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A community outbreak of Legionnaires' disease in South Wales, August–September 2010

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During August and September 2010, an outbreak comprising 22 cases of Legionnaires' disease was identified by the public health service in Wales. The cases are distributed over a wide geographical area in South East Wales. There are two space-time clusters centred on the upper Rhymney Valley and the lower Cynon Valley respectively. Epidemiological investigations are compatible with cooling towers in each location as the potential source, but environmental inspections were satisfactory and microbiological investigations are inconclusive.

Outbreak description

In mid and late August 2010, six cases of Legionnaires' disease, with no history of recent travel abroad, were reported to the public health service. All the patients tested positive for urinary antigen for *Legionella pneumophila* serogroup 1 (mAb2 positive), which is the most common cause of Legionnaires' disease in the United Kingdom (UK). There were 24 cases of Legionnaires' disease in Wales in 2009 and an average of 13 cases per year over the past 10 years. A multidisciplinary Outbreak Control Team was convened on 3 September 2010 and an outbreak of Legionnaires' disease was declared.

Epidemiological investigation

Active case finding was undertaken by alerting clinicians throughout Wales and by alerting public health professionals throughout the UK. All cases of Legionnaires' disease reported in Wales from 1 July 2010 to 30 September 2010 were reviewed. A probable outbreak-associated case was defined as a person with a positive urine antigen test for *L. pneumophila* and onset of symptoms after 1 July 2010, who lived in, or had visited, the outbreak area during the 14 days before onset of symptoms. The outbreak case definition was based on the European Union case definitions for Legionnaires' disease [1]. The outbreak area was defined as the 12 km corridor on either side of the Heads of the Valleys Road (A465). This is a major road that links South West Wales with South East Wales and the English Midlands. Over the next two weeks a further 16 cases of Legionnaires' disease were identified.

Environmental health officers from 10 county or city councils interviewed all cases as soon as possible after notification. Information on demographic factors and recent movements within and outside the outbreak area was collected for the 14–day period before the onset of symptoms. Patients' residence and movements, as well as the locations of cooling towers in the area, were mapped using a geographical information system in order to help generate hypotheses about potential sources of exposure.

Environmental and microbiological investigations

By law, all cooling towers and evaporative condensers in the UK are required to be registered with the local council [2]. Owners should also follow the Approved Code of Practice (ACOP) on their operation and maintenance [3]. The Health and Safety Executive (HSE) inspected registered premises in the Merthyr Tydfil, Blaenau Gwent, Rhondda Cynon Taff and Caerphilly county council areas to identify any operating deficiencies. A search was also undertaken for unregistered premises. In addition, other potential sources within the outbreak area that might generate aerosols such as car wash and jet wash facilities were visited and inspected by local authority environmental health officers. Water samples were taken from a wide variety of sources at all sites that were found to have operating deficiencies or that were epidemiologically linked to the outbreak and analysed for legionella by PCR and culture.

Environmental samples were sent to the Severn Trent Water Company laboratory for testing and to the Respiratory and Systemic Infection Laboratory (RSIL) of the Health Protection Agency for further typing. Patient samples were also collected and sent to RSIL

for testing and typing, in order to identify a match with the potential environmental source.

Results

Thirty-one patients with Legionnaires' disease with onset since 1 July 2010 were identified, 22 of whom met the outbreak case definition [1]. Dates of onset of symptoms ranged from 4 August to 10 September 2010 (Figure 1) and none had travelled abroad in the two weeks beforehand.

Cases had a median age of 65 years (range 38-86) and most had underlying medical risk factors that are known to be associated with Legionnaires' disease. There were 15 males and seven females. All 22 cases were admitted to hospital and two died (case fatality rate 7%). There were two distinct spatio-temporal clusters (Figure 2): a cluster of seven people in the upper Rhymney Valley (cluster A) and a cluster of six people in the lower Cynon Valley (cluster B) including one case linked to both. The clusters are located around 15km apart but both are within a 5 km radius of a cooling tower. Of the remaining 10 cases, two were epidemiologically linked to a retail premises outside the outbreak area and one was microbiologically linked to another premises outside the outbreak area. The source for the remaining cases is unknown.

Laboratory results

All cases tested positive for urinary antigen for *L. pneumophila* serogroup 1 (mAb2 positive). Respiratory samples were available for typing from 11 patients. *L. pneumophila* has so far been cultured/typed from four patients and typed directly from sputum in a further three. Six strains are different subtypes and/or genotypes and neither cluster A nor B can be clearly characterised (Table).

Environmental samples

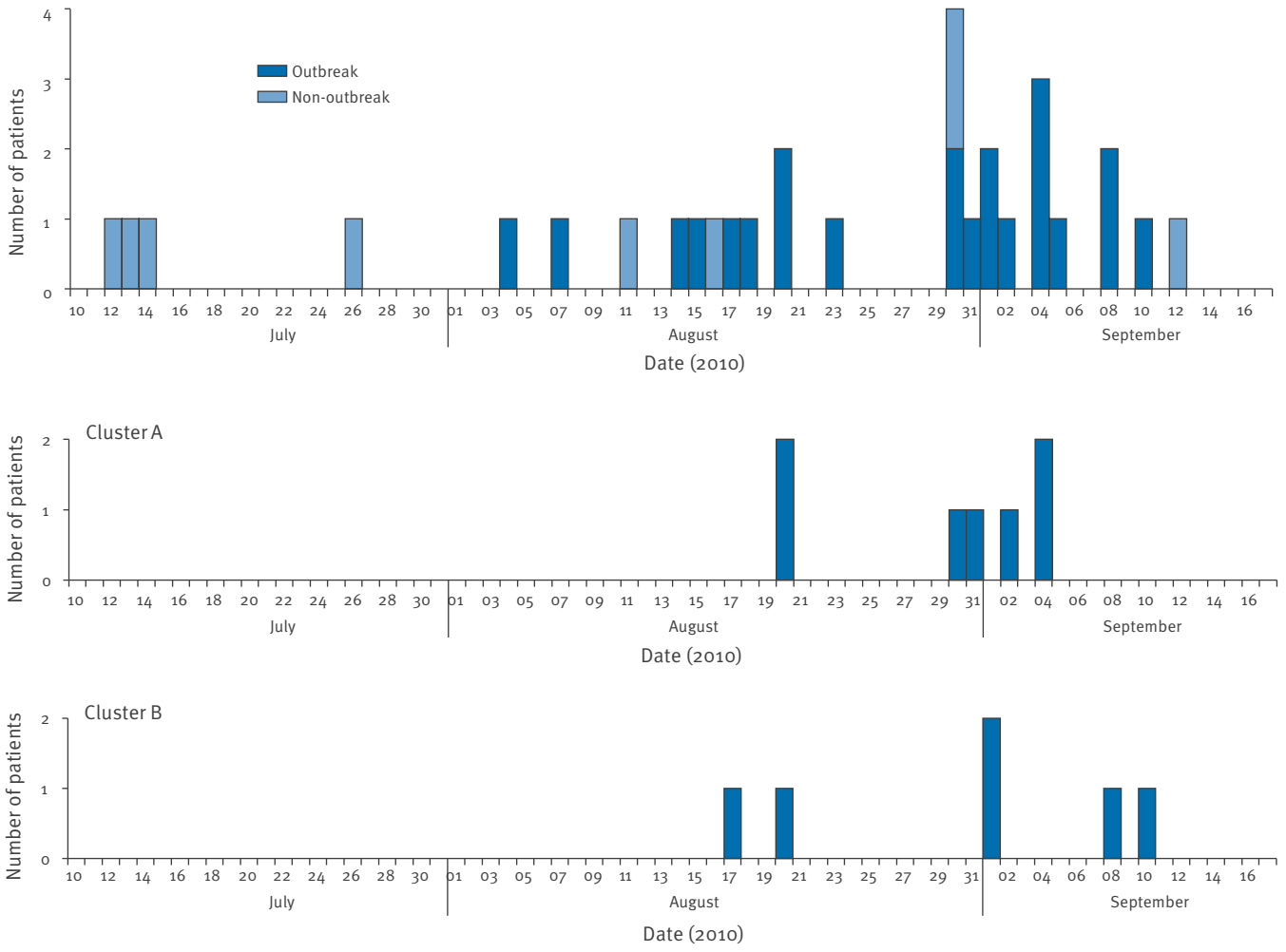
In total, 28 registered premises were visited by the HSE. Another three unregistered premises were identified and visited. A total of 26 environmental samples were collected and tested and all but one (linked to a single case not associated with either cluster) were negative on culture.

Control measures

In the vicinity of cluster A, there is a cooling tower and an air scrubber. Both were voluntarily closed down after inspection and the cooling tower was cleaned and

Figure 1

Patients with Legionnaire's disease by date of symptom onset, South Wales, July-September 2010 (n=31)

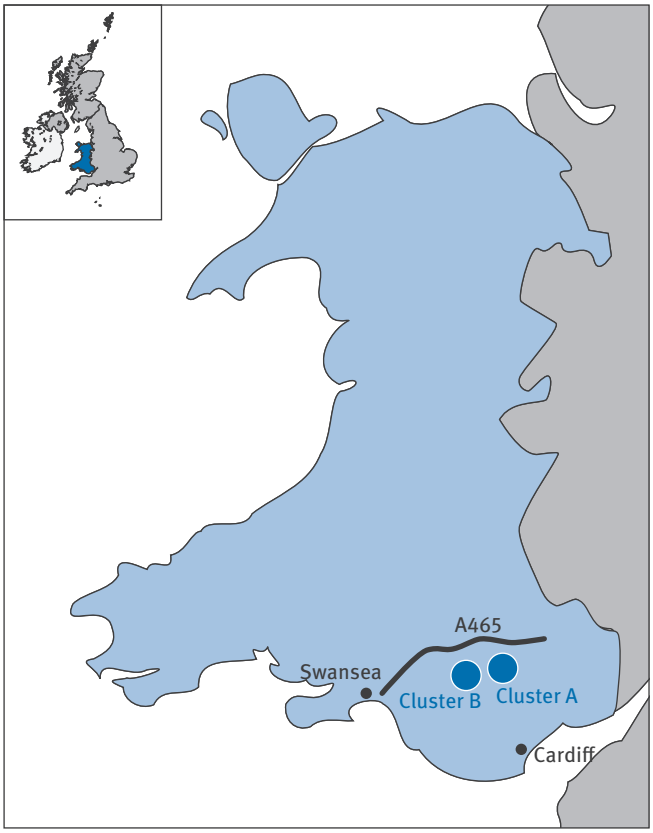


disinfected. Both have resumed normal operation following microbiological clearance. A cooling tower in the vicinity of cluster B was also closed, disinfected and re-opened following microbiological clearance. None of the sites has been definitively identified as the source of the outbreak.

In addition, a Prohibition Notice was served by the HSE at a site in Merthyr Tydfil. The notice was served as the cooling towers were not being operated in accordance with the Approved Code of Practice. Improvement Notices were also served on six further companies that were found to have minor deficiencies in their training, risk management policies, or maintenance procedures requiring them to improve the operation of their systems. None of these companies were located in the vicinity of Cluster A or B.

Figure 2

Location of clusters of Legionnaires' disease cases South Wales, August–September 2010



Discussion and conclusion

The outbreak investigation has so far identified two time-space clusters compatible with cooling towers in each location as the potential source. The outbreak has proved a particular challenge to investigate by virtue of the wide geographical distribution of cases, the identification of two distinct spatio-temporal clusters, the existence of different strains of *L. pneumophila* in cases, and the absence of *L. pneumophila* in environmental samples.

Previous outbreak investigations have identified geographical spread of *Legionella* up to 10 km from an industrial source [4,5]. However, even this would not explain the wide geographical distribution in this outbreak. Although the two clusters are only 15 km apart in a straight line, the topography comprises a series of hills and valleys and the road distance is considerably greater.

When microbiological results do not confirm a single source, or are contradictory, it can be difficult to decide if an outbreak is actually taking place [6]. The number of cases in this outbreak is clearly in excess of what would normally be observed in South Wales at this time of year. Some of this may be the consequence of heightened awareness and active case finding after declaration of the outbreak, but this does not explain the clustering of cases.

Investigations are continuing and some typing results are still awaited. So far, there has been only one successful match between an environmental and human sample. This highlights the importance of isolating and typing *Legionella* from as many clinical and environmental samples as possible to help identify the source [7,8]. The fact that no further cases have been detected since mid September 2010 indicates that the control measures taken appear to have been successful.

Table

Microbiological results from clinical samples, Legionnaires' disease outbreak, South Wales, August–September 2010

Case	Cluster	Sero-group	mAb[a]	Monoclonal antibody subgroup	Sequence-based type (SBT)
Case a	None	1	2	-	ST62[b]
Case b	Cluster A and B	1	2	Knoxville	ST615
Case c	Cluster A	1	2	Benidorm	ST898
Case d	None	1	2	Knoxville	ST902
Case e	None	1	2		SBT 12,0,0,0,0,0,0[b,c]
Case f	Cluster B	1	2	Knoxville	ST20
Case g	Cluster A	1	2		SBT 12,29,0,10,3,20,9[b,d]

[a] Monoclonal antibody type.
[b] By direct-nested PCR typing.
[c] Data obtained were insufficient to distinguish this strain from Case b or Case g.
[d] Unique strain.

Acknowledgements

We would like to thank all the members of the South Wales Legionnaires' Disease Outbreak Control Team: the Public Health Wales health protection teams, communications team, and microbiology services; environmental health departments of Blaenau Gwent, Caerphilly, Merthyr Tydfil, Monmouthshire, Neath Port Talbot, Rhondda Cynon Taff, Torfaen and Vale of Glamorgan County Borough Councils and of Swansea and Cardiff City Councils; the Health and Safety Executive; Environment Agency; microbiology departments of Aneurin Bevan and Cwm Taf Health Boards; the Scottish Legionella Reference Laboratory and the Health Protection Agency's Centre for Emergency Preparedness and Response, Porton Down and Centre for Infections, Colindale who participated in the outbreak investigation. We also thank Clare Elliott for assistance in producing the map.

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A new pandemic influenza A(H1N1) genetic variant predominated in the winter 2010 influenza season in Australia, New Zealand and Singapore

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Pandemic H1N1 influenza virus is of global health concern and is currently the predominant influenza virus subtype circulating in the southern hemisphere 2010 winter. The virus has changed little since it emerged in 2009, however, in this report we describe several genetically distinct changes in the pandemic H1N1 influenza virus. These variants were first detected in Singapore in early 2010 and have subsequently spread through Australia and New Zealand. At this stage, these signature changes in the haemagglutinin and neuraminidase proteins have not resulted in significant antigenic changes which might make the current vaccine less effective, but such adaptive mutations should be carefully monitored as the northern hemisphere approaches its winter influenza season.

Since its emergence in early 2009 [1] the pandemic influenza A(H1N1) virus has remained closely related to one of the earliest viruses detected, A/California/7/2009, with little change in the viruses' genetic makeup in even the most variable genes, haemagglutinin (HA) and neuraminidase (NA). This lack of drift was reflected in the World Health Organization's (WHO) Vaccine formulation decisions which recommended an A/California/7/2009-like pandemic influenza A(H1N1) virus for both the southern hemisphere 2010 and the northern hemisphere 2010-11 influenza vaccines [2]. While some genetic variants have been reported such as the D222G (D239G numbering if starting at the first methionine) HA mutation which was linked with more severe outcomes following pandemic influenza virus infection [3] and a more commonly seen E391K change in the HA gene [4] during late 2009, no clear variant has predominated in a country or region and no vaccine update has been forthcoming. This report, however, describes the recent emergence in Singapore and subsequent spread of a genetic variant of the pandemic influenza A(H1N1) virus to Australia and New Zealand during their 2010 winter influenza season, where it now predominates and has been detected in some vaccine breakthroughs and fatal cases.

Genetic characterisation of the pandemic influenza A(H1N1) variant

We sequenced the HA, NA and other genes of 2010 pandemic influenza A(H1N1) viruses from Singapore, Australia, New Zealand and elsewhere using conventional Sanger sequencing. Viruses early in 2010 (January to April) from Singapore and Australia showed the E391K (numbering beginning at the first methionine in HA; equivalent to E374K if starting after the signal peptide sequence in HA at DTLC) change in the HA but were scattered throughout the phylogenetic trees for HA (Figure 1) and the whole genome (Figure 2).

On 13 April 2010 an influenza A(H1N1) strain, A/Singapore/CC01/2010, was detected in Singapore that had further changes in HA (N142D; numbering beginning at the first M in HA; equivalent to N125D if starting after the signal peptide sequence in HA at DTLC) and in NA (M15I, N189S). Viruses with these changes then increased in frequency during May and June 2010 in Singapore and became the predominant viruses by mid-2010. Of the pandemic influenza viruses sequenced in Australia in 2010, those sampled in January and February mostly had the E391K change.

Viruses with the dual HA mutation (E391K and N142D) were first detected in late April 2010 (e.g. A/Brisbane/10/2010, sample date 29 April 2010), and by June 2010, viruses with these HA (and NA) changes predominated. In the North and South Islands of New Zealand, viruses that were collected in July and August 2010 also showed this dual change in the HA along with the NA changes. Viruses with these genetic characteristics in the HA protein have only been detected sporadically in some other countries (e.g. Guam; Figure 1, Table 1), and the complete set of changes in HA and NA has not yet been reported in the northern hemisphere to date in 2010.

These variant viruses have also been associated with several vaccine breakthroughs and a number of fatalities in both Singapore and Australia (labelled 'dec' in Figure 1). Examination of other gene segments of

several pandemic influenza A(H1N1) variants showed that the other six segments were all very similar to the A/California/7/2009 strain (nucleotide identity ranged from >99% to 100%) with no evidence of gene reassorting between the pandemic influenza (H1N1) virus and seasonal influenza A(H1N1) or H3N2 viruses or another influenza A subtype. Nevertheless, as marked in the whole genome phylogenetic tree (Figure 2), some additional mutations in the other gene segments (PB2, PB1, NP, NS1) appeared commonly among the recent variant strains, but the significance of these changes remains to be determined.

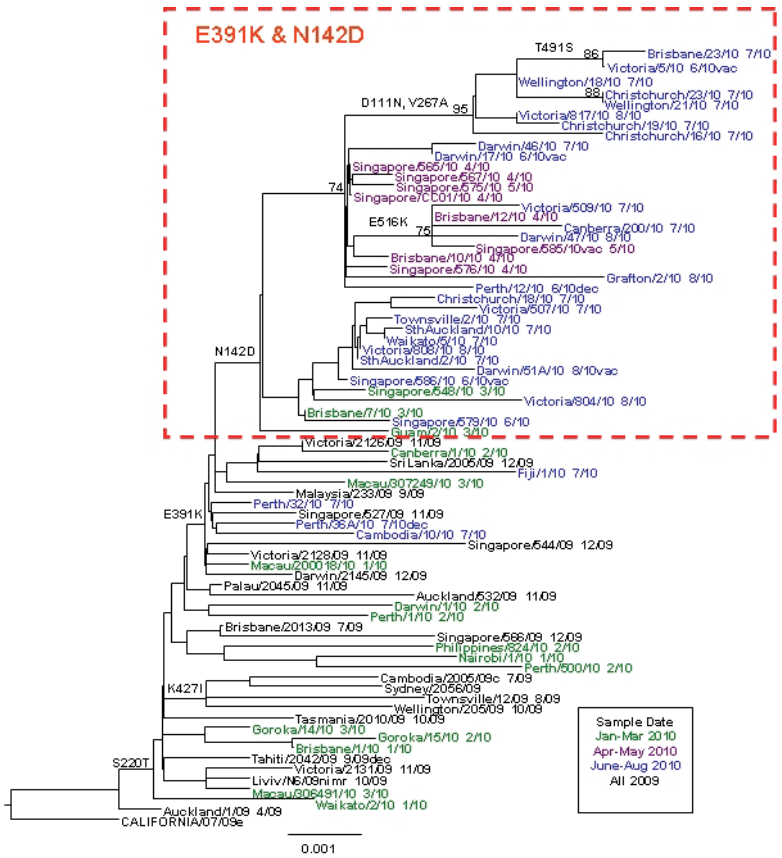
To further investigate the importance of these surface antigen mutations, we built a structural homology model of HA from the A/Brisbane/10/2010(H1N1) virus based on the template structure of A/California/04/2009(H1N1) (PDB:3LZG) [5] using MODELLER with loop refinement [6] and ProQ [7] for model quality control. In Figure 3, we superimpose our model with the complex of the antigenically similar HA of the 1918 influenza A(H1) virus bound to an antibody that recognises the classical Sa epitope (PDB:3LZF) [5]. We show that N142D is centrally located in this epitope, which led us to further investigate the effect of the mutation on antigenic properties with haemagglutination inhibition assays.

Adding to the possibility of the N142D mutation affecting antigenicity, the equivalent mutation N129D(H3)/N124D(H5) in influenza A/Mallard/Pennsylvania/10218/84(H5N2) virus was previously reported to cause antigenic drift as an escape mutant [8]. However, the findings in the context of avian H5 may not be easily transferable to the swine-origin H1. Generally, a single mutation will only partially affect antigenicity as typically several mutations in the same epitope are needed to seriously alter vaccine efficacy.

An additional mutation in the HA sequence, D111N, was common among samples from New Zealand, and an equivalent mutation in avian influenza has been reported to be related to a shift in host specificity (from avian towards human) which could hypothetically mean a small fitness advantage in the human host [9]. The equivalent mutation (referred to as D94N in [9]) enhanced binding of HA to the human-type SA-α-2,6-Gal receptor and decreased binding to the avian-type SA-α-2,3-Gal receptor. It was also observed that the mutation was able to enhance HA-mediated membrane fusion in mammalian cells. Structurally, D111N

Figure 1

Phylogenetic analysis of haemagglutinin sequences from recent pandemic influenza A(H1N1) viruses

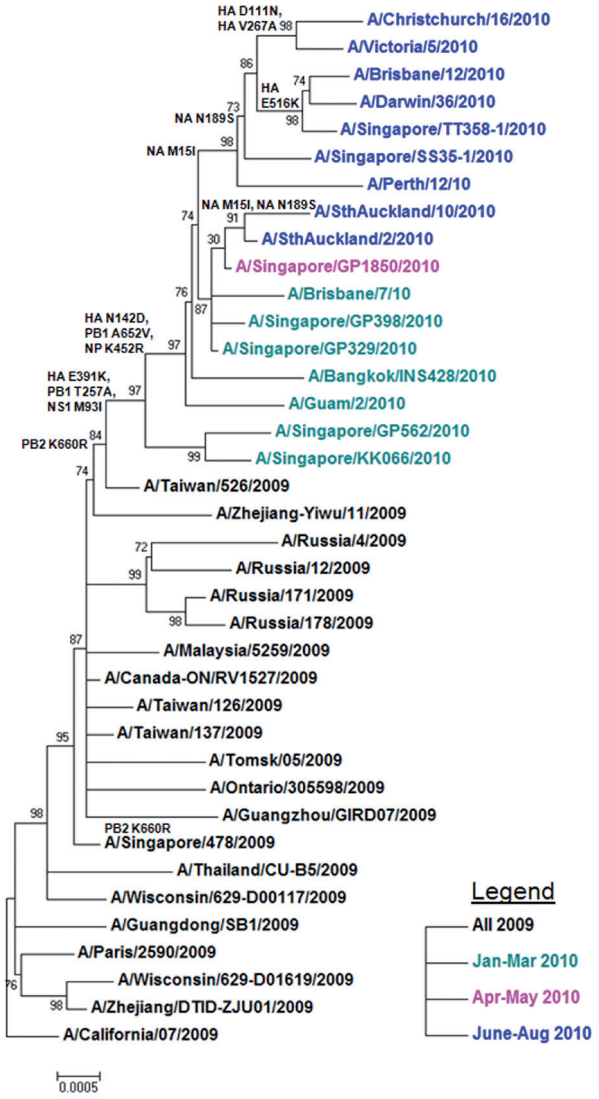


2010 viruses are colour-coded by sample date, e.g. 7/10 representing month/year; dec: a virus obtained from a fatal case; vac: a virus obtained from a person vaccinated with pandemic influenza A(H1N1) monovalent vaccine.

The neighbour-joining phylogenetic tree was constructed using the PAUP (v4.0) plugin on Geneious (v5.0.4) and FigTree (v1.3.1) was used to display the summarised and annotated trees with boostrap values >70 shown on the nodes.

Figure 2

Full genome maximum likelihood phylogenetic analysis of pandemic influenza A(H1N1) variants from Singapore, Australia and New Zealand and other non-redundant (<80% identity) strains



Naturally occurring 2009 pandemic influenza A(H1N1) viral sequences available in GenBank as of 23 August 2010 were downloaded from the NCBI Influenza Virus Resource (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html). Phylogenetic analysis was conducted on 1,877 strains with full-length nucleotide sequences available for all eight segments. The protein-coding nucleotide sequences for these strains were concatenated such that a single sequence representing a single strain contained nucleotide sequences encoding all 10 proteins. The vaccine strain A/California/07/2009 and recent Singaporean, Thai, New Zealand and Australian strains were set aside and redundancy was removed within the remaining strains with Cd-hit [13] by allowing a maximal sequence identity of 80% to reduce the set to 20 non-redundant strains. This representative set was aligned with the vaccine strain and 16 other strains from Singapore, Thailand, New Zealand and Australia using MAFFT [14] with the FFT-NS-2 option. Sequences flanking the coding region were removed from the alignment. A maximum likelihood tree was created using PhyML [12] with the approximate likelihood ratio test, the HKY85 substitution model and parameters such as for the shape of the gamma distribution (0.258) were estimated by the programme. Substitutions discussed in this analysis were identified and marked in the resulting phylogenetic tree using the MEGA software package [15].

Amino acid changes in the various genes are indicated by name of protein and mutation e.g. PB1 A652V.

is located on the outside of the bottom of the sialic acid binding pocket with the side chain pointing to the outside (Figure 3) and the mechanism that causes the reported effect is not fully clear.

HA D111N is almost exclusively found in combination with another mutation, HA V267A, which is located at an internal beta sheet below the receptor binding pocket facing the Sa epitope (Figure 2). Exchanging valine for the smaller alanine at this position creates a small cavity which may slightly alter the surface of the epitope on top and could add to the effects of N142D. However, so far the HA D111N and V267A mutations have only occurred in a close temporal and geographic context (four in New Zealand and three in eastern Australia in July and August 2010, see Figure 1) and their increased local occurrence may simply be due to founder effects.

Two additional mutations occurred in the NA sequence, M15I and N189S, which were predominant in viruses from Singapore and Australia by mid-2010. NA M15I is located in the signal peptide region. The signal peptide is the motif required for cell surface expression of the viral protein and its existence and quality can be predicted with the programme SignalP 3.0 [10]. For the NA M15I mutation, the prediction score (D-score) increases from 0.326 for M15 to 0.404 for I15. This could hypothetically indicate that the mutated version represents a better signal peptide with potentially increasing secretion and surface expression efficiency, but this needs to be further tested experimentally. NA N189S on the other hand is located at the bottom side of the NA structure, far away from the sialic acid- and drug-binding pocket and any phenotypic change cannot easily be predicted.

Antigenic analysis of variant viruses

While genetic differences were apparent in this variant group of pandemic influenza A(H1N1) viruses, when they were assessed for antigenic variation in haemagglutination inhibition assays (HI) using ferret antisera raised to A/California/7/2009-like viruses and viruses from the new variant group (e.g. A/Singapore/548/2010, A/Brisbane10/2010), no differences in titres were apparent, indicating that these viruses were not antigenically distinguishable from the reference and vaccine virus A/California/7/2009 (Table 2).

Further antigenic analysis was performed using a small human serum panel (n=48) containing pre- and post-vaccination sera from Australian adults (24 subjects between the age of 18 and 59 years) and elderly subjects (24 subjects over the age of 60 years) who were given a single dose of inactivated 2010 Australian seasonal influenza vaccine (CSL Fluvax; CSL Limited, Australia) which contained an A/California/7/2009-like pandemic influenza A(H1N1) virus, an A/Perth/16/2009-like A(H3N2) virus and a B/Brisbane/60/2008-like B virus. The geometric mean HI titre (GMT) in the sera of all vaccinated subjects was reduced by 53% when

tested against an egg-grown A/Brisbane/10/2010 virus (one of the genetically variant viruses) compared to the GMT obtained against egg-grown wildtype A/California/7/2009 virus. Despite some reduction in HI titres with human post-vaccination sera, there were no clear differences with ferret sera, suggesting that there are no major antigenic differences in these variant viruses at this stage in their evolution and that they still share most of their antigenic properties with the early pandemic influenza A(H1N1) viruses.

Discussion

While the 2009 pandemic has recently been downgraded by the WHO [11], the pandemic influenza A(H1N1) virus still remains the predominant influenza virus in most countries including those in the southern hemisphere that recently experienced their winter influenza season (with the exception of South Africa where influenza B and A(H3N2) viruses have predominated in 2010) [11]. To date there has been little change detected in either the genetic or antigenic characteristics of the pandemic H1N1 influenza virus in the nearly 18 months that it has infected humans. No clear variant has appeared apart from minor changes occurring in the HA, NA and other viral genes during this time. Recently however a genetically distinct variant containing several signature amino acid changes in both the HA and NA genes has emerged in Singapore, Australia

Table 1

Frequency of amino acid changes at positions 391 and 142 in the haemagglutinin gene of pandemic influenza A(H1N1) viruses obtained in 2010 (n=172)

Country	HA sequences analysed	No. (%) E391K only	No. (%) N142D only	No. (%) E391K and N142D	No. (%) neither E391K nor N142D
Singapore	85	32 (37.6)	0 (0)	39 (45.9)	14 (16.5)
Australia	36	7 (19.4)	0 (0)	25 (69.5)	4 (11.1)
New Zealand	26	1 (3.85)	0 (0)	24 (92.3)	1 (3.85)
Papua New Guinea	8	0 (0)	0 (0)	0 (0)	8 (100)
Other[a]	17	3 (17.7)	0 (0)	1 (5.9)	13 (76.4)
Total	172	43 (25.0)	0 (0)	89 (51.7)	40 (23.3)

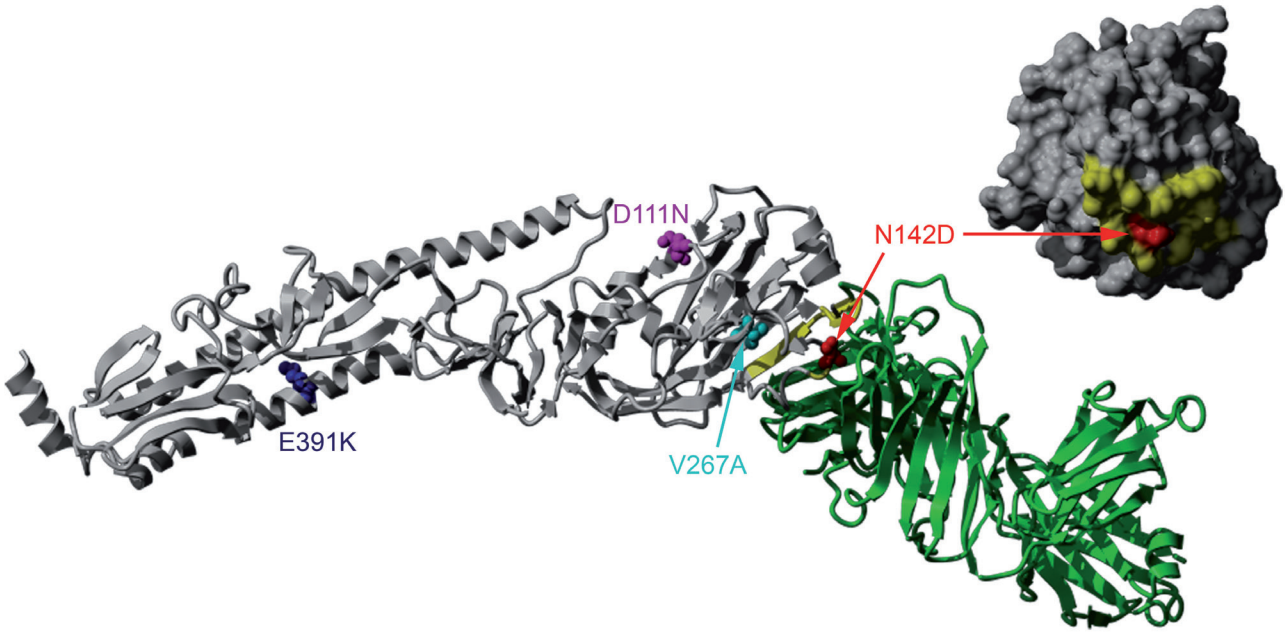
HA: haemagglutinin.

[a] Includes 2010 pandemic influenza A(H1N1) viruses from Brunei (n=4), Philippines (n=1), Thailand (n=2), Macau (n=3), Guam (n=2), Cambodia (n=4) and Fiji (n=1).

Sequenced by the World Health Organization Collaborating Centre Melbourne and Singapore Public Health Laboratory.

Figure 3

Structural model of influenza A/Brisbane/10/2010(H1N1) haemagglutinin



HA: haemagglutinin.

Grey ribbons: influenza A/Brisbane/10/2010(H1N1), sites of all mutations of interest indicated including the positions of the additional ones from the New Zealand strains D111N and V267A
Green ribbons: a bound antibody in the orientation resulting from superimposition of our HA model with that of the antigenically similar 1918 influenza A(H1) HA in complex with this antibody.
Upper right corner: surface representation of the HA model rotated by 90 degrees with the residues of the Sa epitope coloured yellow.

and New Zealand, coinciding with the winter influenza season in the latter two countries. While the combination of HA mutations at E391K and N142D has been seen sporadically in isolates in Korea and the United States in November 2009 (ADM21270, ADM21278, ADL59660, ADD74728), the first appearance of the double HA and double NA change has been in April in Singapore (A/Singapore/CC01/2010). Similar viruses have also been detected in Guam (A/Guam/2/2010(H1N1)) and Thailand (A/Bangkok/INS428/2010(H1N1)) in March 2010; they were lacking the NA mutations but at least partially shared the changes in other segments (PB2 K660R, NS1 M93I, PB1 T257A and A652V, NP K452R) which makes these strains closely related to A/Singapore/GP329/2010 which was isolated in January 2010. Strains with the full set of characteristic mutations of the new variant have not yet been reported in other regions and have not appeared in genetic databases to date.

It remains to be seen whether this variant will continue to predominate for the rest of the influenza season in Oceania and in other parts of the southern hemisphere and then spread to the northern hemisphere or merely die out. Already this variant virus has been associated with several vaccine breakthroughs in teenagers and adults vaccinated in 2010 with monovalent pandemic influenza vaccine as well as a number of fatal cases from whom the variant virus was isolated. Unfortunately we did not have access to the comprehensive patient records that may have enabled us to determine the relative frequency of vaccine breakthroughs with this variant compared to the non-variant. This information is important to eliminate other confounding factors such as the age of the vaccinee, time since they were vaccinated or if they were taking immuosuppressive drugs, all of which might impact on their level of protection following vaccination. It is therefore not known at this time if the amino acid changes in this variant virus are responsible for these vaccine breakthroughs or deaths, or if they are simply a result of this virus genotype being the predominant virus in circulation during this period. The HA and NA amino acid changes seen in the variant are present both in the original clinical samples and in viruses isolated in MDCK cells and are retained in viruses isolated directly in embryonated hens eggs. Careful studies are underway to determine if the variant viruses grow better than other influenza A/California/7/2009-like viruses but preliminary data indicate that these variant viruses grow as well or better than viruses without these characteristic amino acid changes both in MDCK cell culture and in embryonated hens eggs (data not shown) and this may make them useful vaccine virus seeds. If confirmed this may also explain the rapid spread and predominance of this variant virus. Further animal and human transmission and growth studies will be required to support this initial finding.

Conclusions

A new genetic variant of the pandemic influenza (H1N1) virus has emerged in Singapore, Australia and New Zealand in the second and third quarters of 2010 that does not appear at this stage to represent a significant antigenic change for the virus. However, it may represent the start of more dramatic antigenic drift of the pandemic influenza A(H1N1) viruses that may require a vaccine update sooner than might have been expected, with a new human influenza virus.

Table 2

Antigenic reactivity of pandemic influenza A(H1N1) variants compared to the A/California/7/2009-like viruses using haemagglutination inhibition assay

	Ferret antisera						Passage details	Specimen/date	HA amino acid changes
Reference viruses	CAL	ILL	BRI	LVI	SIN	BRI			
A/CALIFORNIA/7/2009	640	160	640	160	320	320	E5	3/2009	-
A/ILLINOIS/9/2007	320	320	320	80	160	160	XMDCK3	2007	-
A/BRISBANE/2013/2009	320	160	320	320	320	320	MDCK7	1/7/2009	-
A/LVIV/N6/2009	80	<40	40	160	80	160	XMDCK1	2009	-
A/SINGAPORE/548/2010	640	160	320	160	1280	1280	E2	26/3/2010	391/142
A/BRISBANE/10/2010	320	80	320	160	1280	1280	E2	29/4/2010	391/142
Test viruses									
A/WAIKATO/2/2010	640	320	640	320	640	640	XMDCK2	4/1/2010	-
A/SINGAPORE/527/2009	320	160	320	160	320	320	MDCK2	17/11/2009	391
A/SINGAPORE/544/2009	320	160	320	80	160	160	MDCK2	10/12/2009	391
A/FIJI/1/2010	640	320	320	160	320	320	MDCK1	12/5/2010	391
A/BRISBANE/12/2010	640	160	320	160	1280	1280	MDCK2	3/6/2010	391/142
A/DARWIN/46/2010	640	160	320	160	1280	1280	MDCK1	28/7/2010	391/142
A/CHRISTCHURCH/23/2010	640	160	320	320	1280	640	XMDCK1	14/7/2010	391/142
A/WELLINGTON/21/2010	320	80	320	160	640	640	XMDCK1	26/7/2010	391/142

HA: haemagglutinin.
Note: Turkey RBC used.

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Urban–rural differences of age- and species-specific campylobacteriosis incidence, Hesse, Germany, July 2005 – June 2006

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Campylobacter **infection is the most common cause of bacterial gastroenteritis worldwide. This study examines the association between campylobacteriosis incidence and degree of urbanicity in Hesse, Germany, by age and *Campylobacter* species. During a one-year period (July 2005–June 2006), Hessian local health authorities provided information on municipality of residence for 3,315 campylobacteriosis cases. We calculated age- and *Campylobacter* species-specific incidences for six levels of urbanicity, as defined by population density and accessibility of centres. For children under five years old, living in inner rural areas (incidence rate ratio (IRR): 2.9; 95% confidence interval (CI): 1.9 to 4.4) and for children aged 5–14 years living in inner rural (IRR: 2.1; 95% CI: 1.3 to 3.1) or intermediate areas (inner intermediate area IRR: 1.8; 95% CI: 1.2 to 2.7; outer intermediate area IRR: 2.1; 95% CI: 1.3 to 3.3) was associated with a statistically significantly higher campylobacteriosis risk (reference category: inner urban area). Calculations by *Campylobacter* species showed a higher risk of gastroenteritis due to *C. coli* for inhabitants (all ages) of non-urban areas. This study suggests that differences in risk factors by age, *Campylobacter* species and degree of urbanicity do exist. For children contact with animals or the environment may be responsible for a substantial proportion of sporadic *Campylobacter* infections.**

Introduction

Campylobacter infections resulting in gastroenteritis are recognised as an emerging problem worldwide. With 47 cases per 100,000 population campylobacteriosis is the most commonly reported gastrointestinal disease in the European Union (data from 2007) [1]. Notification rates differ markedly, ranging from zero per 100,000 population in Romania to 95 per 100,000 population in the United Kingdom in 2007 [1]. In Germany, the annual number of reported cases rose from 47,937 in 2003 (58 per 100,000 population) to 62,807 in 2009 (79 per 100,000 population) [2]. A number of case–control studies identified travelling abroad, eating poultry, pork and sausages, drinking untreated water or unpasteurised milk, barbecuing and having contact with domestic animals as risk factors for infection [3-5], but most infections are believed to result from the ingestion of contaminated food [6]. The primary source of food contamination is believed to be animal faeces. This is consistent with high *Campylobacter* carriage rates in poultry, pigs and cattle and the fact that similar *Campylobacter* genotypes have been identified in farm animals and humans [7-9]. Contamination of the environment by faeces of domestic and wild animals presents an alternative exposure pathway for human infection, for example, via contamination of drinking and recreational water sources [10]. Humans may also be exposed to animal faeces in the environment through other outdoor activities such as playing, camping, walking and picnicking.

Since 2001, when the country's disease reporting system was reorganised, specific notification data on human campylobacteriosis have been available in Germany. Using national case definitions [11], local health authorities verify locally identified notifiable diseases and send case reports electronically via state health departments to the national surveillance unit at the Robert Koch Institute in Berlin [12]. For campylobacteriosis, data collected in this system include demographic characteristics, dates of illness, county and – for internationally imported cases – country of infection, diagnostic procedure used (bacterial culture or enzyme-linked immunosorbent assay (ELISA)), and if performed, results of species differentiation, but not the techniques used) and association with outbreaks.

Hesse is one of the 16 German Laender, with a population of 6.1 million in 2007. In Hesse, the annual number of notified cases of campylobacteriosis rose from 3,000 in 2001 to 4,029 cases in 2009, corresponding to an incidence of 49 per 100,000 population and 66 per 100,000 population, respectively. County-specific incidence of *Campylobacter* infection in Hesse ranged from 19 per 100,000 inhabitants in 2001, 2002 and 2004 to 113 per 100,000 inhabitants in 2007. While the annual campylobacteriosis incidence varied widely between Hessian counties, intracounty incidence changed little

from 2001 to 2007. We investigated the association between campylobacteriosis incidence and living in Hessian urban, intermediate and rural environments by cases' age, sex and *Campylobacter* species.

Figure 1

Geographical distribution of *Campylobacter* infections, by municipality, showing (A) degree of urbanicity, (B) incidence in children aged 0–14 years and (C) incidence in people aged 15 years and above, Hesse, Germany, July 2005 – June 2006

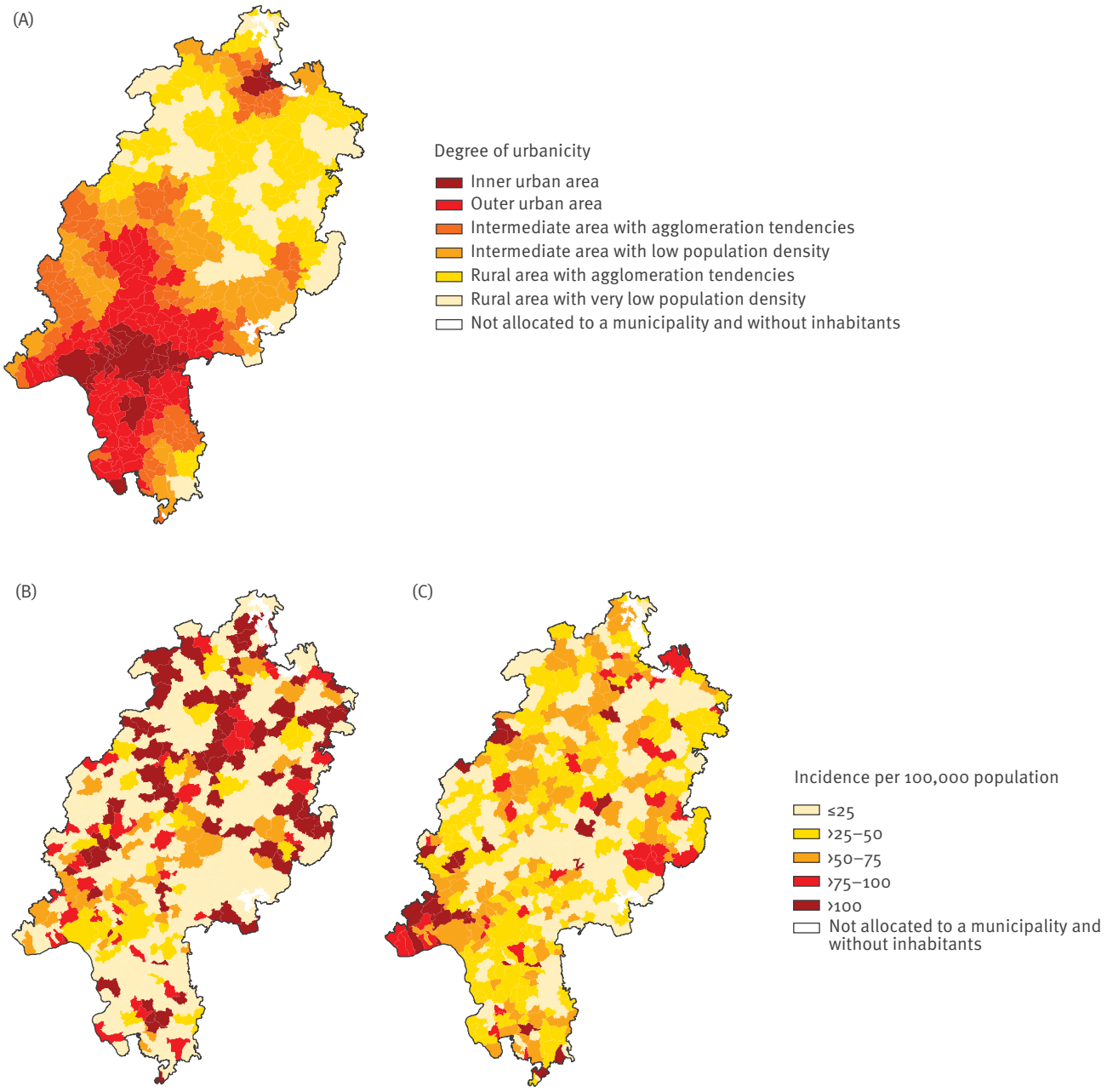


Table 1

Population density and percentage population, by degree of urbanicity, Hesse, Germany, July 2005 – June 2006

Degree of urbanicity	Population density (number of inhabitants per km²)	Percentage of population
Inner urban area	1,441	35
Outer urban area	447	27
Inner intermediate area (has agglomeration tendencies)	274	16
Outer intermediate area (has low population density)	122	7
Inner rural area (has agglomeration tendencies)	136	11
Outer rural area (has very low population density)	66	4

Methods

Data on age, sex and *Campylobacter* species of all campylobacteriosis cases with disease onset (or if missing, date of diagnosis) from July 2005 to June 2006 were extracted from the state surveillance database. In addition, Hessian local health authorities provided information on the municipality of the cases' residence (postal code and/or name of municipality) for all cases included in the study. Population data were provided by the Hesse Statistical Office. The 426 Hessian municipalities were grouped into six categories according to their degree of urbanicity, as defined by the Federal Office of Building and Regional Planning based on population density (urban, intermediate and rural) and accessibility of centres (inner and outer) [13]. The spatial distribution of categories of urbanisation in Hesse is shown in Figure 1. In Hesse, the total inner urban area (IUA) has a population density of 1,441 inhabitants per km². Some 35% of the Hessian population live in municipalities of the IUA (Table 1).

Age-specific campylobacteriosis incidences for the six categories of urbanisation and five age groups (under 5 years, 5–14 years, 15–44 years, 45–64 years, more than 64 years) were calculated. In our study, *C. coli* and *C. jejuni* together represent over 96% of campylobacteriosis reports with species information. Due to the small numbers of *C. coli* cases, species-specific campylobacteriosis incidences for *C. jejuni* and *C. coli* were calculated only for three categories of urbanisation (urban, intermediate and rural areas). We also

Table 2

Association between campylobacteriosis cases and degree of urbanicity, by age group, Hesse, Germany, July 2005 – June 2006 (n=2,710)

Age group and degree of urbanicity	Number of cases (n=2,710)	Population (N=6,097,765)	Incidence (per 100,000 population)	95% CI
0–4 years				
Inner urban area	51	100,800	51	37.7–66.5
Outer urban area	33	76,200	43	29.8–60.8
Inner intermediate area	26	42,700	61	39.7– 89.1
Outer intermediate area	8	19,900	40	17.3–79.1
Inner rural area	44	30,000	147	106.8–197.2
Outer rural area	9	10,100	89	40.7–168.7
5–14 years				
Inner urban area	55	194,300	28	21.3–36.8
Outer urban area	47	174,700	27	19.8–35.8
Inner intermediate area	54	103,600	52	39.2–68.0
Outer intermediate area	30	50,400	60	40.2–85.0
Inner rural area	43	74,100	58	42.0–78.2
Outer rural area	12	26,700	45	23.2–78.5
15–44 years				
Inner urban area	479	901,000	53	48.5–58.1
Outer urban area	374	671,500	56	50.2–61.6
Inner intermediate area	231	388,000	60	52.1–67.7
Outer intermediate area	115	174,700	66	54.3–79.0
Inner rural area	145	264,300	55	46.3–64.6
Outer rural area	41	93,000	44	31.6–59.8
45–64 years				
Inner urban area	236	558,300	42	37.1–48.0
Outer urban area	153	437,800	35	29.6–40.9
Inner intermediate area	94	244,600	38	31.1–47.0
Outer intermediate area	36	114,300	31	22.1–43.6
Inner rural area	49	174,400	28	20.8–37.1
Outer rural area	19	60,800	31	18.8–48.8
>64 years				
Inner urban area	128	385,600	33	27.7–39.5
Outer urban area	71	281,400	25	19.7–31.8
Inner intermediate area	56	170,100	33	24.9–42.8
Outer intermediate area	26	85,200	31	19.9–44.7
Inner rural area	37	138,800	26	18.8–36.7
Outer rural area	8	50,100	16.0	6.9–31.4

CI: confidence interval.

calculated 95% confidence intervals (CI) for incidence rates and incidence rate ratios (IRRs) and the attributable fraction among the exposed and the population attributable fraction for living in non-urban areas according to Boice and Monson, using the incidence in urban areas as reference for calculation [14].

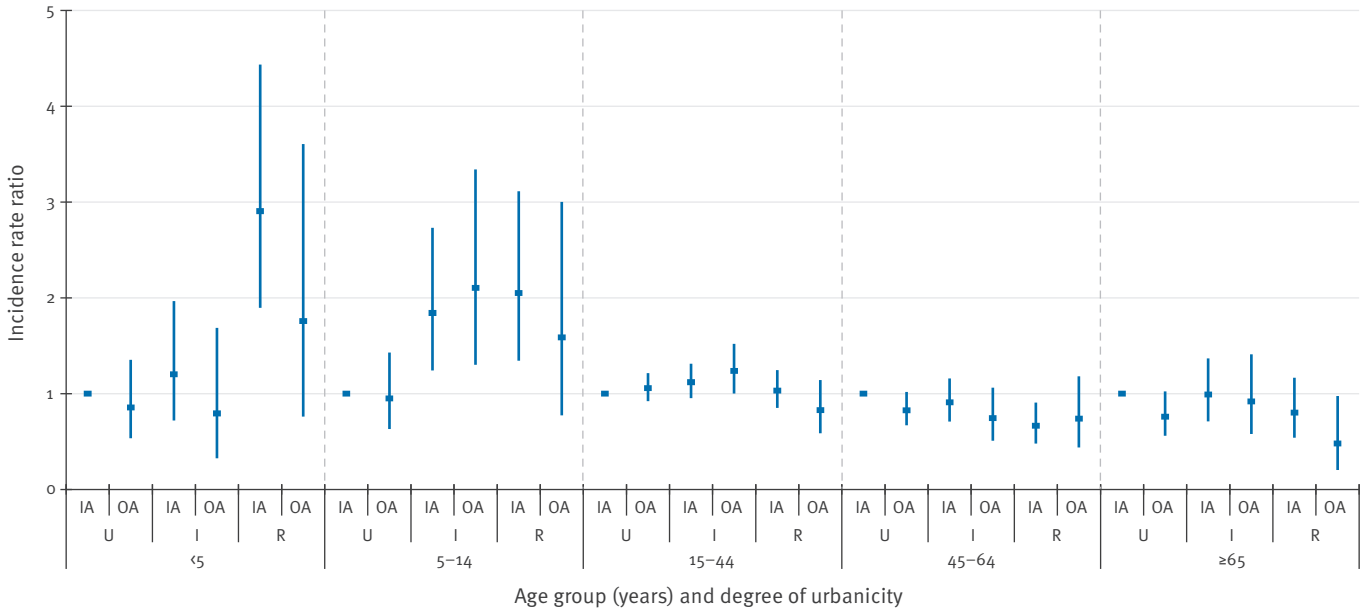
As species information was not available for all cases and in order to take into account variation in the frequency of species differentiation according to degree of urbanicity, the number of species-specific cases was estimated using the formula: corrected number of species-specific cases = (number culture confirmed/ number differentiated to species level) x (reported number of species-specific cases). Corrected incidence rates and IRRs were then calculated. For corrected incidences, 95% CI were not calculated, due to the additional uncertainty resulting from incompleteness of species differentiation. Data were analysed with Stata version 10.0.

Results

From July 2005 to June 2006, 3,331 campylobacteriosis cases were reported in Hesse. Of these, 2,710 cases (81.4%) were reported to have acquired their infection in Hesse. Of the remaining 621 campylobacteriosis cases, 377 (60.7%) were infected outside Germany, 74 (11.9%) were infected in German Laender other than Hesse, and for 170 (27.4%) details of the place of infection were not available. Only the 2,710 cases reported

Figure 2

Age-specific incidence rate ratios and 95% confidence intervals, by degree of urbanicity, Hesse, Germany, July 2005 – June 2006



I: intermediate; IA: inner area; OA: outer area; R: rural U: urban.

Table 3

Association between campylobacteriosis cases and degree of urbanicity, by *Campylobacter* species, Hesse, Germany, July 2005 – June 2006

Campylobacter species and degree of urbanicity	Population	Reported cases			Corrected estimate of cases[a]		
		Number of cases	Incidence (per 100,000 population)	Incidence rate ratio (95% CI)	Number of cases	Incidence (per 100,000 population)	Incidence rate ratio
Campylobacter coli							
Urban areas	3,781,800	88	2	1 (reference)	164	4	1 (reference)
Intermediate areas	1,393,600	91	7	2.8 (2.1–3.8)	115	8	1.9
Rural areas	922,400	64	7	3.0 (2.1–4.2)	90	10	2.3
Campylobacter jejuni							
Urban areas	3,781,800	698	18	1 (reference)	1,300	34	1 (reference)
Intermediate areas	1,393,600	352	25	1.4 (1.2–1.6)	443	32	0.93
Rural areas	922,400	232	25	1.4 (1.2–1.6)	325	35	1.1

CI: confidence Interval.

[a] Number of cases corrected for incomplete differentiation to species level, which differed by level of urbanicity.

to have acquired their infection in Hesse were included in this study. Of these, 2,673 (98.6%) were laboratory confirmed, for 1,581 (58.3%) species information was available, and 43 (1.6%) cases were part of nine small clusters, each of three to six cases.

Campylobacteriosis incidence for Hesse and all age groups was 44 per 100,000 population, (2,710 of 6,095,055) and did not differ by degree of urbanicity (urban area: 43.0, 95% CI: 41.0–45.2; intermediate area: 48.5, 95% CI: 44.9–52.3; rural area: 44.1, 95% CI: 40.0–48.7). Of the 2,710 infections acquired in Hesse, 53.3 % (1,445) were male and 46.5% (1,259) were female. For six cases details of their sex were not available. For males and females campylobacteriosis incidence did not vary by degree of urbanicity (data not shown).

Campylobacteriosis incidence was highest in children under five years of age (annual incidence: 61 per 100,000 population) and in people aged 15–44 years (annual incidence: 56 per 100,000 population), and lowest in people older than 65 years (annual incidence: 29 per 100,000 population). In the age groups under five years and 5–14 years, campylobacteriosis incidence varied largely by degree of urbanicity (Table 2). When compared with living in IUAs, living in IRAs was significantly associated with a higher campylobacteriosis incidence in children aged under five years of age (IRR: 2.9, 95% CI: 1.9–4.4). For children aged 5–14 years, living in IRAs (IRR: 2.1, 95% CI: 1.3–3.1) and in intermediate areas (IIA IRR: 1.8, 95% CI: 1.2–2.7; OIA IRR: 2.1, 95% CI: 1.3–3.3) was significantly associated with a higher campylobacteriosis incidence. For children aged under five years and those aged 5–14 years, the association between living in the ORAs and higher campylobacteriosis incidence was not statistically significant (children under five years IRR: 1.8, 95% CI: 0.8–3.6; children aged 5–14 years IRR: 1.6, 95% CI: 0.8–3.0) (Figures 1 and 2).

We then calculated the attributable risk of living in non-urban areas for children aged 0–14 years In the exposed children, the risk was 46%; the population attributable risk was 25%.

While urban–rural differences were most pronounced in children 14 years of age and younger, they were also seen in the three older age groups. In these age groups there was a tendency towards lower campylobacteriosis incidences for persons living in more rural areas (Figure 2). However, only for those aged 65 years and above living in ORAs did this difference reach statistical significance (IRR: 0.5, 95% CI: 0.2–1.0).

Of 1,581 cases with species information, 15% (n=243 were infected with *C. coli*, 81% (n=1,282) with *C. jejuni*, 3% (n=49) with *C. lari*, and less than 1% (n=7) with other *Campylobacter* species. The proportion of culture-confirmed cases did not differ by degree of urbanicity: 88.7%, 87.4% and 89.4% of cases were culture confirmed in urban, intermediate and rural areas, respectively. However, a higher proportion of isolates from patients in non-urban areas were differentiated to species level: 53.7%, 79.4% and 71.3% of culture-confirmed cases were differentiated to species level in urban, intermediate and rural areas, respectively (Pearson's chi-square test p<0.001). When compared with urban areas, species-specific incidences for *C. coli* and *C. jejuni* were higher in rural and intermediate areas. However, when the number of *C. coli* and *C. jejuni* cases was corrected for incomplete differentiation to species level, only incidence of *C. coli* infections differed by degree of urbanicity (Table 3). In addition, a relatively higher proportion of *C. coli* cases lived in non-urban areas: the ratio of the *C. coli* to *C. jejuni* cases (corrected) was 0.13 in urban areas, 0.26 in intermediate areas and 0.28 in rural areas.

Discussion

In this analysis, degree of urbanisation was found to be associated with campylobacteriosis in children under 15 years of age. Calculation of the attributable risk indicated that 25% of all reported cases of campylobacteriosis aged under 15 years were associated with living in non-urban areas. These attributable risk calculations reflect the degree to which the true, unknown sources of infection are more abundant in non-urban areas than in urban areas.

Recent studies investigated urban–rural differences in campylobacteriosis incidence in Canada [15], the Netherlands [16], Scotland [17], Denmark [18] and Sweden [19]. Four of these studies found higher incidences in rural environments [15, 17-19] and one in urban and urbanised environments [16]. The authors of the last study suggested the higher incidence of campylobacteriosis in urban and urbanised areas could be related to higher consumption of ready-to-eat foods. Of the two studies that presented age-specific data, one reported the greatest urban–rural differences for children 0–4 years-old [15] and one that urban–rural differences were limited to children 0–14 years of age [18]. Authors of both studies suggested that contact with farm animals and the environment were the source of a substantial proportion of sporadic *Campylobacter* infections.

When interpreting age-specific differences in urban–rural gradients of campylobacteriosis incidence, two main factors need to be considered: age-specific risk factors for infection and immunity acquired during childhood towards local, i.e. rural sources of *Campylobacter* infection. Few studies reported on age-specific risk factors for campylobacteriosis [20-25]. However, in case–control studies on risk factors for the disease in infants and young children food exposures explained less than 40% of the infections [20,22,23]. *Campylobacter* infections in these age groups have been associated with contacts with diarrhoeic pets [20,23,24] and live chickens [24,25], drinking water from a well, lake or river [20,23], riding in a shopping

trolley next to meat or poultry [23], visiting or living on a farm [23], ownership of farm animals or visiting farm animals outside the household [22] as well as different food exposures. Among these food exposures were the consumption of fruits and vegetables prepared at home [23], mayonnaise [24], butter [25], porridge [25], undercooked meat [22], products containing raw eggs [22] and grilled meat [20,22]. In contrast to many other published case–control studies [4-5], these studies did not find an association between eating chicken and *Campylobacter* infection. In the light of these findings we believe that environmental exposure accounts for a considerable part of *Campylobacter* infections in children and that children living in non-urban areas have more opportunities for direct or indirect contact with animals or their excrement. In addition, children living in urban and rural environments may differ in their eating and drinking habits. *C. jejuni* outbreaks, for example, have been repeatedly related to the consumption of raw milk [26]; children living in non-urban areas may drink raw milk more frequently.

The absence of urban–rural differences in campylobacteriosis incidence in persons aged 15 years and above may be related to differences in behaviour and/or a higher level of immunity from previous exposures [27]. In developing countries, clinical disease due to *C. jejuni* is common among children younger than two years, but rare among individuals later in life [28]. This relative absence of disease is thought to be related to acquired immunity [29]. If a higher proportion of inhabitants of Hessian rural areas aged 15 years and above are immune to *Campylobacter* infection, then the association between living in rural areas and campylobacteriosis may be decreased or even reversed. These questions should be addressed in *Campylobacter* seroprevalence studies or the inclusion of only non-immune controls in future case–control studies for the identification of risk factors.

In our study, 15% of all campylobacteriosis cases with species information were due to *C. coli*. Germany is one of the European Union Member States with the highest proportion of cases due to *C. coli* [30]. Within Germany, the proportion of cases due to *C. coli* differs widely between States and is higher in the former East German or new Laender (in 2006, 14% of all cases (n=7,494) with species information) than in the former West German or old Laender (in 2006, 6% (n=26,205). For whole of Germany, the proportion in 2006 was 8% (n=33,699) [2]. The new Länder are more rural, i.e. the population density is lower [31], a greater proportion of the total area is agricultural [32] and a smaller proportion of the total area is inhabited [33].

When analysing species–specific differences in campylobacteriosis incidence in urban, intermediate and rural areas and correcting for differences in frequency of species differentiation, we found a higher incidence for *C. coli* in non-urban areas. Microbiological data show that the prevalence of different *Campylobacter* species varies between different potential sources of infection, including animal species, food and water [10,34,35]. Poultry has been recognised as the primary reservoir of *C. jejuni*, while pigs are mostly implicated as reservoirs of *C. coli* [36-38]. Differences in food-borne exposures between *C. coli* and *C. jejuni* have been shown to exist [22,39] and differences in consumption habits between persons living in urban and rural areas may contribute to the observed difference in species distribution. However, it has been suggested that *C. coli* may survive in the environment better than *C. jejuni* [10] and people living in non-urban areas may be exposed more frequently to environmental sources of *C. coli*.

This study is limited by constraints inherent to all ecological analyses: a sample size limiting detailed subgroup analysis and a limited availability of further data on municipality level (for example, information on water supply, animal density or consultation of health services and diagnostic practices). However, our analysis suggests that differences in risk factors by *Campylobacter* species, cases' age and degree of urbanicity do exist.

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