London, United Kingdom
7. Department of Clinical Microbiology, Assaf Harofeh Medical Centre, Zerifin, Israel
8. Institute of Medical Microbiology and Hygiene, University of Technology, Dresden, Germany

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Sequence-based typing (SBT) for *Legionella pneumophila* (Lp) has dramatically improved Legionnaires' disease (LD) cluster investigation. Microbial whole genome sequencing (WGS) is a promising modality for investigation but sequence analysis methods are neither standardised, nor agreed. We sought to develop a WGS-based typing scheme for Lp using de novo assembly and a genome-wide gene-by-gene approach (core genome multilocus sequence typing, cgMLST). We analysed 17 publicly available Lp genomes covering the whole species variation to define a core genome (1,521 gene targets) which was validated using 21 additional published genomes. The genomes of 12 Lp strains implicated in three independent cases of paediatric humidifier-associated LD were subject to cgMLST together with three 'outgroup' strains. cgMLST was able to resolve clustered strains and clearly identify related and unrelated strains. Thus, a cgMLST scheme was readily achievable and provided high-resolution analysis of Lp strains. cgMLST appears to have satisfactory discriminatory power for LD cluster analysis and is advantageous over mapping followed by single nucleotide polymorphism (SNP) calling as it is portable and easier to standardise. cgMLST thus has the potential for becoming a gold standard tool for LD investigation. Humidifiers pose an ongoing risk as vehicles for LD and should be considered in cluster investigation and control efforts.

Introduction

Legionellae are Gram-negative rods found in aqueous environments [1]. Humans become infected through exposure to contaminated aerosols originating from man-made water systems, such as spa pools, cooling towers or showering facilities in various settings such as healthcare, public and domestic facilities as well as occupational and travel-related settings. Clinical manifestation varies from a mild illness (Pontiac fever) to potentially fatal pneumonia known as Legionnaires' disease (LD) [2]. Among nearly 70 *Legionella* species described, *Legionella pneumophila* (Lp) causes the vast majority of LD and of 16 known serogroups, Lp serogroup 1 accounts for over 80% of LD cases and almost all clusters and outbreaks [3,4].

LD is a notifiable disease in all European Union and European Economic Area countries. Surveillance coordinated by the European Legionnaires' Disease Surveillance Network (ELDSNet) of the European Centre for Disease Prevention and Control (ECDC) demonstrates a mild increase in incidence of LD since 2001 [5,6]. The standardised sequence-based typing (SBT) scheme for Lp developed by the European Working Group for Legionella Infections (EWGLI, now European Society for Clinical Microbiology Study Group on Legionella Infections, ESGLI) marked an important advancement in the study of the molecular epidemiology of LD [7,8]. Implementation of this typing scheme, similar to multilocus sequence typing (MLST) has yielded useful and comparable data worldwide [9] and has been shown to be applicable in the investigation of unusual LD cases such as humidifier-associated LD [10] and legionellosis outbreaks [11].

The advent of next-generation sequencing (NGS) has revolutionised microbiology by making whole genome sequencing (WGS) of pathogens of public health importance, readily available [12]. The currently most significant role for NGS in microbiology is communicable disease surveillance and outbreak investigation. Many studies have demonstrated that whole genome comparisons provide far greater resolution for outbreak detection and microbial strain tracking than gold standard typing methods of different bacteria [13-15]. While most studies using WGS-based molecular epidemiology have relied on mapping of read data against

Table 1

Finished genomes[a] and assembled raw reads[b] used for *Legionella pneumophila* core genome definition (n=17)

Strain	SBT BAPS cluster	SBT ST (bySanger sequencing)	Mean coverage	NCBI/EBI accession number
Philadelphia[c]	13	36	N/A	NC_002942.5
Lens	2	15[d]	N/A	NC_006369.1
Lorraine	3	47	N/A	NC_018139.1
Paris	6	1[d]	N/A	NC_006368.1
Alcoy	11	578[d]	N/A	NC_014125.1
Corby	11	51	N/A	NC_009494.2
H093620212	1	46[d]	350.32	ERR315646
H090500162	4	611[d]	447.33	ERR315652
RR08000760	5	376[d]	359.47	ERR315654
H093380153	7	179	38.27	ERR315657
H100260089	8	44	486.84	ERR315660
Lansing-3	9	336	354.57	ERR315662
H063280001	10	23	387.44	ERR315663
H070840415	12	59[d]	463.52	ERR315666
H044500045	13	28[e]	520.93	ERR315669
H074360710	14	68[d]	418.93	ERR315671
H091960009	15	707[e]	391.10	ERR315672

BAPS: Bayesian analysis of population structure; EBI: European Bioinformatics Institute; N/A: not applicable; NCBI: National Center for Biotechnology Information; SBT: sequence-based typing; ST: sequence type.

[a] Finished genomes were from the NCBI database.

[b] Assembled raw reads were from EBI.

[c] Reference genome.

[d] Extraction of *mompS* allele from genomic data not possible due to multi-copy occurrence.

[e] Whole genome sequencing analysis corrected erroneous allelic profile of ST707 compared with original publication [24].

a reference followed by analysis of single nucleotide polymorphisms (SNPs), a de novo assembly and genome-wide gene-by-gene approach looking at allelic differences in core genome (cg) genes (cgMLST, or MLST+ as called in the SeqSphere+ software used for analysis) has been suggested as an alternative to SNP mapping [16,17].

There are limited data regarding the application of WGS for investigation of LD. Moreover, current experience is limited to analyses of SNPs and thus there is an unmet need for a cgMLST typing scheme for Lp that would enable a portable global nomenclature. Therefore, the goal of the current study was to set up, validate and apply a cgMLST scheme for Lp.

Methods

Standard laboratory work up of Legionella pneumophila strains

Isolates were cultured on BCYEα plates (Oxoid, Basingstoke, United Kingdom) for 48–72h at 35 °C before phenotypic and molecular tests were performed. Presumptive identification as Lp was confirmed using MonoFluo *Legionella pneumophila* indirect fluorescent antibody (IFA) Test (Biorad, Hemel Hempstead, United Kingdom). Lp serogroups and immunological subgroups (for selected serogroup 1 isolates) were determined using the Dresden panel of monoclonal antibodies [18]. Strains not readily confirmed as Lp were identified to species level by sequencing the *mip* gene as described by Ratcliff et al. [19] and comparing the sequence to the *mip* database [20].

Between two to three single colonies per Lp strain were selected and DNA extracted using the InstaGene Matrix (Biorad, Hemel Hempstead, United Kingdom). The genotype of each strain was determined using the M13 modification of the ESGLI SBT method by Sanger sequencing [21]. All alleles and sequence types (ST) were determined using the Legionella Sequence Quality Tool [22,23]. SBT was attempted on sputum in culture-negative cases using the direct or nested-SBT approach [21].

Whole genome sequencing and assembly

Whole genome shotgun sequencing was performed on 15 strains recovered from clinical and environmental samples in Israel. High molecular weight and quality DNA was extracted using the Wizard DNA purification kit (Promega, Madison, WI, United States). Sequencing libraries were prepared using the Nextera chemistry (Illumina Inc., San Diego, California, United States) for a 250 bp paired-end sequencing run on an Illumina MiSeq sequencer. Samples were sequenced to aim for a minimum coverage of 75-fold using Illumina's recommended standard protocols. All generated raw reads were submitted to the European Nucleotide Archive (ENA) (http://www.ebi.ac.uk/ena/) of the European Bioinformatics Institute under the study accession number PRJEB7140. After sequencing, the reads were quality-trimmed using the CLC Genomics Workbench software version 6.0 (CLC bio, Aarhus, Denmark) and then assembled de novo using CLC Genomics Workbench with default settings. The resulting assembly files were exported as ACE files and imported into SeqSphere+ software version 2.1 (Ridom GmbH, Germany).

Core genome multilocus sequene typing scheme definition and validation

For determining a cgMLST or MLST+ target set we aimed to cover the whole Lp species variation. By Bayesian Analysis of Population Structure (BAPS) based on more than 800 SBT STs, Underwood et al. recently reported 15 such Lp BAPS SBT clusters [24]. Therefore, we used for the cgMLST scheme definition, six finished genomes available from GenBank and 11 raw read datasets from the ENA archive that cover all BAPS SBT clusters (Table 1). ENA raw read data were again de novo assembled into draft genomes with CLC Genomics Workbench. The genome of strain Philadelphia (NC_002942.5) was used as a reference. To determine the cgMLST target gene set, a genome-wide gene-by-gene comparison was performed using the MLST+ target definer function of SeqSphere+ with default parameters. These

Table 2

Finished genomes[a] and assembled raw reads[b] used for *Legionella pneumophila* core genome validation (n=21)

Strain	SBT BAPS cluster	SBT ST (by Sanger sequencing)	Mean coverage	% MLST+ good targets	NCBI/EBI accession number
Thunder Bay	13	187[c]	N/A	99.34	NC_021350
HL06041035	7	734[c]	N/A	98.29	NC_018140
ATCC43290	13	187	N/A	99.67	NC_016811
LPE509	Not known	New ST (3,10,1,1,–[c],9,1)[d]	N/A	99.67	NC_020521
H053260229	1	74	72.66	97.76	ERR315647
H043940028	2	84	379.34	98.42	ERR315648
LP617	3	47	83.46	98.82	ERR164430
H064180002	3	62	73.28	96.98	ERR315651
H065000139	3	54	283.37	97.57	ERR315650
H063920004	3	47	271.57	98.82	ERR315649
H071260094[e]	5	87	485.82	98.29	ERR315653
LP423	6	1	46.26	98.75	ERR164431
EUL00013	6	5	364.53	98.75	ERR315655
H074360702	6	152[c]	343.23	98.62	ERR315656
RR08000517	7	337[c]	339.81	97.24	ERR315658
LC6774	9	154[c]	356.65	96.32	ERR315661
LC6451	10	78	74.39	97.63	ERR315664
H091960011	11	454[c]	433.78	98.62	ERR315665
H075160080	12	188	388.03	99.01	ERR315667
H034680035	13	37	84.00	97.96	ERR315668
RR08000134	14	34	435.23	99.80	ERR315670

BAPS: Bayesian analysis of population structure; EBI: European Bioinformatics Institute; N/A: not applicable; NCBI: National Center for Biotechnology Information; SBT: sequence-based typing; ST: sequence type.

[a] Finished genomes were from the NCBI database.

[b] Assembled raw reads were from EBI.

[c] Extraction of *mompS* allele from genomic data not possible due to multi-copy occurrence.

[d] Ordered in accordance with SBT scheme [21]: *flaA, pilE, asd, mip, mompS, proA, neuA*.

[e] Wrongly stated as LC6677 in Underwood et al. [24].

parameters comprised the following filters to exclude certain genes of the Philadelphia reference genome from the MLST+ scheme: a 'Minimum length filter' that discards all genes shorter than 50 bp; a 'Start codon filter' that discards all genes that contain no start codon at the beginning of the gene; a 'Stop codon filter' that discards all genes that contain no stop codon, more than one stop codon or if the stop codon is not at the end of the gene; a 'Homologous gene filter' that discards all genes with fragments that occur in multiple copies within a genome (with identity 90% and >100 bp overlap); and a 'Gene overlap filter' that discards the shorter gene from the MLST+ scheme if the affected two genes overlap>4 bp. The remaining genes were then used in a pairwise comparison using Basic Local Alignment Search Tool (BLAST) version 2.2.12 (parameters used were: 'Word size: 11', 'Mismatch penalty: -1', 'Match reward: 1', 'Gap open costs: 5', and 'Gap extension costs: 2') with the 16 query Lp chromosomes [25]. All genes of the reference genome that were common in all query genomes with a sequence identity≥90% and 100% overlap, and with the default parameter 'Stop codon percentage filter' turned on (this discards all genes that have internal stop codons in more than 20% of the query genomes) formed the final MLST+ scheme (downloadable from SeqSphere+ software).

To validate the applicability and representativeness of the Lp MLST+ target gene set, a total of 21 published high-quality genomes [24,26] – four finished genomes and 17 raw read ENA datasets that were first de novo assembled – representing 12 of the 15 BAPS SBT clusters were chosen for SeqSphere+ cgMLST analysis (Table 2) performed as below. It was assumed that a well-defined cgMLST scheme should reach at least 95% of the MLST+ genes present in each of the 21 validation genomes.

Core genome multilocus sequence typing analysis of humidifier related cases

To calibrate the cgMLST scheme for micro-evolutionary change, 15 newly generated Lp genomes (Table 3) representing three epidemiologically unrelated humidifier-associated paediatric LD clusters from Israel were analysed together with the finished Philadelphia and Paris strain genomes.

Thus SeqSphere+ extracted the defined MLST+ core genome genes from each assembly with default parameters, mainly consisting of the following settings: (i) processing options: 'Ignore contigs shorter than 200 bases'; (ii) scanning options: 'Matching scanning thresholds for creating targets from assembled genomes' with 'required identity to reference sequence of 90%' and 'required aligned to reference sequence with 100%'; (iii) BLAST options: 'Word size: 11', 'Mismatch penalty: -1', 'Match reward: 1', 'Gap open costs: 5', and 'Gap extension costs: 2'. In addition, the MLST+ scheme genes were assessed for quality, i.e. the absence of premature stop codons, ambiguous nucleotides, and support of variants to reference sequence by 75% or more read nucleotide.

A core genome gene was considered a 'good target' only if all of the above criteria were met, in which case complete sequence was analysed in comparison to the Philadelphia reference and SeqSphere+ assigned a numerical allele type. The combination of all core genome alleles in each strain formed an allelic profile per the proposed new scheme. From these allelic profiles a minimum spanning tree was calculated and drawn using SeqSphere+. In order to maintain backwards compatibility with Lp SBT, sequences of the seven genes comprising the allelic profile of the SBT schemes were separately extracted from finished genomes and WGS data and then queried against the SBT database in order to assign classic STs in silico.

Table 3

Whole genome sequencing data of *Legionella pneumophila* strains included in the study[a]

Strain	Source	Epidemiological context	SBT ST (by Sanger sequencing)	Mean coverage	Conting count	MLST* good targets %	ENA accession number
Lp-001	Clinical	Unrelated case; ST40 'outgroup' strain	40	131.51	43	99.54	ERR593560
Lp-012	Clinical	Unrelated case	23[b]	48.09	69	98.75	ERR593561
Lp-032	Environmental	Routine inspection; ST1 'outgroup' strain	1	43.61	70	98.29	ERR593562
Lp-56207	Clinical	Case 1; epidemiologically linked to strain Lp-2002694p8	1	93.20	66	98.55	ERR594281
Lp-2002694p7	Environmental	Case 1; concurrent isolate from humidifier; unrelated 'innocent bystander'	40	50.53	39	99.74	ERR593569
Lp-2002694p8	Environmental	Case 1; isolate from humidifier; last stage in transmission chain	1	74.11	57	98.62	ERR593570
Lp-119	Environmental	Case 2; isolate from humidifier; last stage in transmission chain	1	77.40	367	98.29	ERR632205, ERR632206
Lp-120	Environmental	Case 2, isolate from domestic water filtering device; middle stage in transmission chain	1	49.73	92	98.49	ERR593565
Lp-121	Environmental	Case 2; isolate from domestic water; initial stage in transmission chain	1	34.62	89	98.49	ERR593566
Lp-122	Environmental	Case 2, isolate from domestic water filtering device's filter; middle stage in transmission chain	1	109.62	409	98.22	ERR593567, ERR593568
Lp-282-1	Environmental	Case 3; isolate from domestic water; middle stage in transmission chain	1	68.83	113	98.75	ERR593571
Lp-283	Environmental	Case 3; isolate from domestic water; initial stage in transmission chain	1	42.20	77	98.22	ERR593572
Lp-284	Environmental	Case 3, isolate from domestic water filtering device's filter; middle stage in transmission chain	1	52.66	233	97.57	ERR593573
Lp-285	Environmental	Case 3, isolate from domestic water filtering device's filter; middle stage in transmission chain	1	55.89	284	98.49	ERR593574
Lp-286-1	Environmental	Case 3; isolate from humidifier; last stage in transmission chain	1	122.55	87	98.55	ERR593575

ENA: European Nucleotide Archive; MLST: multilocus sequence typing; SBT: sequence-based typing; ST: sequence type.

[a] ENA study number PRJEB7140.

[b] Extraction of *mompS* allele from whole genome sequence data not possible due to multi-copy occurrence.

Results

Setting up and validation of core genome multilocus sequence typing for Legionella pneumophila

Six finished genomes available from GenBank and 11 raw read datasets from ENA that cover all BAPS SBT clusters (Table 1) were used for cgMLST definition. ENA raw read data were de novo assembled into draft genomes. The Philadelphia strain (NC_002942.5) served as reference for core genome gene definition. The resulting cgMLST scheme consisted of 1,521 genes (ca 47.2% of the complete Philadelphia strain chromosome nucleotide; list of core genes available upon request from the authors). The SBT alleles were extracted from the genomes and generated correct ST

Table 4

Characteristics of paediatric humidifier-associated Legionnaires' disease cases included in the study

Case number / Year	Outcome	Setting	Recovered strains	Comments
1 / 2012 [10]	Fatal	Domestic free-standing cold-water humidifier serving as vehicle	ST1 from clinical (sputum) and environmental (humidifier) samples; ST40 concurrently recovered from environmental (humidifier) sample	Infection diagnosed by sputum culture; ST40 considered an innocent bystander not implicated in infection
2 / 2013	Mild	Domestic free-standing cold-water humidifier serving as vehicle; humidifier filled with water from a filtrating machine	ST1 from various environmental samples (domestic water, domestic water filtering device's filter, domestic water filtering device's water and humidifier)	Infection diagnosed by urinary antigen
3 / 2013	Severe Severe Legionnaires' disease	Domestic free-standing cold-water humidifier serving as vehicle; humidifier filled with water from a filtrating machine	ST1 (and also ST93) from various environmental samples (domestic water, domestic water filtering device's filter, domestic water filtering device's water and humidifier)	Infection diagnosed by PCR on sputum; direct sequencing on sputum confirmed ST1 infection; ST93 co-infection documented

PCR: polymerase chain reaction; ST: sequence type.

designations for nine strains. In eight strains, six of seven alleles were called correctly but the allele number for the *mompS* gene could not be determined due to presence of more than one copy of *mompS* in the genome (Table 1).

The cgMLST scheme was validated using 21 additional genomes derived from recent publications (Table 2). All 21 strains showed›96% good MLST⁺targets and resulted on average in 98.4% MLST⁺targets. Of 20 strains for which the ST designation was available, 14 were fully extracted from the WGS data, whereas in the six remaining strains, only six of seven alleles were called correctly due to multiple *mompS* gene copies (Table 2).

Investigation of humidifier-associated Legionnaires' disease

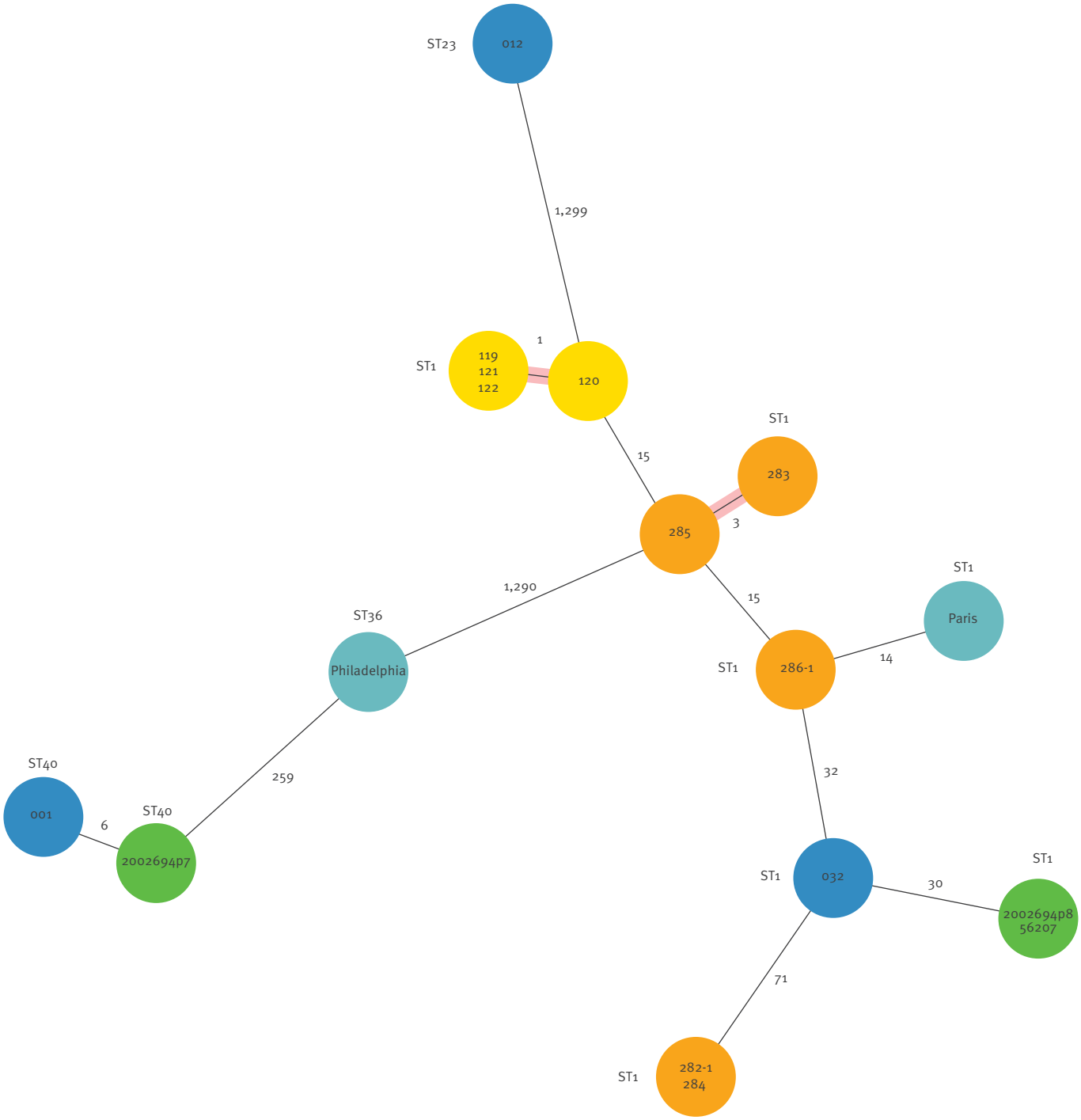
To calibrate the cgMLST scheme for micro-evolutionary change and to define a cluster type (CT) threshold, 15 newly generated Lp genomes representing three epidemiologically unrelated humidifier-associated paediatric LD clusters from Israel were analysed together with the finished Philadelphia and Paris strain genomes. Characteristics of sequenced strains are summarised in Table 3. Analysis involved 11 ST1 strains from the three incidents, a concurrent ST40 strain and three 'outgroup' strains including unrelated ST1, ST40 and ST23 strains. The median coverage was 55.9 (range: 34.6–131.5) and on average 98.6% of the MLST⁺targets could be called. The SBT ST was called complete and correct for 14 of the 15 draft genomes.

The three incidents are described in Table 4. All three cases involved children below one year of age exposed to domestic free-standing cold-water humidifiers. In case 1 the humidifier was filled with tap water whereas in cases 2 and 3 humidifiers were filled with water dispensed through domestic filtrating machines (water bars) that used charcoal filters and ultraviolet light. In case 1 Lp ST1 was detected by culture and polymerase chain reaction (PCR) of the patient's sputum and Lp ST1 was also recovered from humidifier residual water. Notably, environmental sampling revealed a ST40 strain which was not present in clinical samples. In case 2, diagnosis was made using urinary antigen testing and no sputum was available for analysis. Multiple environmental samples obtained from the water system, water filtrating machine, and humidifier were all positive for Lp ST1. In case 3, sputum was culture negative but PCR was positive for Lp. Direct SBT performed on sputum revealed a co-infection with Lp serogroup 1 ST1 and Lp serogroup 3 ST93. Environmental samples obtained from the water system, water filtrating machine and humidifier were all positive for Lp ST1 and some were also ST93 positive.

All 17 analysed genomes (including the 15 from Israel as well as the Philadelphia and Paris strain) shared in total 1,446 of the 1,521 defined core genome genes (data not shown – allelic profiles available upon request). From these allelic profiles SeqSphere⁺ calculated and drew a minimum spanning tree where the number of differing alleles is given along the branches (Figure). For case 1, identical clinical and environmental ST1 strains (Lp-2002694p8 and Lp-56207) were found (no differing alleles) and a concurrent ST40 (Lp-2002694 p7), which as expected, did not cluster with implicated ST1 strains. This ST40 strain exhibited a difference of six alleles (of the 1,521 core genome genes) from an unrelated ST40 strain serving as an 'outgroup' for ST40. Of the four environmental strains representing

Figure

Use of a minimum spanning tree generated from allelic profiles of 1,446 core genome genes shared by 17 *Legionella pneumophila* strains analysed, to investigate paediatric humidifier-associated Legionnaires' disease cases



Lp: *Legionella pneumophila*; ST: sequence type.

The Lp strain numbers are described inside the circles. Finished genomes of the 'Philadelphia' (ST36) and 'Paris' (ST1) strains obtained from the National Center for Biotechnology Information (NCBI) were used as reference (turkoise blue). Strains corresponding to the three ST1 humidifier-associated epidemiological clusters are designated in green (epidemiological cluster 1; includes also one ST40 'bystander' strain), yellow (epidemiological cluster 2) and orange (epidemiological cluster 3). Epidemiologically unrelated strains, including ST1 and ST40 'outgroup' strains (Lp-032 and Lp-001, respectively) and a ST23 (Lp-012) are designated in dark blue. The number of differing alleles is stated along the branches of the tree. Lines connecting strains within cluster type distance are highlighted by pale red background shading. The lengths of the branches reflecting distances between strains are drawn in a logarithmic scale.

the chain of transmission in case 2, three were identical ST1 strains (Lp-119, Lp-121 and Lp-122) and one had only one differing allele (Lp-120). Case 3 demonstrated more complex clustering of environmental strains into

two pairs (one identical pair formed by Lp-282-1 and Lp-284, and one pair with three differing alleles formed by Lp-285 and Lp-283) and a fifth distinct strain (Lp-286-1) which on epidemiological grounds was considered the most likely cause of infection (Lp strain recovered from humidifier residual water and therefore most likely to have been aerosolised during humidifier use). A subsequent cloning experiment performed on the patient's sputum extract, revealed sequences unique to at least two of the environmental strains (Lp-286–1 and Lp-285), suggesting co-infection (data not shown).

Discussion

WGS-analysis is emerging as the optimal molecular epidemiology tool for microbial genotyping but its application and implementation are limited by challenges in timely analysis of data and standardised integration into scalable classification schemes. While most WGS-based epidemiological investigations published to date have relied on mapping of SNPs, extension of the classical MLST approach [27] to a gene-by-gene typing scheme based on the entire core genome [16] is a promising approach for a standardised, portable and expandable typing method. The current study presents a novel core-genome allele-based typing scheme for Lp based on a standardised analysis of WGS of an internationally representative and biologically diverse Lp collection of genomes. The proposed scheme follows several recently proposed cgMLST schemes for pathogens of public health importance including *Staphylococcus aureus, Listeria monocytogenes, Escherichia coli, Neisseria meningitidis* and *Mycobacterium tuberculosis* [13-15,28,29].

Only a few clusters of LD have been investigated to date using a WGS-based approach. One study provided a retrospective analysis of a community-acquired LD cluster in which WGS yielded comparable results to that of conventional SBT [26]. Notably, WGS could not identify the most likely source of infection. The two clinical and three environmental isolates analysed in that study were not more than 15 SNPs apart [26]. Another study provided a real time investigation of a nosocomial LD cluster involving two patients [30] in which WGS had a greater resolution as compared with conventional typing and was able to link the two cases with an environmental strain and possibly to a past case. Related strains in that study were 17 SNPs apart. The reliance on SNP mapping makes those two reports difficult to reproduce and to compare, especially given the differences in reference genomes and bioinformatics pipelines used, as well as software parameter selections. Nevertheless, both papers contribute to the proof of concept of harnessing WGS for Lp investigation.

In our report, WGS of Lp strains related to three independent LD incidents was successful in demonstrating the phylogeny of implicated clinical and environmental strains. We chose to focus on Lp ST1 which is one of the most abundant ST globally and by far the most common cause of LD in Israel [31] and thus conventional tools such as SBT are not always powerful enough for epidemiological purposes. Moreover, it has recently been shown that Lp ST1 could be further characterised using additional typing methods such as spoligotyping [32]. Using the 'Paris' ST1 type strain and an 'outgroup' ST1 strain our analysis shows that the cgMLST scheme of ca 1,500 genes has an adequate discriminatory power and could resolve clustering of multiple strains in the ST1 complex. Discriminatory power in concordance with epidemiological data are among the most important performance criteria set to evaluate proposed typing methods [33]. In that respect, the observed clustering pattern of our study isolates suggests that a difference of up to four alleles between strains may serve as a preliminary threshold value for defining a WGS cluster. Nevertheless, this should be further evaluated and fine-tuned as additional genomic epidemiology data on Lp accumulates.

We included in our analysis LD cases diagnosed by three accepted laboratory modalities, being sputum culture, urinary antigen and sputum PCR in order to demonstrate the usefulness of WGS-based typing in all typical epidemiological scenarios. Of note is that case 3 was more difficult to resolve as strain clustering yielded three unrelated ST1 groups. While spontaneous mutations could provide a possible explanation, we believe that this is the result of infection with multiple ST1 strains. This reflects the inherent limitation of culture-based methods used in water testing for Lp, where picking out a single colony from similar morphotypes may overlook the presence of multiple strains. This limitation could be resolved by liberal use of SBT target screening before colony picking and in the future via metagenomic approaches.

One notable hindrance to routine application of WGS for Lp genotyping is the failure to determine the *mompS* allele number (and as a result determine the ST) for some strains, regardless of whether SNP mapping or cgMLST is being used. This phenomenon results from the presence of multiple copies of the *mompS* gene in many Lp strains, which are commonly non-identical, a fact not known when the SBT scheme was initially designed [7]. Current SBT primers used for Sanger sequencing amplify only a single copy of the gene due to sequence variation in the noncoding flanking region and thus generate consistent ST designations [7]. Therefore, in the future, tools for extraction of the correct *mompS* allele from finished genomes harbouring multiple gene copies must take synteny information, e.g. the primer sequences, into consideration to choose the correct gene copy for allele calling. Remediation of the problem for draft genomes is more difficult to achieve as the rather short second generation sequencing reads from both copies are assembled or mapped into a single contig. Notably, resolution of this limitation would be highly desirable for routine WGS application for Lp as backwards compatibility would be maintained.

Our report also highlights humidifier-associated paediatric LD as a continuously emerging risk for LD. After the first paediatric case was acknowledged and reported in Eurosurveillance [10], additional cases have been reported in Europe including Spain [34] and Cyprus [35], the latter involving a nosocomial outbreak in a nursery. The public health response to the first case in Israel was coordinated by the National Programme for Legionellosis Prevention. As part of this response, the Israeli Ministry of Health released specific guidance to professionals and members of the public. Thereafter, scheduled press releases occur every winter. As mandated, cold water humidifiers sold in the country are also labelled with a safety hazard warning through the National Standards Institute of Israel. Moreover, the Israeli Paediatrics Association has released a position paper regarding domestic humidifier use highlighting the benefits and risks. Nevertheless, paediatric humidifier-associated LD is still a public health challenge and deserves more attention.

In conclusion, we devised a WGS-based cgMLST scheme for typing of Lp, which provided high-resolution analysis of Lp strains within the same clonal complex. cgMLST appears to have satisfactory discriminatory power for LD cluster analysis and is advantageous over mapping followed by SNP calling as it is easier for standardisation and dissemination. cgMLST thus has the potential for becoming a gold standard tool for LD investigation. Humidifiers pose an ongoing risk as vehicles for LD and should be considered in cluster investigation and control efforts.

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Conflict of interest

D. Harmsen has declared a potential conflict of interest. He is one of the developers of the Ridom SeqSphere+ software mentioned in the manuscript, which is a development of the company Ridom GmbH (Münster, Germany) that is partially owned by him. All other authors have declared that no competing interests exist.

Author's contribution

JM-G initiated the study, interpreted data and drafted the manuscript; KP performed genome sequencing, bioinformatics analysis, and contributed to manuscript drafting; FK also conducted genome sequencing; EY, TL, LV and VA collected strains and performed traditional and molecular laboratory analyses; TGH performed microbiological analyses and participated in creation of typing scheme and drafting of manuscript; AU performed BAPS analysis and characterisation of Lp strains. CL participated in creation of typing scheme; IG contributed to interpretation of data and related public health policy; DH conceptualized the analysis of the genomic data, the creation of the Lp typing scheme, and contributed to manuscript drafting.

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Improving national surveillance of Lyme neuroborreliosis in Denmark through electronic reporting of specific antibody index testing from 2010 to 2012

R B Dessau (ramd@regionsjaelland.dk)[1], L Espenhain2, K Mølbak[2], T G Krause[2], M Voldstedlund[2]
1. Department of Clinical Microbiology, Slagelse Sygehus, Slagelse, Denmark
2. Department of Infectious Disease Epidemiology, Statens Serum Institut, Copenhagen, Denmark

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Our aim was to evaluate the results of automated surveillance of Lyme neuroborreliosis (LNB) in Denmark using the national microbiology database (MiBa), and to describe the epidemiology of laboratory-confirmed LNB at a national level. MiBa-based surveillance includes electronic transfer of laboratory results, in contrast to the statutory surveillance based on manually processed notifications. Antibody index (AI) testing is the recommend laboratory test to support the diagnosis of LNB in Denmark. In the period from 2010 to 2012, 217 clinical cases of LNB were notified to the statutory surveillance system, while 533 cases were reported AI positive by the MiBa system. Thirty-five unconfirmed cases (29 AI-negative and 6 not tested) were notified, but not captured by MiBa. Using MiBa, the number of reported cases was increased almost 2.5 times. Furthermore, the reporting was timelier (median lag time: 6 vs 58 days). Average annual incidence of AI-confirmed LNB in Denmark was 3.2/100,000 population and incidences stratified by municipality ranged from none to above 10/100,000. This is the first study reporting nationwide incidence of LNB using objective laboratory criteria. Laboratory-based surveillance with electronic data-transfer was more accurate, complete and timely compared to the surveillance based on manually processed notifications. We propose using AI test results for LNB surveillance instead of clinical reporting.

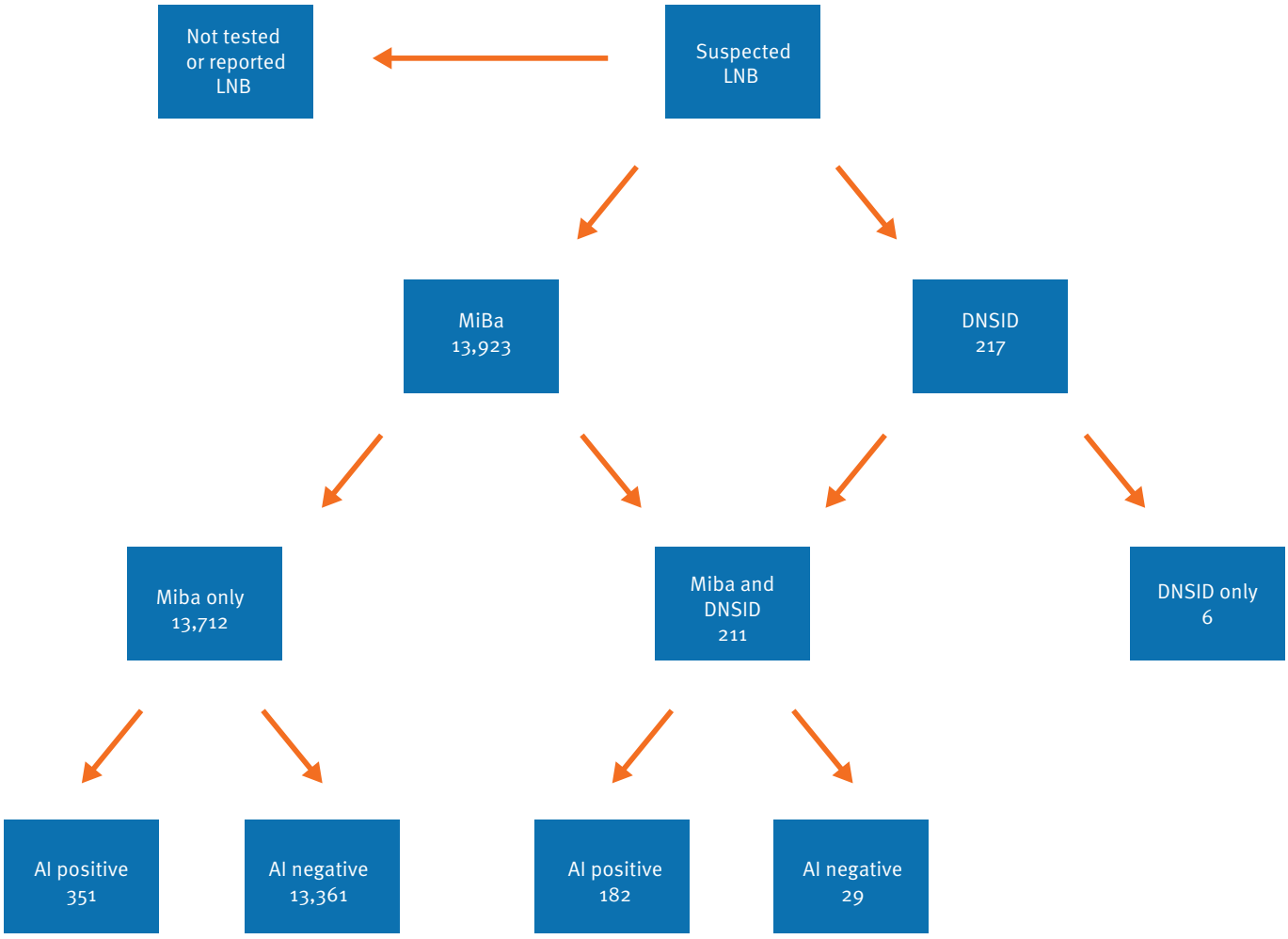
Introduction

Borrelia species, known to cause Lyme borreliosis, are collectively known as *Borrelia burgdorferi sensu lato*. *B. burgdorferi sensu stricto* is the cause of Lyme borreliosis in North America, whereas *B. afzelii* and *B. garinii* cause most European cases [1]. Differences in the clinical presentation of Lyme borreliosis in North America and Europe are ascribed to differences in the predominant species. One important systemic manifestation of Lyme borreliosis is Lyme neuroborreliosis (LNB), which is a clinically characteristic neurological syndrome. Notably LNB is much more common in Europe where *B. garinii* is frequently the causative agent. In Denmark *B. afzelii* has been isolated from patients with erythema migrans and *B. garinii* from patients with LNB [2]. The disease is transmitted by the hard tick (*Ixodes ricinus* or *I. persulcatus*). The abundance of *I. ricinus* is determined by complex interaction with many factors including wildlife, geography and climate [3]. According to the clinical European case definition developed by the European Federation of Neurological Societies (EFNS), and also written stated by the European Study group for Lyme Borreliosis (ESGBOR) under the European Society for Clinical Microbiology and Infectious Diseases, demonstration of intrathecal antibody production (antibody index, AI) is essential for the diagnosis of LNB [4,5]. In patients with short duration of clinical disease, antibody response may be weak or absent [5]. The original development of a new assay and diagnostic criteria to diagnose patients with LNB with AI in Denmark was described by Klaus Hansen and co-workers [6-9] and this assay has been found sensitive and specific [5,8-10].

In Denmark, surveillance of LNB started in 1991 when the Danish Health and Medicines Agency decided to make LNB a mandatory clinical notifiable disease to Statens Serum Institut (SSI), as LNB was considered to be the most common severe clinical manifestations of Lyme borreliosis. LNB is relevant for surveillance, as it is of public health interest to follow trends due to climate change or altered outdoor behaviour in order to adopt appropriate preventive measures. The incidences of the other disseminated manifestations of Lyme borreliosis such as acrodermatitis, carditis, Lyme arthritis and lymphocytoma are not known in Denmark. The incidence of patients suspected of these

Figure 1

Flowchart of patients suspected for Lyme neuroborreliosis (LNB) included in the study, Denmark, 2010–2012 (n=13,929 suspected patients)



AI: antibody index; DNSID: Danish notification system for infectious diseases; MiBa: Danish microbiology database

other manifestations was however 47/100.000, but a low seropositivity rate, similar to healthy controls, was found in consecutive patients seen in general practice, which suggests a low incidence and poor positive predictive value of serology in most cases [11].

The current statutory Danish notification system for infectious diseases (DNSID) is based on collection of paper forms completed by the physician treating the patient; the forms are sent by mail to the Department of Infectious Disease Epidemiology at SSI and the Regional Medical Officer of Health. SSI sends reminders to the clinicians if intrathecal antibody production has been detected by the SSI laboratory and no notification has been received within a certain timeframe. From 2011 to 2012, 44% of the notifications was received only after a reminder had been sent. Due to increased testing at the regional microbiological laboratories, SSI is responsible for a decreasing fraction of the AI tests in Denmark, and therefore the current surveillance system for LNB is neither timely nor complete, even considering the sending of reminders.

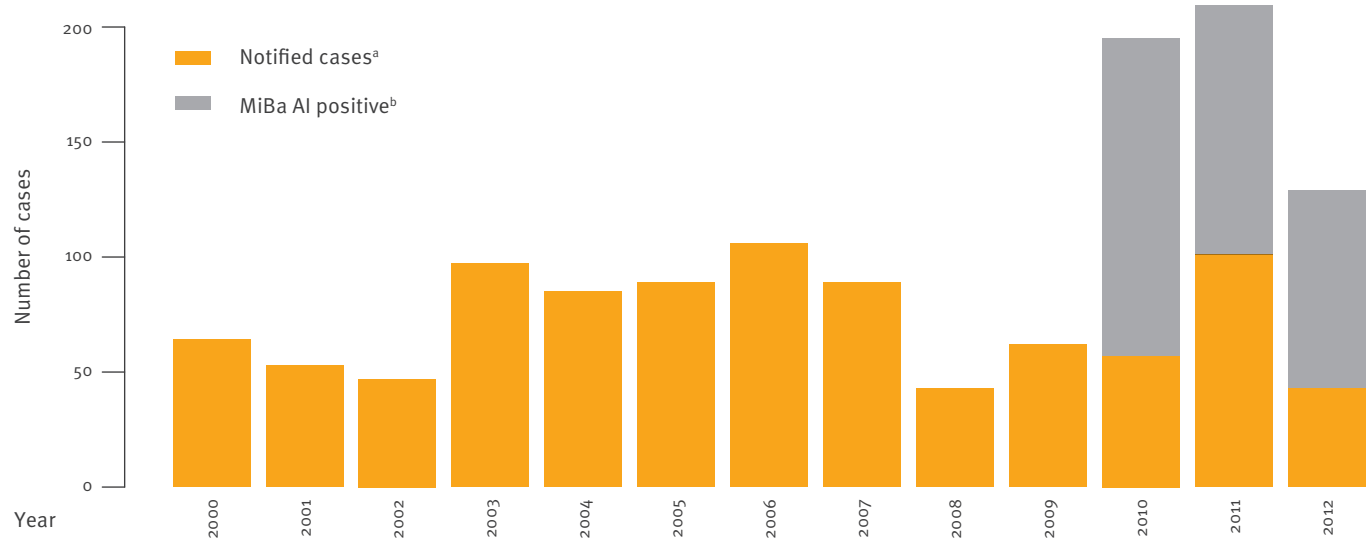
The Danish microbiology database (MiBa) has recently been developed and has since 2010 received electronic copies of all reports from all Danish departments of clinical microbiology [12]. MiBa has several objectives including improving the national laboratory-based surveillance [10]. The aim of the present study was to assess MiBa for laboratory-based surveillance of LNB, including a comparison with the prevailing system based on manually-processed clinical notifications. Furthermore, we use the data from MiBa to describe the epidemiology of laboratory-confirmed LNB in Denmark.

Methods

The study period was from January 2010 to December 2012 and the study population was the population of Denmark.

Figure 2

Annual numbers of Lyme neuroborreliosis cases identified in the Danish microbiology database (MiBa) from 2010 to 2012 and annual numbers of notified cases, Denmark, 2000–2012



AI: antibody index.

From 2010 to 2012, six cases were notified but not included in MiBa. These cases were not subjected to AI testing and are not visible on the Figure.

[a] Notified cases are based on clinical reporting.

[b] Except for 35 notified cases, all notified cases in the period 2010–2012 are included in the MiBa-AI positive cases.

Data sources

The statutory Danish notification system for infectious diseases

DNSID is a case-based database including information on the civil registration number (CPR number) of the case, date of disease-onset, symptoms, laboratory results as well as sequelae. The CPR number is a unique identification number given to each person living in Denmark.

Both confirmed and probable cases (see below) were included in the DNSID dataset extracted for the present study, as both, so far, have been included in the national surveillance.

The Danish microbiology database

MiBa receives real-time electronic copies of all reports from all Danish departments of clinical microbiology [12]. The data model and basic principles have been described previously [12,13]. Within MiBa local codes are automatically mapped to national shared codes before data extraction. All reports include the CPR number of the patient.

In Denmark a total 11 laboratories performed AI tests. At the time of the study, nine of these reported to MiBa through their microbiology laboratory information systems. The two remaining laboratories included one biochemistry laboratory that did not report to MiBa, and one laboratory that had technical problems with the transfer protocol during the first two years. To obtain complete nationwide data, data on AI test results were acquired directly from the two latter laboratories and merged with the MiBa data. This merged dataset is referred to as the MiBa dataset hereafter.

The Danish civil registration system

Using the CPR number information on age, sex, address, and municipality was obtained. Information on population size was obtained from Statistics Denmark (www.dst.dk). To represent the middle of the study period, data was retrieved for the first quarter of 2011, were Denmark had a total population of 5.56 million people.

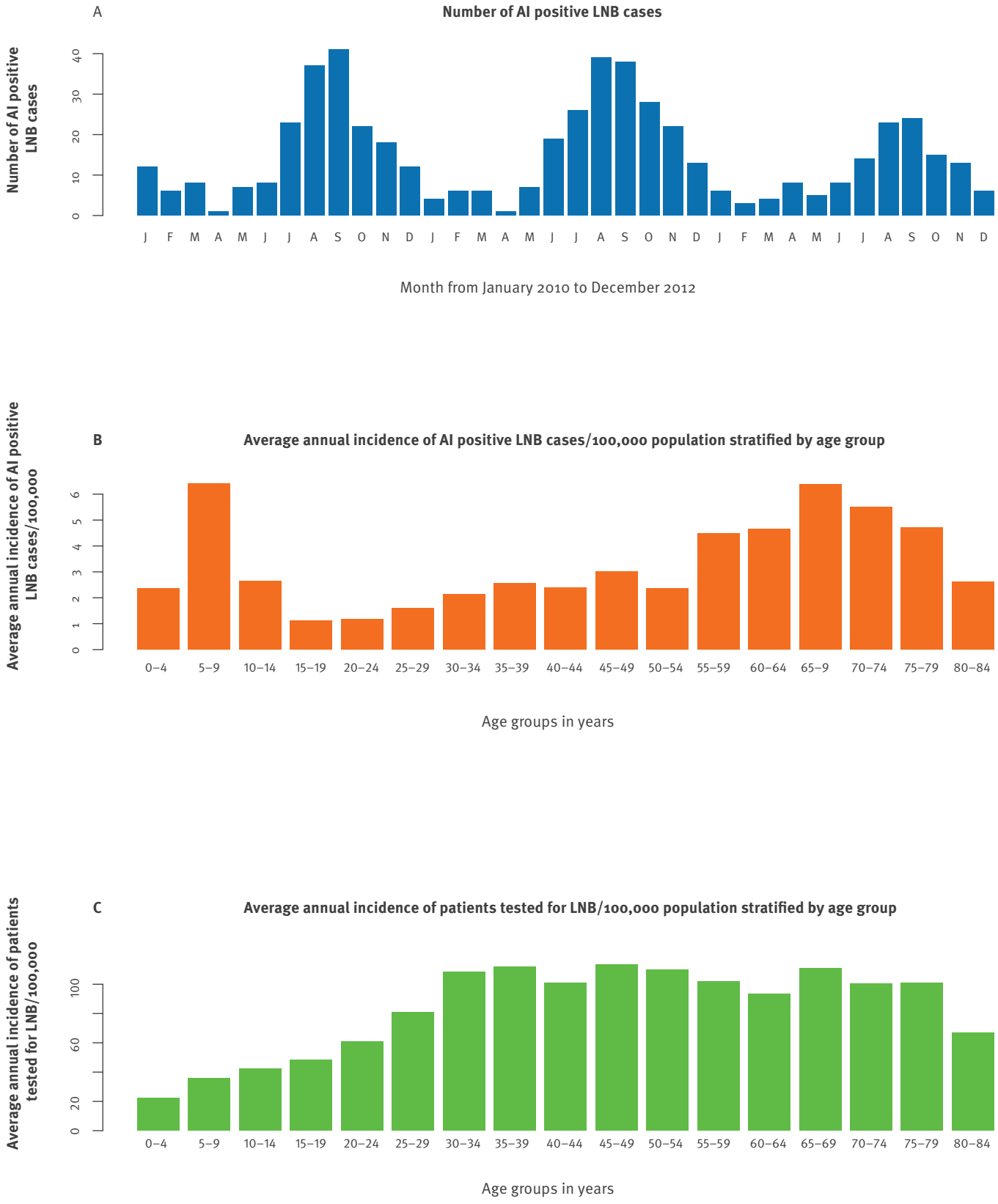
Definitions and data management

The statutory case definition for LNB in DNSID is as follows:

- Confirmed case: patient with clinical symptoms con sistent with LNB and a positive AI test.
- Probable case: patient with clinical symptoms consistent with LNB and borrelia antibodies in serum.
- Concerning the probable cases the AI is either negative or not done, but the detection of serum-antibodies is required.
- Case definition for LNB in MiBa:
- A patient with one or more AI tests positive for Borrelia IgG or IgM antibodies or both.

Figure 3

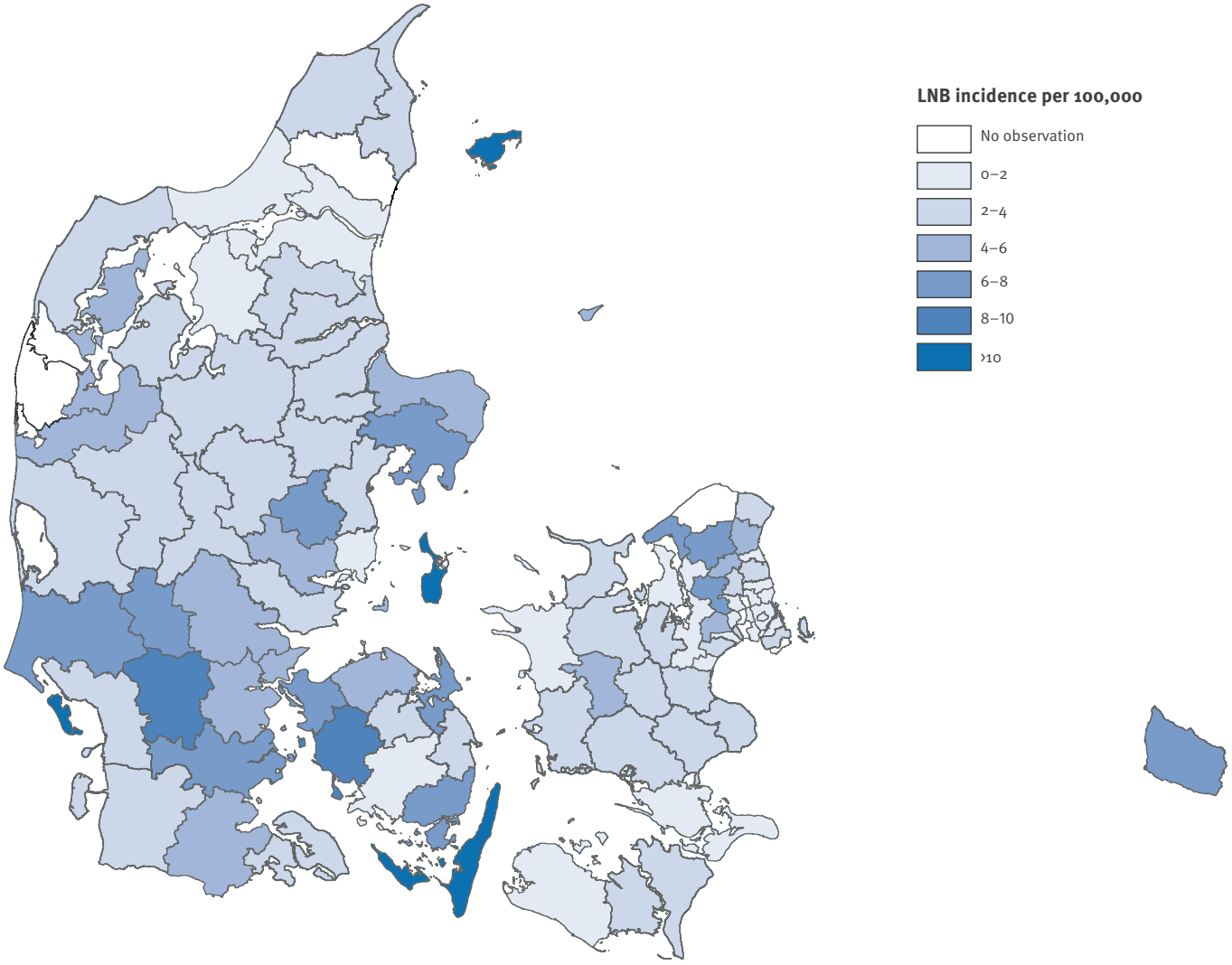
Monthly numbers of antibody index (AI) positive Lyme neuroborreliosis (LNB) cases and average annual incidence of cases and patients tested, Denmark, 2010–2012



Months are abbreviated by their first letter.

Figure 4

Three year average of annual incidence of Lyme neuroborreliosis antibody index-positive cases, according to municipalities, Denmark, 2010–2012



Data management

Patients residing in Greenland and patients with temporary CPR numbers, including foreign travellers, were excluded from all datasets.

The raw MiBa dataset included results from more than one test report per patient. This raw dataset was transformed into a dataset with only one record per CPR number (case) according to the following rules:

Each patient was classified as IgG positive, IgM positive or both IgG-and-IgM positive, based on the accumulated AI-results of one or more reports. If a test result was stated as inconclusive it was considered as negative. A patient was only included once during the three year study period with the first positive test as a case-defining event. A patient with only negative AI tests was included as a LNB negative patient with the date of the first test performed. The total number of tests performed per patient was registered. The age was calculated as age at the date of disease.

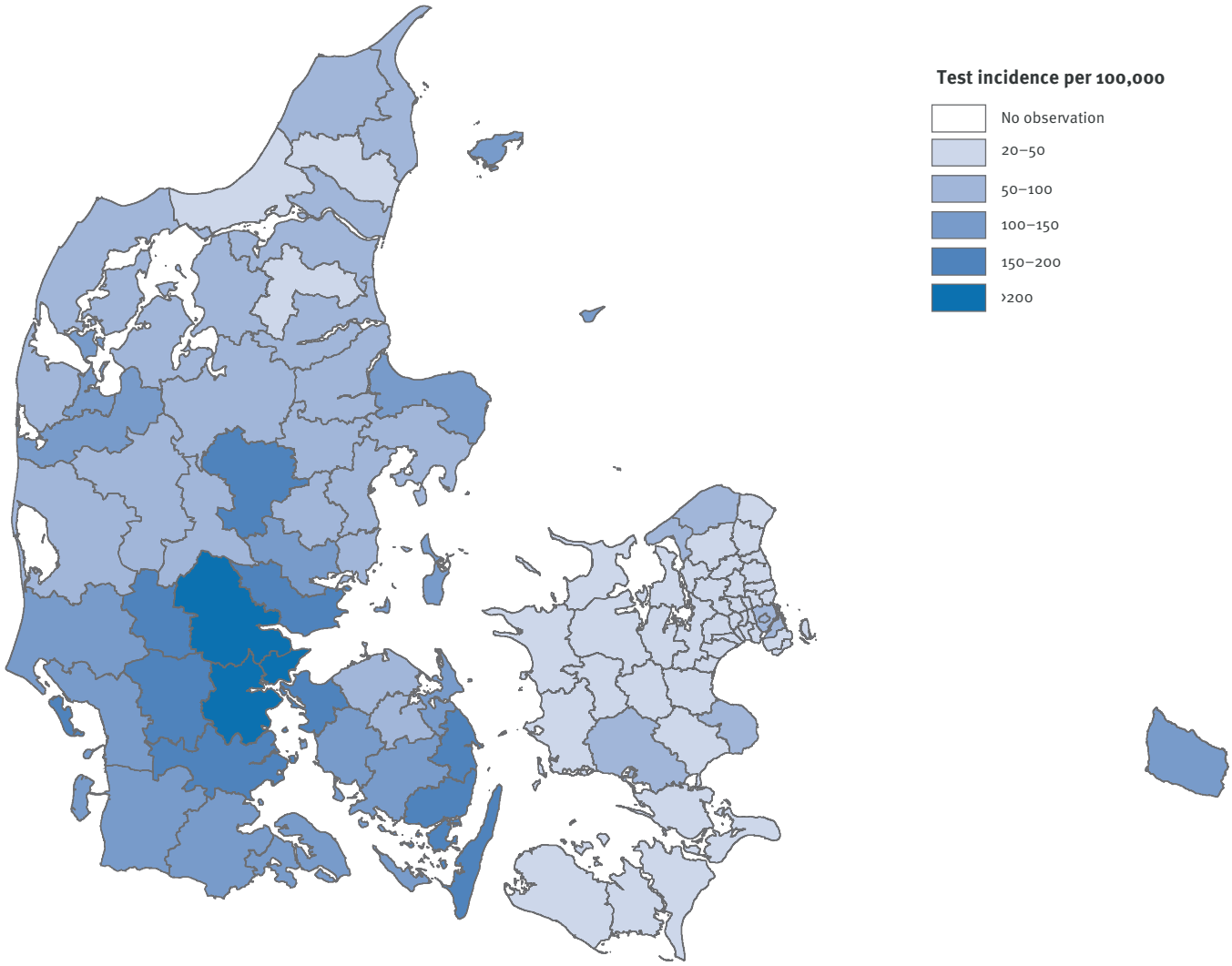
Reports on tests that for some reason were not performed (e.g. sample tube broken during transport) were excluded from the dataset.

Intrathecal antibody production

For this study, with the purpose of surveillance, the conclusion by the laboratory in the report was considered valid regardless of the type of assay and method of index calculation. All laboratories except one used an assay based on native purified flagella antigen (IDEIA LNB IgG/IgM assay, Oxoid, Cambridgeshire, United Kingdom). The index calculation is specified by the manufacturer with the formula: Index = (ODcsf/ODserum)*(ODcsf – -ODserum), where ODcsf and ODserum is the optical absorbance in the cerebrospinal fluid and the serum, respectively. An index above 0.3 was considered as positive. If the absolute absorbance (abs.) in the spinal fluid was below 0.150 the result was negative in any case. The assay is a capture

Figure 5

Three year average of antibody index-tested patients for Lyme neuroborreliosis per 100,000 population according to municipalities, Denmark, 2010–2012



enzyme-linked immunosorbent assay (ELISA) thus the relative abundance of Borrelia specific antibodies compared with the total IgG is important for a positive result and measurements of total IgG are not needed in the algorithm.

Data linkage

Data from DNSID, MiBa, and the Danish civil registration system was linked using the CPR number.

Timeliness

The timeliness of the two systems was calculated as a technical time lag in days from sampling date to the date, where information on the case was received at SSI, either in the form of a notification or when the test result in MiBa was available for data extraction. From the DNSID surveillance only confirmed cases were included (182 observations). For the MiBa surveillance AI positive LNB cases were included.

Statistical methods

Data management was performed using SAS (SAS Institute Inc., Cary, North Carolina, United States) and statistical analyses were performed in R [14] using chi-squared tests and confidence intervals for proportions. Total number of tested patients and LNB cases were stratified by age group, sex, and municipality of residence and presented per 100,000 population. Also annual (cumulative) incidences were calculated per 100,000 population. For the geographical presentation of data QGIS (version 2.4.0) was used.

Ethical considerations

This study was approved by the Danish data protection authority as part of a general permission for performing surveillance studies (registration number 2008–54–0472) and falls within the regulatory community framework for the national surveillance in Denmark

Table 1

Number of patients tested for Lyme neuroborreliosis (LNB) in the Danish microbiology database and number of notified cases of LNB in Denmark, 2010–2012

Year	MiBa[a]		DNSID
	Tested[b]	Cases[c] (% positive)	Notified cases[d]
2010	4,347	195 (4.5)	57
2011	4,957	209 (4.2)	101
2012	4,619	129 (2.8)	59
Total	13,923	533 (3.8)	217[e]

DNSID: Danish notification system for infectious diseases; MiBa: Danish microbiology database.

[a] Numbers are based on the MiBa-case dataset.

[b] Number of patients tested by LNB specific antibody index tests.

[c] Number of cases which are antibody index positive for borrelia IgM, IgG or both.

[d] Number of cases clinically reported on notifications.

[e] Of the total 217 notified patients in DNSID six were probable cases, which were not subjected to antibody index testing and 29 had a negative antibody index test. The remaining 182 notified patients had a positive antibody index test.

(the National Board of Health Statutory Order on Physicians' Notification of Infectious Diseases).

Results

Comparison between MiBa and the mandatory notification system

According to MiBa from 2010 to 2012 a total of 13,923 patients were tested by AI and LNB was confirmed in 533 (4%) patients (Table 1). Of these 172 (32%) were only IgG positive, 103 (19%) only IgM positive and 258 (48%) cases were found positive in both IgM and IgG. By contrast, in DNSID only 217 patients were notified as LNB in this period (Figure 1, Table 1). Among the 217 DNSID patients, AI tests were positive for 182, while 35 patients were either AI negative (29 patients) or not tested by AI (6 patients).

When data from DNSID and MiBa were linked, we found that of 13,923 tested patients in MiBa, 211 were also in DNSID and 182 (83%) of these were AI positive (Table 1, Figure 1).

Thus in MiBa 351 cases of LNB were identified, which had not been registered by the national surveillance. These cases were compared with the notified cases in Table 2. Among the 351 MiBa cases not notified 65 (19%) were children (0–15 years of age) and this was significantly lower compared with 64 children (29%) of 217 cases notified in DNSID (relative risk (RR): 0.63; 95% confidence interval (CI): 0.47–0.84). Also twelve (34%) children were found among the 35 cases notified where AI was either negative or not performed, which may be compared with 65/351 (19%) which were AI positive but not notified in DNSID (RR: 1.8; 95% CI: 1.1–2.9).

The regional distribution of LNB cases was different between notified and unnotified cases identified in MiBa.

Since 2000 the average number of notified cases was 73 per year (Figure 2), corresponding to an annual incidence of LNB of 1.3 cases/100,000 population. The average annual number of notified cases from 2010 to 2012 was 67 and thus comparable to the previous years. In the same period, the average number of cases identified in MiBa was 178 per year, which yielded an annual incidence, of 3.2/100,000.

The median time lag from sampling date to reception of the notification by SSI was 58 days (range: 6–613), whereas the median time lag from sampling date to availability for data extraction in MiBa was five days (range: 1–106). The reports with more than 20 days delay were due to transfer of samples from some local laboratories, for testing at the reference laboratory at SSI.

Descriptive epidemiology of Lyme neuroborreliosis in Denmark

The data from MiBa show a clear seasonal variation of AI-confirmed LNB cases, with lower numbers in the period between February to May and peaks in August and September (Figure 3A). Also the average number of patients tested for AI exhibited seasonal variation with lowest number in April (n=278) and highest in August (n=468). The average monthly percentage of positive test results among patients tested ranged from 1.2% in April to 7.4% in September. Thus during winter and early spring the diagnostic yield is low.

The age-specific annual incidences of LNB were highest among children (5–10 years) and the older age groups (55 to 79 years-old) with a peak at 65 to 69 years of age (Figure 3B). In contrast, the average annual cumulative age-specific incidence of patients being tested, increased with age until 30 years of age, over which the incidence of testing was stable and caabout 100/100,000 population (Figure 3C).

Geographical distribution

For the geographical distribution of LNB, the DNSID data were not used as it they wereas considered unreliable due to differences found in reporting frequency between regions (Table 2). Based on MiBa data, the average annual incidence of LNB stratified by municipality ranged from none to more than 10/100,000 (Figure 4). 'Hot spots' were found in northern Zealand, Funen and parts of southern Jutland, and interestingly in many of the 'smaller' islands (Bornholm, Læsø, Samsø, Langeland, Ærø and Fanø). These results were not adjusted for differences in age and sex distribution between municipalities.

Table 2

Comparison of the 351 Lyme neuroborreliosis cases identified in the Danish microbiology database (MiBa) but not notified, with the 217 notified cases, and annual incidences of all antibody index positive cases in MiBa, Denmark, 2010–2012

Characteristics of cases	Notified cases of neuroborreliosis		MiBa cases not notified	MiBa cases not notified vs notified cases RR (95% CI)	All cases in MiBa: average annual incidence per 100,000 population
	Positive AI	No result or negative AI result			
Age group in years					
0–15	52	12	65	0.63 (0.47–0.84)	3.7
16–64	90	17	188	1.08 (0.92–1.29)	2.6
≥65	40	6	98	1.32 (0.97–1.80)	5.0
Sex					
Male	106	18	200	1.08 (0.94–1.24)	3.7
Female	76	17	151	1.00 (0.82–1.22)	2.7
Region of residence					
Capital	76	18	39	0.26 (0.18–0.36)	2.3
Zealand	19	2	46	1.35 (0.84–2.20)	2.6
Southern Denmark	44	7	138	1.67 (1.28–2.21)	5.1
Middle Jutland	23	1	106	2.73 (1.83–4.12)	3.4
Northern Jutland	20	6	15	0.36 (0.20–0.65)	2.0
Unknown[a]	0	1	7	NA	NA
Total	**182**	**35**	**351**	**1.00**	**3.2**

AI: antibody index; CI: confidence interval; NA: not applicable; RR: relative risk.

[a] Unknown address of residence.

The geographical distribution of LNB (Figure 4) did not just reflect differences in frequency of testing (Figure 5). For example, absence of cases in three municipalities was not explained by lack of testing. Also municipalities with the highest incidence of AI testing (Figure 5) did not have the highest incidence of LNB cases.

Discussion

We compared the existing surveillance system, DNSID, based on manually processed notifications with a new laboratory-based system, MiBa, automatically compiling all AI test performed on a national level. In spite of fundamental differences between the systems, the data are comparable since the case definitions for both the MiBa-based-system and for confirmed cases in the DNSID-based surveillance rely on the AI test for LNB. However, the Danish clinical guideline on Lyme borreliosis recommends, not only a positive AI, but also a lumbar puncture with leucocytosis, for the diagnosis of LNB (www.dskm.dk). In MiBa there is no access to spinal-fluid leucocyte counts. Leucocytosis in the spinal fluid is an important marker of active disease and, if absent, a positive AI probably indicates past infection. In an earlier study of 3,756 AI tests, the sensitivity was estimated to be 88% and specificity 99.7% for LNB [8,9,15]. Of 125 AI positive patients, seven patients did not show leucocytosis, indicating previous LNB. Thus, a case definition solely based on AI testing, would provide an adequate specificity for surveillance in a Danish context, where previous LNB is rare. The risk of reporting past infections as active LNB would be ca 6%. We therefore consider this concern as less important compared with the advantages of an automated system.

The existing surveillance includes notifications of both probable cases as well confirmed cases (AI-positive). On average 12 patients (35 cases/3 years) were notified as probable cases per year; these were not captured by MiBa. The probable cases were more frequent in children less than 16 years-old (Table 2), thus if these 12 were all true cases, some children would be missed by a surveillance based on laboratory reporting of AI positive results only. In addition, there could be a number of patients treated for LNB on a putative diagnosis, which were neither tested nor reported. However, this is not limitation for surveillance as this group of patients may contain misclassified non-LNB patients as well. A surveillance of LNB based on AI results would have a low risk of including misclassified cases. We assume that a lumbar puncture is only performed, when neurological

symptoms and objective findings justify this invasive procedure. Thus testing, without relevant indication, which could lead to a low positive predictive value, is considered rare.

MiBa receives in principle all test reports from all departments of clinical microbiology. However, a few tests for infectious diseases are for historical reasons still reported by a biochemistry laboratory. This biochemistry laboratory and one department of clinical microbiology were unable to report electronically to MiBa in the study period. Prospectively, MiBa will be complete on LNB AI reports at the national level. The automated transfer of all reports frees physicians and laboratories from active reporting; and makes surveillance independent of local healthcare personnel remembering to do the notification. The present study demonstrated underreporting as only 34% of the 533 AI positive LNB were notified. This probably reflects both the workload associated with filling in and sending paper forms and uncertainty on whether LNB is notifiable or not. The 533 cases over three years, might be slightly underestimated, as we did not take into account that it is possible to have more than one episode of LNB during this period.

The present study described an improved system for surveillance of LNB based on objective criteria. It also provided an important insight into the epidemiology of LNB in Denmark. The incidence of LNB in Denmark was found to be more than twice as high as previously estimated. An average annual incidence of 3.2/100,000 in Denmark is comparable to a one year Swedish study from May 1992 to April 1993 in which the incidence of LNB with lymphocytic pleocytosis was found to be 2/100,000 [16]. The incidence of AI positivity was not reported in this study. In the Würzburg region of Germany a yearly incidence for LNB of 3/100,000 may be calculated from the study from May 1996 to April 1997, however the AI was not specified as part of the case definitions [17]. In Norway, surveillance of LNB is based on a broader case definition comprising clinical symptoms with IgM antibodies in serum or spinal fluid or evidence of intrathecal production. The Norwegian Public Health Institute reports an average incidence of 6/100,000 per year (www.msis.no). However, including cases based on IgM antibodies in serum alone may confer problems with specificity and risk of overreporting. LNB is rare in the US with an incidence of 0.07/100,000 per year (www.cdc.org). This is due to the occurrence of B. burgdorferi sensu stricto in the US and the absence of B. garinii, which is the principal cause of LNB in Europe [18]. In 2013 the reported annual incidence of other causes of bacterial meningitis reported in Denmark was 3.0/100,000 and of these, pneumoccal meningitis represented 1.4/100,000 [19]. Thus LNB with an incidence of 3.2/100,000 per year was the most frequent bacterial cause of infection affecting the brain.

LNB is a relatively acute infection and this was reflected in the seasonal variation found in the present study. The number of LNB cases peaked in August and September, in line with a previous Danish study on LNB [8].

An advantage of the MiBa-based surveillance is that it also includes data on negative test results. This makes it possible to study healthcare practices including testing activity on an individual level in a defined population and calculate positive rates. We found that testing for LNB is common and that 96% were negative. During the late winter and the early spring, the diagnostic yield is very low. The low positivity rates suggest that symptoms suggestive of LNB are caused by other diseases, reflecting the non-specific nature of symptoms compatible with LNB.

Interestingly, the population-based LNB incidences stratified by age, sex and geography showed a different pattern compared with the incidences of patients being tested. Testing activity was highest in the age group 30 to 79 years, whereas the annual incidence of LNB peaked in childhood and again in the older age groups (55 to 79 years). Children are presumably exposed when they are playing, and a Dutch study found that people over 60 years were often bitten by ticks in their gardens [20].

The geographical distribution of AI-confirmed LNB has not been described previously in Denmark. In the present study, the results from MiBa indicated that annual incidence varied substantially across the country from zero to more than 10/100,000. The finding of hot spots on smaller islands has also been described for the Finish archipelago forming the region of Åland in the Baltic Sea [21]. Three Danish municipalities had no LNB cases; this absence could not be explained by lack of tested persons as the incidence of tested persons was 20–100/100,000 (Figure 4 and 5). Compared with the statutory notifications, the MiBa-based surveillance showed a different geographical distribution of LNB. The statutory system is biased by regional differences in notification rates; this bias is in part explained by SSI only sending reminders for missing notifications, based on laboratory results performed at SSI, which mainly received samples from the Capital region. This was not the case if testing was done at a regional laboratories because the epidemiological department at SSI then would be unaware of a possible positive AI. One limitation in the description of the geographical distribution is that we only have information on place of residence, which is not necessarily the place of exposure to tick bites.

Whereas the statutory notification system asks for information on clinical manifestation and sequelae, this information is not available in MiBa. In any case, the reporting of this additional clinical information was too incomplete to be useful (data not shown). Also asking for sequelae does not make sense in a reporting

system concerning acute disease as this requires a longer follow up. However, as the LNB cases in MiBa have a unique identifier that allows for linkage with other health registries, it will be possible to further explore risk factors for developing LNB, clinical manifestations and long-term sequelae in ad hoc projects. It will also be possible to compare testing for LNB with testing for other tick-borne diseases.

In time of writing, MiBa has accumulated data for four years, and procedures for data cleaning, analysis and aggregation are being automatised. Soon we will be able to calculate baselines and analyse for trends.

It may be relevant also to have surveillance in place for other clinical manifestations of Lyme borreliosis such as erythema migrans in order to estimate the burden of Lyme borreliosis. This may be possible in the future through data-capture from general practitioners' databases and the national patient register, as active reporting from clinicians would be difficult to motivate and organise. Other systemic manifestations in Denmark are rare and have been shown not to contribute significantly to the burden of Lyme borreliosis in clinical practice [11]. It was shown that consecutive patients with suspected Lyme arthritis had the same level of IgG seropositivity as had been found in Danish blood donors, indicating that the positive predictive value of the serology was negligible [11]. Thus being a rare unspecific clinical syndrome lacking a specific diagnostic test, Lyme arthritis is not a candidate for national surveillance.

Conclusion

In the present study, we found that a surveillance of LNB based on data from MiBa was more timely and more complete compared with the statutory surveillance based on manually processed notifications. We propose electronic reporting using the AI to replace clinical reporting as the basis of future LNB surveillance.

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Conflict of interest

None declared.

Authors' contributions

R Dessau has performed the statistical data analysis and drafted the first versions. Marianne Voldstedlund has performed data extraction with L Espenhain. L Espenhain has created the incidence maps of Denmark. All authors have contributed to the conception of the study and the revisions of the final manuscript.

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Estimating influenza vaccine effectiveness in Spain using sentinel surveillance data

S Jiménez-Jorge (silviajimenezjorge@gmail.com)[1,2], S de Mateo[1,2], C Delgado-Sanz[1,2], F Pozo[3], I Casas[3], M García-Cenoz[2,4], J Castilla[2,4], C Rodriguez[5], T Vega[5], C Quiñones[6], E Martínez[6], J M Vanrell[7], J Giménez[7], D Castrillejo[8], J M Altzíbar[2,9], F Carril[10], J M Ramos[11], M C Serrano[11], A Martínez[2,12], N Torner[2,12], E Pérez[13], V Gallardo[13], A Larrauri[1,2], on behalf of the Spanish Influenza Sentinel Surveillance System[14]

1. National Centre of Epidemiology, Institute of Health Carlos III, Madrid, Spain
2. CIBER Epidemiología y Salud Pública (CIBERESP), Institute of Health Carlos III, Madrid, Spain
3. National Centre for Microbiology, National Influenza Reference Laboratory, WHO National Influenza Centre, Institute of Health Carlos III, Madrid, Spain
4. Instituto de Salud Pública de Navarra, Navarre, Spain
5. Dirección General de Salud Pública, Consejería de Sanidad de Castilla y León, Valladolid, Spain
6. Servicio de Epidemiología y Prevención Sanitaria, Dirección General de Salud Pública y Consumo de La Rioja, La Rioja, Spain
7. Servicio de Epidemiología, Dirección General de Salut Pública, Mallorca, Baleares, Spain
8. Servicio de Epidemiología. Dirección General de Sanidad y Consumo, Consejería de Bienestar Social y Sanidad, Ciudad Autónoma de Melilla, Melilla, Spain
9. Dirección de Salud Pública de Gipuzkoa, País Vasco, San Sebastián, Spain
10. Servicio de Salud Pública, Departamento de Salud, Gobierno del País Vasco, Álava, Spain
11. Dirección General de Salud Pública, Servicio Extremeño de Salud, Junta de Extremadura, Mérida, Spain
12. Servicio de Vigilancia Epidemiológica, Dirección General de Salud Pública, Departament de Salut, Generalitat Catalunya, Barcelona, Spain
13. Servicio de Epidemiología y Salud Laboral, Secretaría General de Salud Pública y Participación, Consejería de Salud de Andalucía, Sevilla, Spain
14. Institutions involved in the network are listed at the end of the article (vgripe.isciii.es/gripe/)

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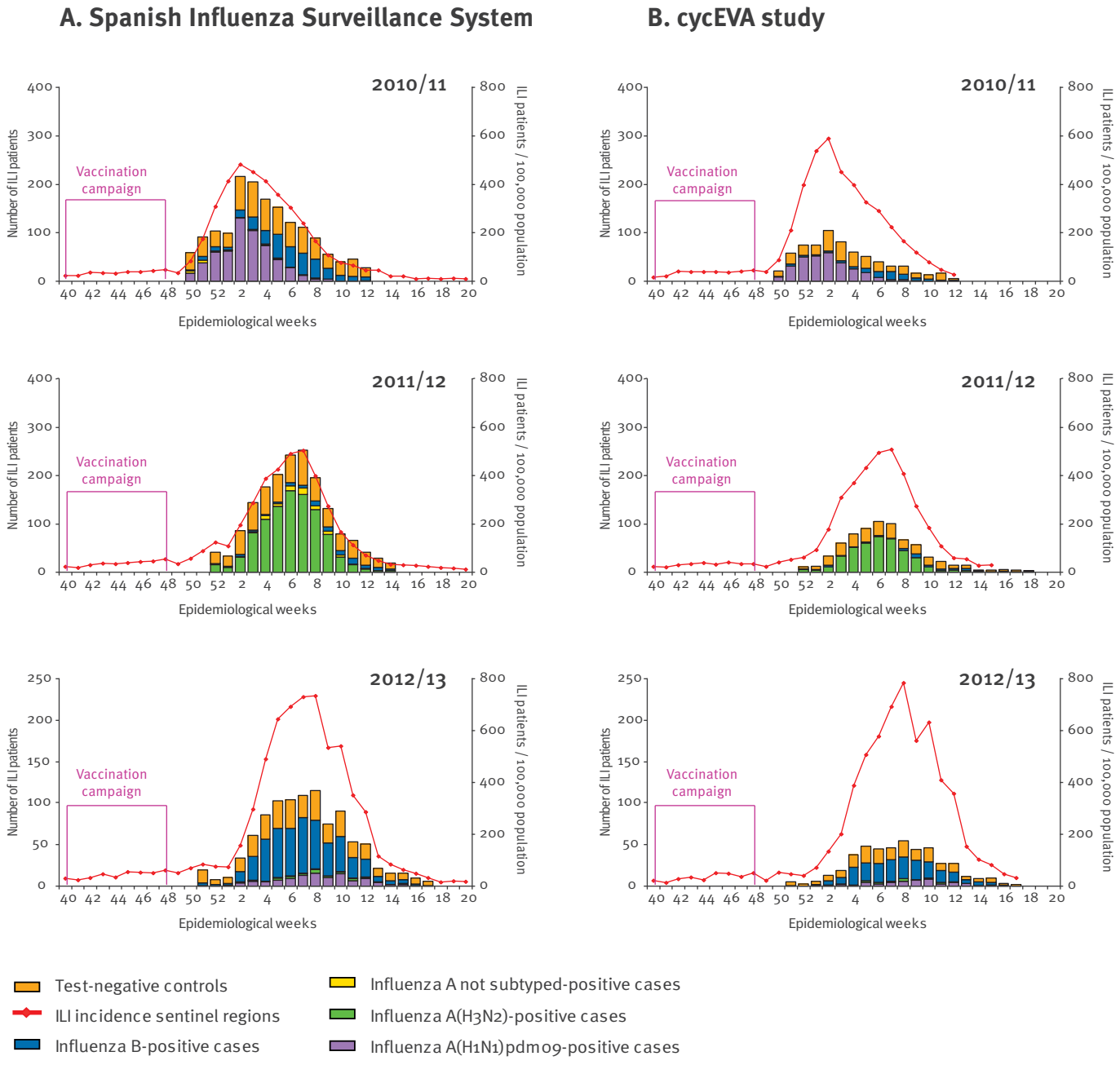
We aimed to estimate influenza vaccine effectiveness (VE) against laboratory-confirmed influenza during three influenza seasons (2010/11 to 2012/2013) in Spain using surveillance data and to compare the results with data obtained by the cycEVA study, the Spanish component of the Influenza Monitoring Vaccine Effectiveness (I-MOVE) network. We used the test-negative case–control design, with data from the Spanish Influenza Sentinel Surveillance System (SISS) or from the cycEVA study. Cases were laboratory-confirmed influenza patients with the predominant influenza virus of each season, and controls were those testing negative for any influenza virus. We calculated the overall and age-specific adjusted VE. Although the number of patients recorded in the SISS was three times higher than that in the cycEVA study, the quality of information for important variables, i.e. vaccination status and laboratory results, was high in both studies. Overall, the SISS and cycEVA influenza VE estimates were largely similar during the study period. For elderly patients (> 59 years), the SISS estimates were slightly lower than those of cycEVA, and estimates for children (0–14 years) were higher using SISS in two of the three seasons studied. Enhancing the SISS by collecting the date of influenza vaccination and reducing the percentage of patients with incomplete information would optimise the system to provide reliable annual influenza VE estimates to guide influenza vaccination policies.

Introduction

Influenza causes considerable morbidity worldwide, even among those who are not in vulnerable high-risk groups, and therefore represents a public health problem with socio-economic implications [1]. Influenza vaccination has the potential to prevent annual morbidity and premature mortality. The influenza vaccine is reformulated every year and consequently its effectiveness must be estimated annually [1]. In Europe, seasonal and pandemic influenza vaccine effectiveness (VE) has been monitored since the 2008/09 influenza season through the Influenza Monitoring Vaccine Effectiveness (I-MOVE) project [2], a publicly funded network supported by the European Centre for Disease Prevention and Control (ECDC) and European Union (EU) Member States in the framework of the European sentinel influenza systems. Since the inception of I-MOVE, Spain has participated through an observational case–control study to monitor influenza VE in Spain (cycEVA). This study is conducted within the framework of well-established sentinel influenza networks comprising the Spanish Influenza Sentinel Surveillance System (SISS).

Figure 1

Test-negative controls and laboratory-confirmed influenza cases by type/subtype of influenza virus and epidemiological week, Spanish Influenza Sentinel Surveillance System (A) and cycEVA study (B), 2010/11, 2011/12 and 2012/13 seasons, Spain



cycEVA study: the Spanish component of the Influenza Monitoring Vaccine Effectiveness (I-MOVE) network; ILI: influenza-like illness.

Participating sentinel physicians follow a European protocol specifically designed for this study [3]. The protocol includes systematic swabbing of recruited patients and recording the date of influenza vaccination and information on potential confounding factors that have not been historically collected during influenza surveillance. Through five influenza seasons, the cycEVA study has provided timely and reliable [4-8] influenza VE estimates and has been useful in guiding public vaccination policy at the national and European level [9]. However, after the initial ECDC funding was exhausted (December 2012), the Spanish cycEVA study encountered serious difficulties in continuing to measure influenza VE. Therefore, a major challenge in Spain and the rest of Europe is sustaining these VE studies.

Influenza surveillance data have been used in Australia, the United Kingdom (UK) and Canada [10-12] to monitor influenza VE using the test-negative control approach, an efficient method of estimating VE [13]. Because

surveillance data are already available, this method is less costly than observational studies.

The SISS was established in 1996 to provide timely epidemiological and virological information on influenza activity in Spain [14]. The SISS also participates in the European Influenza Surveillance Network (EISN). After more than 15 years, the SISS has been demonstrated to be a robust system for monitoring seasonal influenza [15]. Since the 2009/10 pandemic season, the SISS has been enhanced by increasing the number of swabs taken for virological confirmation, adopting a systematic sampling procedure and collecting information on the presence of chronic conditions and risk factors [16]. These approaches have positively affected the SISS by improving the quality and accuracy of its surveillance information and, consequently, enabling it to provide estimates of influenza VE [17]. In the present study, we aimed to estimate influenza VE against laboratory-confirmed influenza using surveillance data from the SISS during the three influenza seasons (2010/11 to 2012/13) following the A(H1N1)pdm09 pandemic and to compare these results with data obtained by the cycEVA study, to explore the feasibility and validity of monitoring the effectiveness of the influenza vaccine in Spain using surveillance data.

Methods

The Spanish Influenza Sentinel Surveillance System and cycEVA study

SISS was implemented more than a decade ago, in accordance with established national and international guidelines [18]. The system meets the surveillance requirements (European Influenza Surveillance Scheme, ECDC) regarding the minimum population covered (>1%) and representativeness in terms of age, sex and degree of urbanisation [19].

The SISS comprises 17 networks of sentinel physicians (general practitioners and paediatricians) in 17 of the 19 Spanish regions as well as network-affiliated laboratories, including the National Influenza Reference Laboratory (National Centre for Microbiology, World Health Organization National Influenza Centre in Madrid). Sentinel physicians report cases of influenza-like illness (ILI) detected in their reference populations on a weekly basis according to a definition that is based on the EU ILI case definition [20].

For influenza surveillance, sentinel physicians systematically swab (nasal or nasopharyngeal) the first two patients presenting with ILI each week and send the swabs to the network-affiliated laboratories for influenza virus detection.

The information collected in the SISS includes the patient's sex, age, symptom onset date, swabbing date, clinical symptoms, virological information (type and subtype detected and strain characterisation), chronic conditions (i.e. chronic cardiovascular diseases, chronic pulmonary diseases, congenital or acquired immunodeficiency, diabetes mellitus, chronic hepatic disease and chronic renal disease) and risk factors (i.e. pregnancy (in women aged 15–44 years) and morbid obesity (defined as body mass index (BMI) ≥ 40 kg/m2)).

Vaccination status is collected as a dichotomous variable (yes/no); this information is collected either by asking the patient (or parent/guardian if the patient is too young) whether they have received the current influenza seasonal vaccine ≥ 14 days before the onset of symptoms or from sentinel physician records.

The data are entered weekly by each regional sentinel network in a web-based application and analysed centrally by the National Centre of Epidemiology in Madrid to provide timely information on the evolving influenza activity in Spanish regions and at the national level [15] Physicians from sentinel networks participating in the cycEVA study collect additional information from patients, including date of vaccination, type of vaccine, previous seasonal influenza vaccination and information on confounding factors [8,21].

Study design and population

To measure influenza VE, we conducted two test-negative case–control studies on laboratory-confirmed influenza cases during the 2010/11, 2011/12 and 2012/13 influenza seasons using surveillance data (SISS) and data from the cycEVA study. Most of the patients included in the cycEVA analysis were also included in the SISS analysis, but the information collected in cycEVA was more exhaustive and accurate. The study period used was the same as that previously evaluated in the cycEVA study, i.e. the epidemic weeks of each season: week 50 2010 to week 11 2011 for the 2010/11 season, week 50 2011 to week 14 2012 for the 2011/12 season, and week 51 2012 to week 17 2013 for the 2012/13 season.

The study population using data from the SISS comprised all patients with ILI who consulted sentinel physicians belonging to the SISS. The first two ILI patients each week were swabbed and tested for influenza virus. The targeted vaccination groups were as follows: individuals older than 59 or 64 years (depending on the Spanish region), individuals with at least one chronic condition (i.e. cardiovascular disease, chronic pulmonary disease, congenital or acquired immunodeficiency, diabetes mellitus, hepatic disease or renal disease), pregnant women and/or morbidly obese individuals (BMI ≥ 40 kg/m2).

Cases were defined as patients with ILI with laboratory-confirmed influenza infection, as determined by reverse transcription (RT)-PCR analysis of samples obtained from respiratory specimens and/or cell culture using the Madin–Darby canine kidney (MDCK) cell line. Controls were defined as patients with ILI with who tested negative for any influenza virus strain.

Influenza VE was estimated by comparing the vaccination statuses of influenza virus-positive patients with those of influenza virus-negative patients.

The population, sampling protocol and definitions of cases and controls of the cycEVA study have been previously described [8,21].

Data analysis

Analyses were performed for the study population and for the population targeted for vaccination. Influenza VE estimates were compared using SISS and cycEVA data [8,21,22]. We estimated seasonal influenza VE against laboratory-confirmed influenza with the predominant influenza viruses A(H1N1)pdm09, A(H3N2) and B for the 2010/11, 2011/12 and 2012/13 seasons [15], respectively. We also studied the protective effect of the seasonal influenza vaccine by age group using the same categories used by the I-MOVE network (0–14 years, 15–59 years and ≥ 60 years) in the study population during the three seasons studied. We included in the analyses ILI patients with information available on vaccination status, laboratory confirmation of infection and swabbing date.

To reduce the risk of misclassification over time because of false-negative results, we restricted our analyses to ILI patients with a delay between symptom onset and swabbing: we included those swabbed less than eight days after symptom onset [23] in the 2010/11 and 2011/12 seasons and those swabbed less than four days after symptom onset in the 2012/13 season. For the analysis using SISS data, a sensitivity analysis was also undertaken: if dates of onset and/or swabbing were missing (in 15–17% of patients) then the delay between symptom onset and swabbing was assumed to have been less than eight days (98% of the patients with complete information on dates of symptom onset and swabbing had a delay of less than eight days).

Baseline characteristics of cases and controls were compared using chi-squared or Fisher's exact tests, as appropriate. Chi-squared test was used to compare proportions and $p < 0.05$ was considered to be statistically significant. Odds ratios (ORs) and their corresponding 95% confidence intervals (CIs) were obtained. Influenza VE was calculated using (1 – OR) × 100. Logistic regression models were used to estimate the unadjusted and adjusted ORs. For both the cycEVA study and SISS data, we adjusted for age group, week of swabbing and sentinel region. A comparison between influenza VE estimates for the three influenza seasons studied was performed for each data source (SISS and cycEVA), using a linear regression fit and testing whether the slopes and intercepts were significantly different [24].

Statistical analyses were conducted using STATA/IC 12.1 (StataCorp., College Station, Texas).

This study was performed within the framework of Spanish influenza surveillance activities, with no personal data collected. The patients or patient/guardian provided verbal informed consent to participate in the study. Consequently, the study did not require the approval of the Human Research Ethics Committee.

Results

Influenza season and characteristics of patients with influenza-like illness

The three influenza seasons included in the study in Spain differed in the presentation time of the epidemic, the type and subtype of the dominant virus (Figure 1) and the concordance between the vaccine and circulating influenza strains. On the basis of data from the SISS (Figure 1A) and the cycEVA study (Figure 1B), the weekly number of laboratory-confirmed influenza cases of influenza and test-negative controls recruited into the studies followed the same progression as the weekly ILI incidence in the participating regions during the three seasons studied.

In the 2010/11 influenza season, influenza A(H1N1) pdm09 predominantly circulated until the epidemic peak in week 2/2011 (240 ILI cases per 100,000 population), whereas influenza B virus became predominant after the epidemic period. Both circulating viruses were antigenically similar to the vaccine strains. Influenza activity in Spain during the 2011/12 season was associated with a predominance of circulating subtype A(H3N2) influenza virus and a lower contribution of influenza B virus, which emerged primarily after the influenza epidemic had peaked. The 2011/12 season was a late season, with the maximum peak of influenza activity occurring in mid-February 2012 (Figure 1) and with a limited match between the vaccine and the circulating strains. Influenza activity during the 2012/13 season also occurred late and peaked in February 2013. That season was clearly dominated by circulation of the influenza B/Yamagata lineage virus, co-circulating with both the A(H3N2) and the A(H1N1)pdm09 influenza A subtypes (Figure 1), which were all antigenically similar to the vaccine strains [25].

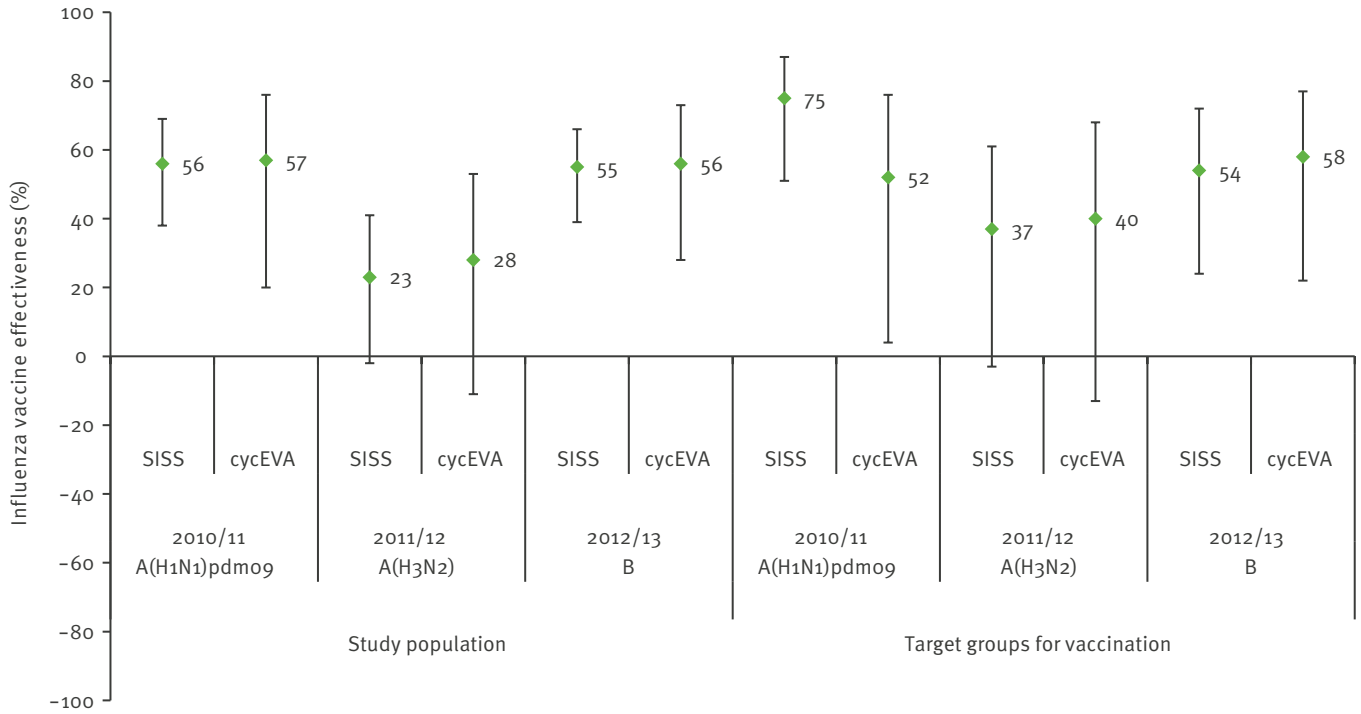
The annual influenza vaccination campaign in Spain lasted from September to November during the three influenza seasons studied (Figure 1).

During those seasons, the number of physicians participating in the SISS ranged from 867 to 885 (including 225–236 paediatricians) covering a population of 2.2–2.6% of the total Spanish population, which was representative in terms of age, sex and degree of urbanisation (Table 1). Of ILI patients visiting physicians who reported to the SISS during the study period (n = 48,000), between 4,454 and 4,583 per season were swabbed and received laboratory confirmation of influenza virus infection, which ranged from 27% in the 2012/13 season to 29% in the 2010/11 season.

The percentage of patients with incomplete information on laboratory results, vaccination status or date of

Figure 2

Adjusted influenza vaccine effectiveness of the seasonal trivalent vaccine against A(H1N1)pdm09 (2010/11 season), A(H3N2) (2011/12 season) and B influenza virus (2012/13 season) in the study population and target groups for vaccination[a], Spanish Influenza Sentinel Surveillance System and cycEVA study, Spain



cycEVA study: the Spanish component of the Influenza Monitoring Vaccine Effectiveness (I-MOVE) network; SISS: Spanish Influenza Sentinel Surveillance System.

The bars represent 95% confidence intervals.

[a] Individuals older than 59 or 64 years (depending on the Spanish region), individuals with at least one chronic condition (i.e. cardiovascular disease, chronic pulmonary disease, congenital or acquired immunodeficiency, diabetes mellitus, hepatic disease or renal disease), pregnant women and/or morbidly obese individuals (body mass index≥40 kg/m2).

symptom onset ranged from 3% to 5% of the patients who were swabbed (Table 1): these patients were excluded from the analysis. We also excluded 15–17% of the patients who were swabbed because the swabbing date was unknown. In addition, patients with laboratory-confirmed influenza A virus infection without any subtype information were not included in the specific analysis of influenza VE against the predominant influenza strain (range of 3.6–6% of the recruited patients in the study period).

After applying the exclusion criteria, we included 93% of the recruited patients from the 2012/13 season in the analysis (restricted to those patients swabbed less than four days after symptom onset) and 98% of the recruited patients for the 2010/11 and 2011/12 seasons (restricted to patients swabbed less than eight days after symptom onset) (Table 1).

From the patients who were included, we collected information on the presence of any chronic conditions or risk factors. This information was missing in 16–18% of the patients in the SISS in the first two seasons studied and only 3% in 2012/13 season (Table 1).

The number of GPs participating in the cycEVA study was 246, 231 and 239 for the 2010/11, 2011/12 and 2012/13 seasons, respectively, covering 2.1% of the total population of the Spanish regions participating in the cycEVA study (Table 1). Compared with the number of cycEVA GPs, the number of participating sentinel physicians within the SISS was more than 3.5 times greater (Table 1). The SISS also included a higher proportion of paediatricians, averaging 26% in the three seasons compared with 19% in the cycEVA study ($p<0.01$) (data not shown). Additionally, the number of patients with ILI determined using the SISS was three times higher than the number in the cycEVA study during the study period (Table 1). However, the information collected in the cycEVA study showed a lower percentage of incomplete information than that in the SISS; therefore, a lower percentage of recruited patients was excluded from the analysis (ranging from 0.1% to 5%, compared with 24–29% for the SISS). Information regarding possible confounding factors, such as the presence of any chronic conditions or risk factors, was also more comprehensive in the cycEVA study, with none of the recruited patients having incomplete data during the last two seasons of the study period (2011/12 and 2012/13).

Table 1

Spanish Influenza Sentinel Surveillance System and cycEVA study data, 2010/11, 2011/12 and 2012/13 seasons, Spain

Characteristic	2010/11 influenza season[a]		2011/12 influenza season[b]		2012/13 influenza season[c]	
	SISS	cycEVA	SISS	cycEVA	SISS	cycEVA
Number of participating Spanish regions	17	7	17	7	17	7
Number of participating GPs (percentage population covered)[d]	885 (2.6)	246 (2.1)	877 (2.4)	231 (2.1)	867 (2.2)	239 (2.1)
Number of ILI patients reported	15,302	1,376	16,286	1,471	16,486	1,471
Number of ILI patients swabbed (%)[e]	4,468 (29)	1,376 (100)	4,583 (28)	1,471 (100)	4,454 (27)	1,471 (100)
Exclusions (n (%))[fz]						
Laboratory result missing	228 (5.1)	0 (0.0)	151 (3.3)	14 (0.9)	184 (4.1)	7 (0.5)
Vaccination status missing	130 (2.9)	1 (0.07)	131 (2.9)	0 (0.0)	151 (3.4)	0 (0.0)
Date of symptom onset missing	234 (5.2)	0 (0.0)	198 (4.3)	0 (0.0)	14 (0.3)	0 (0.0)
Date of swabbing missing	778 (17)	0 (0.0)	686 (15)	0 (0.0)	753 (17)	0 (0.0)
Information on patients included in the analysis (n (%))[f]						
Swabbing restriction: patients with swabbing delay‹8 days[g]	3,180 (98)	1,369 (99)	3,484 (98)	1,446 (98)	3,357 (91)	1,432 (97)
Missing information on chronic conditions[h, i]	571 (18)	280 (20)	541 (16)	0 (0.0)	88 (2.0)	2 (0.13)
Missing information on risk factors[h,j]	574 (18)	83 (6.0)	1,020 (29)	0 (0.0)	615 (18)	1 (0.07)
Genetic characterisation of influenza viruses[k]	274 (11)	119 (15)	422 (14)	145 (16)	447 (17)	142 (16)
Patients with swabbing delay‹8 days[g] included in the subtype-/type-specific analysis[l]	2,480 (78)	1,165 (85)	3,189 (92)	1,325 (92)	2,875 (86)	1,225 (86)

cycEVA study: the Spanish component of the Influenza Monitoring Vaccine Effectiveness (I-MOVE) network; GP: general practitioner; ILI: influenza-like illness; SISS: Spanish Influenza Sentinel Surveillance System.

[a] Week 50 2010 to week 12 2011.

[b] Week 50 2011 to week 14 2012.

[c] Week 51 2012 to week 17 2013.

[d] Of the total Spanish population for the SISS and of the population of the Spanish regions participating in the cycEVA study.

[e] Of the reported patients.

[f] Of the swabbed patients.

[g] Patients with missing date of swabbing not included.

[h] Of patients with swabbing delay ‹ 8 days.

[i] Defined as diabetes mellitus, cardiovascular disease, chronic pulmonary disease, renal disease, hepatic disease, congenital or acquired immunodeficiency.

[j] Pregnancy (women 15–44 years-old) and morbid obesity (defined as body mass index ›40 kg/m^2).

[k] Of the total laboratory-confirmed influenza cases.

[l] A(H1N1)pdm09 in 2010/11 season, A(H3N2) in 2011/12 season and B in 2012/13 season.

Genetic sequencing of the haemagglutinin gene of the circulating influenza viruses isolated from patients in the SISS increased over the seasons studied, from 274 to 447 influenza strains, accounting for 11% and 17% of the total number of laboratory-confirmed influenza cases reported during the first and last seasons, respectively. The proportion of characterised viruses in the cycEVA study (among those included in the analysis) was similar to that in the SISS, with 16% of the viruses characterised in the last two seasons (2011/12 and 2012/13) studied (Table 1).

Taking into account that we estimated seasonal influenza VE against laboratory-confirmed influenza due to the predominant influenza viruses A(H1N1)pdm09, A(H3N2), and B, we finally included SISS data of 2,480, 3,189, and 2,707 patients from the 2010/11, 2011/12 and 2012/13 seasons, respectively, in the analysis. The sample obtained from the cycEVA study was

Table 2

Characteristics of laboratory-confirmed influenza cases and test-negative controls in the study population, Spanish Influenza Sentinel Surveillance System, 2010/11, 2011/12 and 2012/13 seasons, Spain

Characteristic	2010/11 Influenza season (n = 2,480)			2011/12 Influenza season (n = 3,189)			2012/13 influenza season (n = 2,707)		
	Controls	A(H1N1)pdm09 cases	P value[d, e]	Controls	A(H3N2) cases	P value[d,f]	Controls	B cases	P value[d,g]
	n/N (%)[a]	n/N (%)[a]		n/N (%)[b]	n/N (%)[b]		n/N (%)[c]	n/N (%)[c]	
Median age (range years)	22 (0–95)	26 (0–87)	**0.036**	26 (0–88)	31 (0–93)	**0.013**	27 (0–97)	17 (0–84)	**0.009**
Age group in years									
0–4	184/1,319 (14)	132/1,161 (11)	**0.000**	198/1,221 (16)	330/1,968 (17)	**0.021**	210/1,151 (18)	185/1,556 (12)	**0.000**
5–14	359/1,319 (27)	269/1,161 (23)		276/1,221 (23)	451/1,968 (23)		266/1,151 (23)	564/1,156 (36)	
15–64	694/1,319 (53)	733/1,161 (63)		678/1,221 (55)	1017/1,968 (52)		593/1,151 (52)	752/1,556 (48)	
≥65	78/1,319 (5.9)	25/1,161 (2.2)		69/1,221 (5.7)	169/1,968 (8.6)		81/1,151 (7.0)	53/1,556 (3.4)	
Missing information	4/1,319 (0.3)	2/1,161 (0.2)	0.800	0/1,221 (0.0)	1/1,968 (0.05)	**0.000**	1/1,151 (0.09)	2/1,556 (0.1)	0.793
Sex									
Male	665/1,319 (50)	593/1,161 (51)	0.820	638/1,221 (52)	949/1,968 (48)	0.081	600/1,151 (52)	772/1,556 (50)	0.382
Missing information	5/1,319 (0.4)	6/1,161 (0.5)	0.986	3/1,221 (0.2)	4/1,968 (0.2)	**0.000**	4/1,151 (0.3)	4/1,556 (0.3)	1.000
Chronic conditions									
Any chronic condition[h] reported	130/1,319 (10)	88/1,161 (7.6)	**0.000**	139/1,221 (11)	195/1,968 (10)	0.135	165/1,151 (14)	169/1,556 (11)	**0.025**
Missing information	229/1,319 (17)	294/1,161 (25)	**0.000**	182/1,221 (15)	338/1,968 (17)	0.130	27/1,151 (2.3)	38/1,556 (2.4)	0.839
Risk factors									
Any risk factor reported[i]	31/1,319 (2.3)	21/1,161 (1.8)	**0.000**	22/1,221 (1.8)	39/1,968 (2.0)	0.936	14/1,151 (1.2)	17/1,556 (1.1)	0.753
Missing information	432/1,319 (33)	478/1,161 (41)	**0.000**	364/1,221 (30)	587/1,968 (30)	0.998	209/1,151 (18)	267/1,556 (17)	0.518
Vaccine status[j]									
All ages	155/1,319 (12)	64/1,161 (5.5)	**0.000**	149/1,221 (12)	222/1,968 (11)	0.430	142/1,151 (12)	83/1,556 (5.3)	**0.000**
Vaccine eligibility									
Eligible for vaccination	66/183 (36)	27/128 (21)	**0.005**	77/173 (44)	117/284 (41)	0.487	72/176 (41)	39/182 (21)	**0.000**

P values in bold are statistically significant.

[a] Cases and controls recruited between week 50 2010 and week 12 2011 and with an interval between symptom onset and swabbing of less than eight days.

[b] Cases and controls recruited between week 52 2011 and week 14 2012 and with an interval between symptom onset and swabbing of less than eight days.

[c] Cases and controls recruited between week 51 2012 and week 17 2013 and with an interval between symptom onset and swabbing of less than four days.

[d] Non-parametric test of the median or chi-squared test or Fisher's exact test, when appropriate.

[e] A(H1N1)pdm09 cases vs controls p value.

[f] A(H3N2) cases vs controls p value.

[g] B cases vs controls p value.

[h] Defined as diabetes mellitus, cardiovascular disease, chronic pulmonary disease, renal disease, hepatic disease, congenital or acquired immunodeficiency.

[i] Defined as pregnancy (women 15–44 years-old) and/or morbid obesity (body mass index ≥ 40 kg/m^2).

[j] Only patients with known vaccination status were included in the analysis.

1.8–2.4 times smaller in size (1,165, 1,325 and 1,192 patients, respectively). Among the SISS patients analysed, we identified 1,319 controls and 1,161 A(H1N1) pdm09 cases for the 2010/11 season; 1,221 controls and 1,968 A(H3N2) cases for the 2011/12 season; and 1,151 controls and 1,556 B cases for the 2012/13 season (Table 2).

The main characteristics of cases and controls during the study period are shown for the SISS (Table 2) and

Table 3

Characteristics of laboratory-confirmed influenza cases and test-negative controls in the study population, cycEVA study, 2010/11, 2011/012 and 2012/13 seasons, Spain

Characteristic	2010/11 Influenza season (n = 1,165)			2011/12 Influenza season (n = 1,348)			2012/13 influenza season (n = 1,192)		
	Controls	A(H1N1) pdm09 cases	P value[d, e]	Controls	A(H3N2) cases	P value[d,f]	Controls	B cases	P value[d,g]
	n/N (%)[a]	n/N (%)[a]		n/N (%)[b]	n/N (%)[b]		n/N (%)[c]	n/N (%)[c]	
Median age in years (range)	32 (0–95)	31 (0–85)	0.493	33 (0–87)	36 (0–93)	0.067	32 (0–85)	31 (0–84)	0.711
Age group (years)									
0–4	46/591 (8)	43/574 (7)	**0.000**	41/528 (8)	96/820 (12)	**0.001**	75/535 (14)	68/657 (10)	**0.000**
5–14	111/591 (19)	73/574 (13)		90/528 (17)	133/820 (16)		102/535 (19)	197/657 (30)	
15–64	387/591 (65)	441/574 (77)		358/528 (68)	491/820 (60)		323/535 (60)	362/657 (55)	
≥ 65	47/591 (8)	17/574 (3)		39/528 (7)	100/820 (12)		35/535 (7)	30/657 (5)	
Missing information	0/591 (0)	0/574 (0)	NA	0/528 (0)	0/820 (0)	NA	0/535 (0)	0/657 (0)	NA
Sex									
Male	293/591 (50)	271/574 (47)	0.419	265/528 (50)	402/820 (49)	0.676	277/535 (52)	333/657 (51)	0.708
Missing information	0/591 (0)	0/574 (0)	NA	0/528 (0)	0/820 (0)	NA	0/535 (0)	0/657 (0)	NA
Chronic conditions									
Any chronic condition reported	79/591 (13)	60/591 (10)	0.174	78/528 (15)	117/820 (14)	0.797	103/535 (19)	96/657 (15)	**0.033**
Missing information	126/591 (21)	112/574 (20)	0.673	0/528 (0)	0/820 (0)	NA	0/535 (0)	0/657 (0)	NA
Risk factors									
Any risk factor reported[h]	13/591 (2.2)	7/574 (1.2)	0.091	9/528 (1.7)	18/820 (2.2)	0.530	9/535 (1.7)	9/657 (1.4)	0.490
Missing information	30/591 (5)	44/574 (8)	**0.037**	0/528 (0)	0/820 (0)	NA	1/535 (0.2)	0/657 (0)	**0.000**
Vaccine status[i]									
All ages	63/591 (11)	23/574 (4)	**0.000**	69/528 (13)	111/820 (14)	0.805	56/535 (10)	31/657 (5)	**0.000**
Vaccine eligibility									
Eligible for vaccination	57/184 (31)	20/135 (15)	**0.001**	50/123 (41)	88/226 (39)	0.755	45/133 (34)	26/139 (19)	**0.005**

cycEVA study: the Spanish component of the Influenza Monitoring Vaccine Effectiveness (I-MOVE) network; NA: not applicable.

P values in bold are statistically significant.

[a] Cases and controls recruited between week 50 2010 and week 12 2011 and with an interval between symptom onset and swabbing of less than eight days.

[b] Cases and controls recruited between week 52 2011 and week 14 2012 and with an interval between symptom onset and swabbing of less than eight days.

[c] Cases and controls recruited between week 51 2012 and 17 2013 and with an interval between symptom onset and swabbing of less than four days.

[d] Non parametric test of the median or chi-squared test or Fisher's exact test, when appropriate.

[e] A(H1N1)pdm09 cases vs controls p value.

[f] A(H3N2) cases vs controls p value.

[g] B cases vs controls p value.

[h] Defined as pregnancy (women 15–44 years-old) and/or morbid obesity (body mass index ≥ 40 kg/m^2).

[i] Only patients with known vaccination status were included in the analysis.

cycEVA study (Table 3). Regarding the main characteristics of the patients included in the analysis, overall, the most represented age group was 15–64 year-olds, who accounted for 50–58% of all recruited patients from each season according to the SISS data (Table 2) and 57–71% according to the cycEVA data (Table 3).

Vaccine effectiveness*

Adjusted influenza VE estimates for the study population were similar using data from the SISS and cycEVA study: 56% (95% CI: 38 to 69) and 57% (95% CI: 20 to 76), 23% (95% CI: −2 to 41) and 28% (95% CI: −11 to 53), and 55% (95% CI: 39 to 66) and 56% (95% CI:

28 to 73) in the 2010/11, 2011/12 and 2012/13 influenza seasons, respectively (Figure 2). Adjusted influenza VE estimates in the population targeted for vaccination were also consistent using both data sources, although the SISS point estimates were slightly higher for the 2010/11 season (75% (95% CI: 51 to 87)) than the 52% (95% CI: 4 to 76) in the cycEVA study (Figure 2). The comparison analyses showed no statistically significant differences in the slopes of influenza VE estimates along the three studied seasons for the two data sources, either for the study population ($F^1_5 = 0.03$; $p = 0.88$) or for the population targeted for vaccination ($F^1_5 = 0.51$; $p = 0.55$).

On the assumption that patients with missing dates of onset and/or swabbing were swabbed within eight days from symptom onset, we estimated VE for the study population to be 64% (95% CI: 51 to 73), 9% (95% CI: –16 to 27) and 60% (95% CI: 47 to 70) for the 2010/11, 2011/12 and 2012/13 seasons, respectively. For the target groups for vaccination, VE estimates were 70% (95% CI: 48 to 83), 33% (95% CI: –1 to 55) and 62% (95% CI: 38; to 77) for the 2010/11, 2011/12 and 2012/13 seasons, respectively.

The analysis by age group in the study population showed that influenza VE for patients aged 15–59 years and those older than 59 years were similar using data from either the SISS or cycEVA study (Table 4).

For patients aged 15–59 years, the VE estimates using both data sources ranged from 30% to 74%, with a higher and optimal protective effect of the vaccine during the 2012/13 season using the cycEVA study data (74% (95% CI: 38 to 89)) and lower but not statistically significant difference in the 2011/12 season with SISS data (30% (95% CI: –8 to 54)). For elderly patients (›59 years), adjusted VE estimates ranged from 42% to 72%, with a higher and optimal protective effect of the vaccine during the 2012/13 season using the cycEVA study data (72% (95% CI: 15 to 91)). In general, the sample size was 1.7 to 2.4 times higher using SISS compared with cycEVA study data and, consequently, SISS-estimates by age group generally showed narrower confidence intervals (Table 4).

However, estimates from the two data sources for patients aged 0–14 years were not comparable. We did not find any protective effect of the vaccine using cycEVA study data; however, using SISS data, the VE estimates were 31% (95% CI: –17 to 60) and 57% (95% CI: 22 to 76) in 2011/12 and 2012/13, respectively. For the 2010/11 season, the adjusted VE estimates were identical for the two data sources (Table 4). Regarding sample size, using SISS data we included in the specific 0–14 years analysis 2.6–5 times more patients than with the cycEVA study data.

Lower VE estimates in each age group were generally observed during the late 2011/12 influenza season using both data sources (Table 4).

Discussion

Our estimates of influenza VE against laboratory-confirmed influenza using surveillance data were largely similar to those obtained from the observational cycEVA study [8,21,22] and showed a moderate protective effect for the trivalent influenza seasonal vaccine during the study period.

For the 2010/11 season, adjusted VE estimates against the predominant A(H1N1)pdm09 influenza virus, was 56% and 57% using SISS and cycEVA, respectively, in line with those described by the I-MOVE network [26], the UK [27] and the Navarre region of Spain [28], which ranged from 55% to 62%. Lower estimates against A(H3N2) virus (23% (SISS) and 25% (cycEVA)) were also observed in the Navarre region (29%) [29] and in the I-MOVE network (25%) [30] during the late 2011/12 influenza season, as well as in previous A(H3N2) dominant seasons (31%) in Spain [31]. For the 2012/13 season, adjusted VE estimates against B virus were 56% and 62% using SISS and cycEVA, respectively, similar to those observed in the I-MOVE network [32].

Limitations arising from surveillance versus research-oriented systems

When studying the protective effect of the seasonal influenza vaccine among the groups targeted for vaccination, we observed some difference in the influenza VE point estimates using the SISS and cycEVA data, although they were not statistically significant. Some of these could have been caused by limitations arising from use of surveillance data, which will be described below.

The quality of the information collected by the SISS and the cycEVA study on exposure (influenza vaccination) and outcome (laboratory confirmation) was satisfactory, with low percentages of incomplete information in both systems (around 0–3.5%) [11,33].

A more substantial limitation of our surveillance data was missing information on swabbing date (in 15–17% of recruited patients): this information is crucial when restricting the analysis according to time between symptom onset and swabbing, and helps to minimise the possibility of misclassification as false-negative RT-PCR results [23]. However, the sensitivity analyses, which included patients with missing dates of onset and/or swabbing, on the assumption that they were swabbed within eight days from symptom onset (as did 98% of the patients with complete information), showed VE estimates that differed by 4–8% and with narrower CIs. In spite of that, the differences were higher (14%) for the study population for the 2011/12 season, a season characterised by a late epidemic peak and a limited match between the circulating A(H3N2) influenza virus and the vaccine strain, and in which the trivalent seasonal vaccine showed a lower protective effect compared with other influenza seasons [25].

SISS data also contained a high proportion of patients with missing co-morbidity data, which could bias VE

estimates from the surveillance data. Although the SISS point estimates were only 7–8% lower than those observed with cycEVA data during the last two seasons studied (Figure 2), the VE estimates using SISS could be overestimated for the 2010/11 season (75% compared with 52% with cycEVA for the target groups). This discrepancy could be related to a higher vaccine coverage for patients with information on chronic conditions/risk factors (included in analysis for the target groups) than for patients with missing information on chronic conditions/risk factors (not included in the analysis for the target groups), with coverage of 9% and 7%, respectively ($p < 0.05$).

Results of a sensitivity analysis excluding patients with missing information on chronic conditions (data not shown) showed similar adjusted VE estimates (point differences ranged from 4% to 7% in 2010/11 and 2011/12 and an exact point estimate of 55% in 2012/13). In addition, inclusion of the variable chronic conditions into the regression models did not significantly change VE estimates. Therefore, missing information on chronic conditions was unlikely to have biased our VE estimates using SISS data. Imputation techniques will be used in further analyses in order to adjust for missing values in key variables [34,35].

Another reason for the observed discrepancies between the results from the two data sources could be possible differences in the main characteristics of the study populations. The median age of patients in the SISS were 6–10 years younger than that of the patients in the cycEVA study.

Information on vaccine status was collected by the sentinel physicians based on patient self-report at the time of specimen submission, before the test result was known, thus minimising differential recall bias. Although this could generate a potential source of misclassification, studies in other settings have reported consistency between self-reported and registry-based influenza vaccination status [36,37].

A more general limitation of the surveillance strategy is that the system does not currently collect the vaccination dates of patients. However, the likelihood of vaccination status misclassification within the SISS it is low since the seasonal influenza vaccination campaign usually finishes in Spain well before the beginning of the influenza season. Only unusual scenarios, such as influenza pandemics [7,12] or when the influenza season starts early, would require a specific observational study to estimate influenza VE.

A further limitation of our study could arise from the fact that comparison of VE estimates was made among two data sources that are not mutually exclusive. Patients in the cycEVA study were a subset of SISS patients for whom GPs collected additional information on confounders and date of influenza vaccination.

We also have to be aware that the distribution of influenza virus strains might differ by time of the epidemic and region: this could explain certain differences in the VE estimates obtained in this study, which was focused on influenza VE against the predominant circulating influenza subtype.

Age-specific vaccination effectiveness estimates

By age group, the SISS influenza VE estimates were quite similar to those from the cycEVA study for 15–59 age group (±10–13%), who comprised the most represented age group in both study populations, and for the elderly (±5–12%), except for the 2012/13 season, with 19% points of difference between the SISS and cycEVA estimates.

In general, point estimates using the surveillance data were lower for both age groups compared with cycEVA study estimates. Differences in the estimates for elderly patients could be related to different swabbing practices (all patients in cycEVA study but the first two patients of any age each week in the SISS). However, both criteria for selecting patients for swabbing were recently shown to give similar influenza VE estimates [37]. Considering the difference in the extent of data collection for important confounders in elderly patients, the influenza VE estimates for this group could have been under estimated using the surveillance data. By improving the quality of information and the swabbing protocol in the future [30], we should be able to overcome the limited accuracy of our current influenza VE estimates for elderly patients using SISS data.

Comparison with published data

In general, our age-specific VE estimates were comparable to those in other European countries and regions. Point estimates for patients aged 15–59 years were in the range of those described by the Navarre region, the I-MOVE network and the UK [28,32,34]. Point estimates lower than 50% in preventing A(H3N2) infections in the late 2011/12 season (30% with SISS and 41% with cycEVA) were also described in patients younger than 65 years in the UK (19%) [35] and the Navarre region (44%) [29], although a higher protective effect was observed by the I-MOVE network (63%) [30]. In addition, protective estimates against influenza B virus observed during the 2012/13 season, 64% (SISS) and 74% (cycEVA), were comparable to the 64% observed by I-MOVE [32]. Our point estimates for elderly patients were in line with those published by the UK: 48% protection against A(H3N2) virus in the 2011/12 season [35] (47% (SISS), 42% (cycEVA)) and higher protective effect against B virus in 2012/13 season (65% in UK [34] vs 53% (SISS) and 72% (cycEVA)). In the 2010/11 season, our VE estimates for elderly patients were lower than those from the I-MOVE network [26], 47–59% vs 72%. The differences observed could be related to differences in vaccine coverage, vaccine brands used, proportion of people with chronic conditions, and/or characteristics of influenza circulating strains.

Table 4

Adjusted influenza vaccine effectiveness of the seasonal trivalent vaccine against the predominant circulating influenza virus in the study population by age group, Spanish Influenza Sentinel Surveillance System and cycEVA study, 2010/11, 2011/12 and 2012/13 influenza seasons, Spain

Influenza season	Age group in years	Data source	Total number of cases/ controls	Number of vaccinated cases/ vaccinated controls	Adjusted influenza VE % (95% CI)
2010/11	0–14	SISS	476/725	20/58	60 (28 to 78)[a]
		cycEVA	116/157	2/6	60 (–180 to 94)[a]
	15–59	SISS	910/917	35/76	56 (32 to 72)[b]
		cycEVA	419/358	9/18	43 (–41 to 77)[b]
	≥60	SISS	65/146	24/65	47 (–23 to 78)[c]
		cycEVA	39/76	12/39	59 (–19 to 76)[c]
2011/12	0–14	SISS	781/474	37/34	31 (–17 to 60)[a]
		cycEVA	229/128	13/8	2 (–186 to 66)[a]
	15–59	SISS	944/638	59/51	30 (–8 to 54)[b]
		cycEVA	452/334	21/22	41 (–16 to 70)[b]
	≥60	SISS	242/109	126/64	47 (3 to 72)[c]
		cycEVA	141/63	77/39	42 (–29 to 74)[c]
2012/13	0–14	SISS	749/476	24/31	57 (22 to 76)[a]
		cycEVA	265/177	8/5	–22 (–305 to 63)[a]
	15–59	SISS	701/560	28/50	64 (40 to 79)[b]
		cycEVA	334/305	8/22	74 (38 to 89)[b]
	≥60	SISS	104/114	31/60	53 (5 to 77)[c]
		cycEVA	58/53	15/29	72 (15 to 91)[c]

CI: confidence interval; cycEVA study: the Spanish component of the Influenza Monitoring Vaccine Effectiveness (I-MOVE) network; SISS: Spanish Influenza Sentinel Surveillance System; VE: vaccine effectiveness.

[a] Model adjusted for age group (0–4, 5–9 and 10–14 years), week of swabbing and Spanish region.

[b] Model adjusted for age group (15–40 and 41–59 years), week of swabbing and Spanish region.

[c] Model adjusted for age group (60–69, 70–79, 80–89 and 90–105 years), week of swabbing and Spanish region.

Regarding the younger age groups, the SISS estimates were generally higher and more precise than the cycEVA estimates (the sample size of the cycEVA study being 2.5–5 times lower than for the SISS (Table 3)). Children monitored by the cycEVA study were under-represented compared with children in the SISS because of a low proportion of paediatricians among the participating sentinel physicians. We would therefore like to highlight the importance of performing influenza VE analysis by age group, especially in elderly patients, the main group recommended for vaccination. Age group-specific VE estimates shown in this study, although limited by a lack of precision with wide CIs that do not indicate statistical significance, will allow comparisons to be drawn among countries and regions and across seasons.

After five influenza seasons, the cycEVA study has become a system that is capable of rapidly providing and disseminating reliable information on influenza VE on an annual basis at the national and European level [4-8,21,22,26,30,38]. This research-oriented system was able to address ancillary questions, such as the effect of repeated annual vaccination, waning immunity, potential sources of bias and confounding (beyond what is collected by sentinel networks) and other issues. Currently, however, cost is a critical factor limiting the sustainability of this study.

Surveillance networks have been shown to be excellent frameworks for conducting influenza VE studies [10-12,39]. By using data from existing systems, surveillance networks are simpler and less expensive than observational studies. In addition, these networks have the advantage of larger sample sizes and being representative of the entire country. Larger sample sizes would allow increasingly important early in-season estimates to be carried out, when the virus is still circulating. This ability is crucial to contribute to the World Health Organization's seasonal vaccine composition consultation for deliberation on influenza viruses for vaccines for the next season [40] and supports the possibility of obtaining more accurate subgroup

estimates (e.g. for target groups, virus types/subtypes and patient age groups).

Most of the limitations described in the current sentinel surveillance system could be overcome without costly modifications, including collection of the date of influenza vaccination and a reduction in the percentage of patients for whom there is incomplete information. To enhance the exhaustiveness of the data, we recommend emphasising to sentinel physicians the importance of improving the completeness of collected data at the regional level, with subsequent checking and validation of the data at the national level. Although strengthening the national influenza surveillance system does not require extra costs, it will require a long-term commitment of both human and material resources.

In conclusion, while acknowledging a role for a spectrum of VE research approaches, real-time monitoring of influenza VE using routine surveillance data is currently feasible in Spain and meets the minimum requirements described for influenza VE studies [41]. Enhancing the SISS by overcoming the drawbacks mentioned would optimise the system to provide reliable annual influenza VE estimates that guide national health authorities who implement influenza vaccination policies.

The sustainability of the well-established Spanish cycEVA study, as part of the I-MOVE network, is a crucial factor for more efficient validation and optimisation of the Spanish Influenza Sentinel Surveillance System.

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Conflict of interest

None declared.

Authors' contributions

Silvia Jiménez-Jorge and Amparo Larrauri designed the study. Silvia Jiménez-Jorge wrote the first draft of the manuscript and undertook the statistical analysis. Silvia Jiménez-Jorge, Salvador de Mateo and Amparo Larrauri participated in data analysis, writing and interpretation of the results. Francisco Pozo and Inmaculada Casas established the microbiology database and contributed with the interpretation of the virological data. All authors participated in the interpretation of the data, contributed to the revision of the draft manuscript and approved the final version.

Members of the Spanish Influenza Sentinel Surveillance System

Physicians of the influenza sentinel surveillance networks of: Andalucía, Aragón, Asturias, Baleares, Canarias, Cantabria, Castilla la Mancha, Castilla y León, Cataluña, Comunidad Valenciana, Extremadura, Madrid, Navarre, País Vasco, La Rioja, Ceuta and Melilla; Epidemiologists from: Servicio de Vigilancia Epidemiológica y Evaluación, Consejería de Salud, Junta de Andalucía; Servicio de Vigilancia en Salud Pública, Dirección General de Salud Pública, Aragón; Dirección General de Salud Pública y Planificación, Consejería de Salud y Servicios Sanitarios, Asturias; Servicio de Epidemiología, Dirección General de Salut Pública, Baleares; Servicio de Epidemiología y Prevención, Consejería de Sanidad de Canarias; Sección de Epidemiología, Consejería de Sanidad, Trabajo y Servicios Sociales de Cantabria; Servicio de Epidemiología, Consejería de Sanidad de Castilla la Mancha; Dirección General de Salud Pública, Consejería de Sanidad de Castilla y León; Servicio de Vigilancia Epidemiológica, Dirección General de Salud Pública, Departament de Salut, Generalitat Catalunya; Àrea d'Epidemiologia, Conselleria de Sanitat, Comunitat Valenciana; Subdirección de Epidemiología, Dirección General de Salud Pública, Servicio Extremeño de Salud, Junta de Extremadura; Dirección Xeral Saúde Pública de Galicia; Dirección General de Atención Primaria de la Comunidad de Madrid; Servicio de Epidemiología, Consejería de Sanidad de la Región de Murcia; Instituto de Salud Pública de Navarra; Servicio de Vigilancia Epidemiológica, Consejería de Sanidad del País Vasco; Servicio de Epidemiología, Subdirección de Salud Pública de La Rioja; Sección de Vigilancia Epidemiológica, Consejería de Sanidad y Bienestar Social de Ceuta; Servicio de Epidemiología, Dirección General de Sanidad y Consumo, Consejería de Bienestar Social y Sanidad, Ciudad Autónoma de Melilla; Virologists from the following laboratories: National Influenza Reference Laboratory, WHO Influenza collaborating Centre (National Centre of Microbiology, ISCIII, Majadahonda-Madrid); WHO Influenza collaborating Centre, Facultad de Medicina de Valladolid; WHO Influenza collaborating Centre, Hospital Clínico de Barcelona; Hospital Virgen de las Nieves de Granada, Andalucía; Laboratorio del Hospital Miguel Servet de Zaragoza, Aragón; Laboratorio del Hospital Nuestra Senora de Covadonga de Oviedo, Asturias; Laboratorio del Hospital Son Dureta de Palma de Mallorca, Baleares; Laboratorio del Hospital Dr. Negrín de Las Palmas de Gran Canaria, Canarias; Laboratorio del Hospital Universitario Marqués de Valdecilla de Santander; Instituto Valenciano de Microbiología, Valencia, Comunitat Valenciana; Hospital San Pedro de Alcántara de Cáceres, Extremadura; Servicio de Microbiologia del Hospital Universitario Ramon y Cajal, Madrid; Laboratorio de la Clínica Universidad de Navarra, Pamplona, Navarra; Laboratorio de Microbiología del Hospital Donostia, País Vasco; Hospital San Pedro de la Rioja de Logroño, La Rioja; Laboratorio de Microbiología del Hospital de INGESA de Ceuta; Laboratorios de Microbioloxía CH de Vigo y de Ourense (Galicia), and Hospital Virgen de la Arrixaca de Murcia.

* Authors' correction

The adjusted influenza vaccine effectiveness estimates in the text for 2012/13 for the study population obtained from SISS data and cycEVA study data were corrected. These changes were made on 21 July 2015, at the request of the authors.

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