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Isolation of an NDM-5-producing ST16 *Klebsiella pneumoniae* from a Dutch patient without travel history abroad, August 2015

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A New Delhi Metallo-beta-lactamase-5 (NDM-5)-producing ST16 *Klebsiella pneumoniae* strain was isolated from a Dutch patient in a long-term care facility without recent travel history abroad. Core genome multilocus sequence typing (cgMLST) revealed that the Dutch isolate was clonally related to isolates detected in four patients in Denmark in 2014. Public health experts and clinicians need to be informed; repetitive screening may be needed in patients without known risk factors for carbapenemases-producing *Enterobacteriaceae* who have undergone antibiotic treatment.

Here we report of a New Delhi Metallo-beta-lactamase-5 (NDM-5)-carrying ST16 *Klebsiella pneumoniae*, isolated from a hospitalised patient in the Netherlands, with no recent history of travel abroad. Analysis by core genome multilocus sequence typing (cgMLST) based on the core genome sequence of the isolate, showed that it is clonally related to four recently reported isolates cultured from four patients in two hospitals in Denmark, in 2014 [1].

Background

NDM is a carbapenemase that has been detected for the first time in a Swedish patient returning from New Delhi in 2008 [2]. In the first years thereafter, introduction of NDM-producing isolates in European hospitals was associated with returning travellers from India and Pakistan [3]. NDM-producing *Enterobacteriaceae* are rapidly dispersing over the world, and cases without any epidemiolgical links to the Indian subcontinent have been reported [4]. Reports on detection of NDM in environmental samples and in the food chain are worrisome, in particular since this might implicate spread of these resistance genes in the community [5,6]. In northern and western Europe, however, identification of patients with NDM-producing *Enterobacteriaceae* is still rare [7].

Currently, nine types of NDMs have been detected, of which NDM-1 is the most prevalent type [8]. NDM-5 has been isolated for the first time in the United Kingdom in 2011 from a patient returning from India [9]. Cases of NDM-5-carrying *Enterobacteriaceae* are sporadic. Some unrelated cases have been described, for instance three cases in Algeria and a case in Spain, with no recent history of travel abroad [10,11]. The identification of unrelated cases colonised with the same of NDM-5-producing *K. pneumoniae* ST16 clone in two countries with low prevalence, may be cause for concern.

Case description

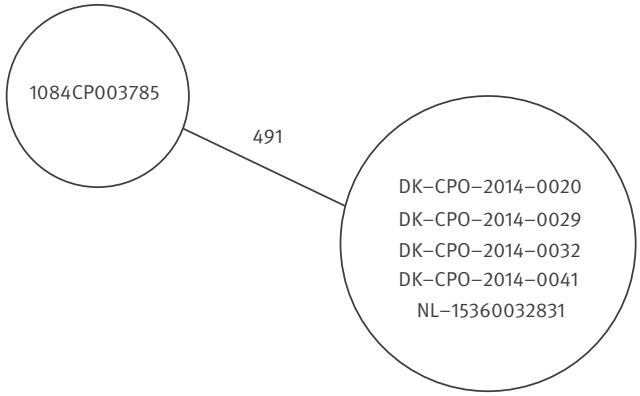
A Dutch patient in their 50s suffering from spinal cord injury had been admitted for rehabilitation in a long-term care facility in a northern region of the Netherlands. Upon admission in August 2015 (day 0), swab cultures taken from throat and rectum were negative for carbapenem-resistant Gram-negatives. The patient was treated upon admission with ceftriaxone 2 gr once per day for seven days, for an infection.

On day 10, an NDM-5-producing *K. pneumoniae* was cultured from a rectal swab taken as part of routine screening. Antibiogram based on automated susceptibility testing (VITEK2, bioMerieux, Marcy l'Etoile, France) and E-tests (AB Biodisk, Mannheim, Germany) showed increased minimum inhibitory concentrations (MICs) to meropenem and imipenem, and susceptibility to gentamicin, fosfomycin, and colistin (Table).

Whole genome sequencing, de novo assembly, and assessment of multilocus sequence typing (MLST), cgMLST, and resistome were performed as described previously [12,13]. The strain harboured beta-lactam

Figure

Minimal spanning tree of the four Danish NDM-5-producing ST16 *Klebsiella pneumoniae* isolates, the Dutch isolate, and reference strain K. *pneumoniae* 1084



NDM: New Delhi Metallo-beta-lactamase.

Sequences of core genome genes of Danish (DK-) isolates, the Dutch (NL-) isolate, and reference strain *K. pneumoniae* 1084 (GenBank accession number CP003785) were compared.

The tree is based on 634 columns, pairwise ignoring missing values. Number of allelic mismatches is presented.

Table

Antimicrobial minimum inhibitory concentrations for the NDM-5-producing *Klebsiella pneumoniae* isolate in a Dutch patient in a long-term care facility, August 2015

Antimicrobial agent	MIC (mg/L)[a]	Interpretation[a]
Piperacillin/tazobactam	≥128	S
Cefotaxime[b]	16	R
Ceftazidime	≥64	R
Cefepime	≥64	R
Meropenem[b]	6	I
Imipenem	≥16	R
Ciprofloxacin	≥64	R
Gentamicin	≤1	S
Tobramycin	≥64	R
Trimethoprim-sulfamethoxazole	≥320	R
Colistin	≤0.5	S
Tigecyclin [b]	2	I
Fosfomycin [b]	3	S

MIC: minimum inhibitory concentration; I: intermediate; R: resistant; S: susceptible.

[a] According to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines.

[b] Measured by E-test.

genes bla_{NDM-5}, $bla_{CTX-M-15}$, bla_{SHV-1}, bla_{OXA-1}, and bla_{TEM-1b}; non-beta-lactam acquired resistance genes included *strB*, *strA*, *aac(6")lb-cr*, *QnrB66*, *fosA*, *catB3*, *sul2*, *tet*(A), and *dfrA14*.

The patient was isolated and their room was cleaned and disinfected once a day. Throat and rectum swabs were taken twice a week from all 25 patients on the ward during the following three weeks. Pooled samples from rectum and throat were tested both by direct PCR (Check-direct CPE, Check-points, Wageningen, the Netherlands), and incubated in Brain-Heart Infusion broth with 0.25 mg/L ceftriaxone. The broth was subsequently cultured on combination Iso-Sensitest agar plates containing ceftriaxone, ceftazidime, tobramycin, or piperacillin/tazobactam (Mediaproducts, Groningen, the Netherlands). All patients were screened likewise upon admission or discharge for a period of three weeks. Ten patients who had been hospitalised on the same ward from day 0, and had already been discharged, were contacted and invited to the ward for taking cultures. Among 45 patients investigated, no additional cases have been detected as at 15 October 2015.

To search for the origin of the NDM-positive *K. pneumoniae* strain, environmental cultures were taken. All healthcare workers on the ward received a questionnaire on the following risk factors: recent stay in a foreign hospital as patient or worker, or being colonised previously with carbapenemase-producing *Enterobacteriaceae* (CPE). We did not advise to take cultures from healthcare workers because the Dutch infection control guidelines recommend only to screen healthcare workers in case of ongoing transmission after implementation of outbreak control measures [14]. We did not identify any likely origins through these investigations.

Literature was searched for common sources of NDM-5-producing *Enterobacteriaceae*. A recently published report on a Danish cluster of four patients with NDM-5-producing *K. pneumoniae* clones presented a similar resistome to our case (bla_{NDM-5}, $bla_{CTX-M-15}$, bla_{SHV-1}, bla_{OXA-1}, and bla_{TEM-1b}) [1]. Sequences of the Danish isolates were compared with the isolate from the Dutch case. All isolates belonged to ST16. The results of cgMLST analysis using a typing scheme described by Bialek-Davenet et al. [15] showed no allelic mismatches between our isolate and the Danish isolates (NCBI BioProject ID PRJNA285138). When comparing with reference strain *K. pneumoniae* 1084 (GenBank accession number CP003785), we found 491 allelic mismatches (Figure). Thus, the isolate of our patient is clonally related to the Danish isolates.

Discussion

We report the sporadic detection of an NDM-5-producing *K. pneumoniae* from a Dutch patient with no risk factors for acquisition. The origin of the strain is unknown. Similar cases have been presented in Denmark with clonally related isolates. There were no known direct epidemiological links between the Danish patients and our case: the Danish positive cases did not travel to the Netherlands, and in our long-term care facility, no Danish patients were hospitalised during the past year.

Unknown links or a common source may be the reason for the clonality of this rare isolate.

Another explanation might be that the clonal strains have been acquired from a foodborne source. *Escherichia coli* carrying bla_{NDM-5}, bla_{CTX-M} and bla_{TEM} have been reported as cause of mastitis in cows, suggesting that bla_{NDM-5}-carrying plasmids might enter the food chain. Moreover, contamination of retail chicken meat with *K. pneumoniae* producing a combination of NDM, CTX-M-15, TEM, and SHV-1 has recently been reported [16]. Typing of bla_{NDM}, plasmid, and MLST of these isolates may reveal a link with a foodborne source.

K. pneumoniae ST16 is a highly prevalent type causing nosocomial infections [17]. It is unsure whether this ST16 clone is newly introduced, or whether this clone was already present in our region, and has now acquired an NDM-5-carrying plasmid. We anticipate a full study, which will assess the relation between the NDM-5-producing isolates and the epidemic CTX-M-15-producing *K. pneumoniae* ST16 isolates in our region, and possible links with the food chain. Since our case was detected by chance and we did not identify any routes of transmission, further cases may be found in the Netherlands or elsewhere. We contacted national reference centers in Europe to find cases retrospectively, and to alert them about potential future cases. Finally, our case suggests that it might be necessary, under specific conditions, to screen for CPE also in patients who do not have a recent history of travel to a CPE-endemic country, and that diagnostics excluding CPE at admission, should be repeated if patients are using antibiotics during hospitalisation.

Conflict of interest

None declared.

Authors' contributions

EB collected the data and drafted the manuscript, JWR supervised the molecular research and analysis, ML organised infection control measurements and risk assessments, AWF participated in the coordination and concept of the manuscript, AMH coordinated and edited the manuscript.

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Research article

Was the increase in culture-confirmed *Campylobacter* infections in Denmark during the 1990s a surveillance artefact?

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In 1991, 1999 and 2006, randomly selected individuals from the Danish Central Personal Register provided a serum sample. From individuals aged 30 years and above, 500 samples from each year were analysed for *Campylobacter* IgG, IgA and IgM antibodies using a direct ELISA method. We applied a seroincidence calculator available from the European Centre for Disease Prevention and Control to perform a mathematical back-calculation to estimate the annual *Campylobacter* seroincidence in the Danish population. The estimated *Campylobacter* seroincidence did not differ significantly between the 1991, 1999 and 2006 studies although the reported number of culture-confirmed cases of *Campylobacter* infection increased 2.5 fold from 1993 to 1999 among individuals aged 30 years and above. This suggests that *Campylobacter* was widely present in the Danish population before the increase in poultry-associated clinical *Campylobacter* infections observed from 1993 to 2001 among individuals of this age groups.

Introduction

Following the description of a simple method for *Campylobacter* spp. isolation in 1977 [1], this genus was recognised as the leading cause of bacterial gastroenteritis in most industrialised countries [2]. The incidence of reported cases of *Campylobacter* infection rose dramatically during the 1990s in many industrialised countries and the reasons for this increase have been discussed in the scientific literature [3,4]. As contaminated poultry, in particular chickens, is expected to be a principal source of *Campylobacter* infections, it has been suggested that the emergence of these infections in a number of industrialised countries was caused by increased consumption of fresh chicken in the 1990s [5]. This hypothesis has been supported by analysis of a 'natural experiment', in which withdrawal of chicken and eggs from the Belgium market in June 1999 due to dioxin-contaminated feed resulted in a 40% decrease in human *Campylobacter* infections, as well as by interventions in New Zealand and Iceland in 1997–2008 and 1995–2007, respectively to reduce contamination of chickens [6-8]. However, it is also likely that increased diagnostic activity after the implementation of routine methods in clinical microbiology to detect *Campylobacter* and increased awareness by clinicians contributed to the increase in the number of *Campylobacter* infections [4].

In Denmark, the annual number of reported *Campylobacter* infections increased during the 1990s and from 1993 to 1999 a 2.5-fold increase in the incidence of *Campylobacter* infection was observed among individuals aged 30 years and above (Figure 1). The aim of our study was to use historical Danish serum collections of individuals representing the adult Danish population to determine the seroincidence of *Campylobacter* infections in 1991, 1999 and 2006. The main hypothesis was that the increase in the number of reported *Campylobacter* infections in Denmark reflects a real increase in incidence, and consequently the seroincidence was expected to be lower in 1991 than in 1999 as well as 2006.

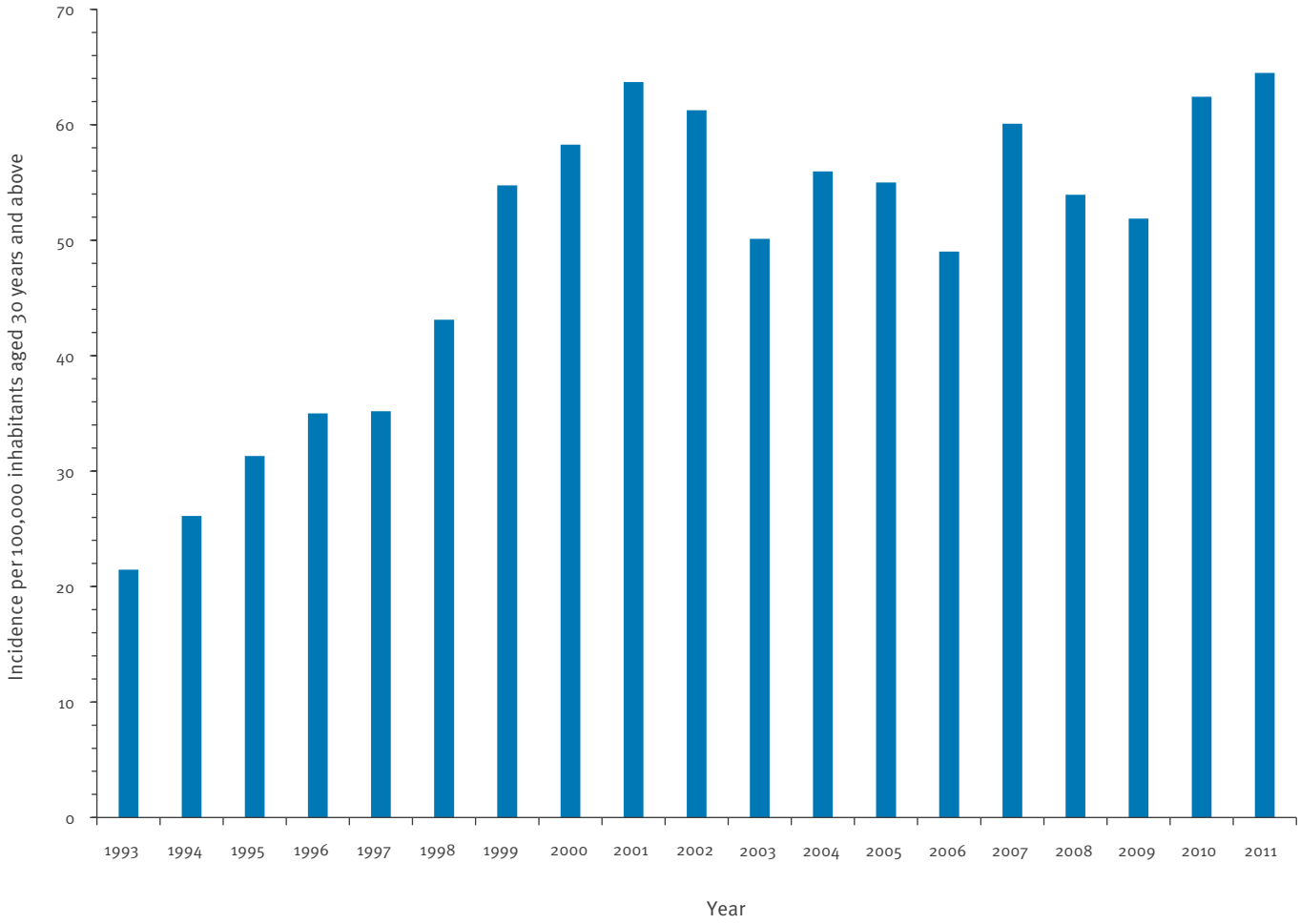
Methods

Study population

In 1991, 1999 and 2006, three consecutive cross-sectional studies were organised by the same group of investigators [9]. Danish adults (with Danish citizenship and born in Denmark) living in one of the 11 municipalities of Copenhagen County were invited to

Figure 1

Incidence of laboratory-confirmed *Campylobacter* infections per 100,000 inhabitants aged 30 years and above, Denmark, 1993–2011



Source: Danish Register of Enteric Pathogens.

participate in a general health examination. The study populations were randomly selected from the Danish Central Personal Register among individuals above 18 years of age.

Blood samples were taken during the following time periods: (i) February 1991 to May 1992, from 2,017 individuals; (ii) March 1999 to January 2001, from 3,501 individuals; and (iii) June 2006 to June 2008, from 3,471 individuals. These three cross-sectional studies are referred to hereafter as the 1991, 1999 and 2006 studies, respectively.

For our study presented here, 500 serum samples were randomly selected using a random-digits algorithm from each cross sectional study. In each of the three studies, 125 individuals from each of the age groups 30–39, 40–49, 50–59 and 60–69 years were included.

Serology

All the serum samples (3 × 500) were tested for IgG, IgM and IgA antibodies against *Campylobacter* at the serological laboratory at SSI using a direct in-house-developed *Campylobacter* ELISA based on a combination of *C. jejuni* O:1,44 and O:53 antigens in the ratio 1:1 [10].

Statistical analyses

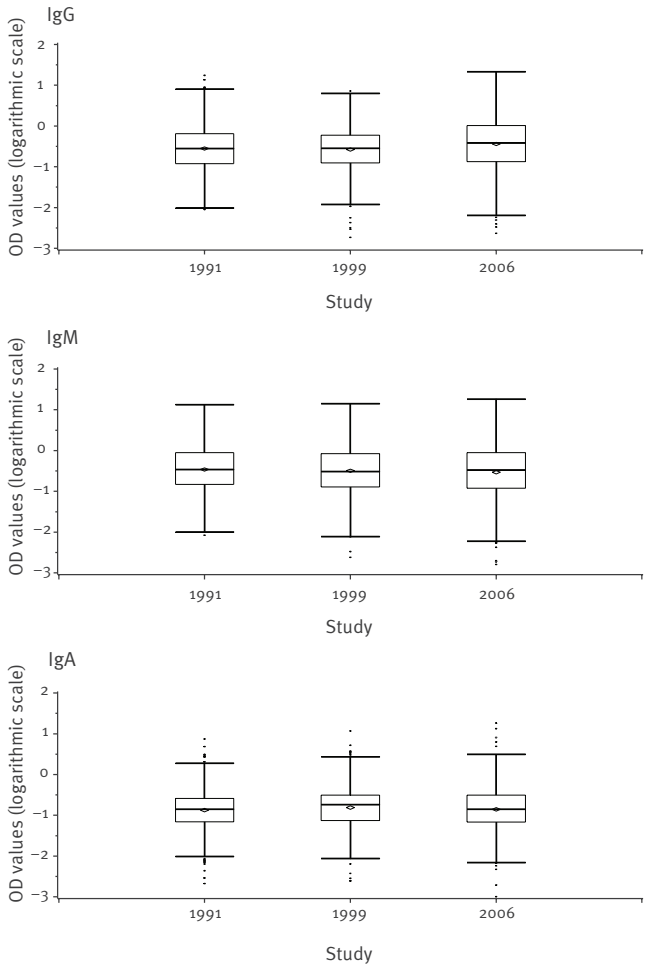
The distribution of IgA, IgG and IgM optical density (OD) values in samples from the three studies were compared overall and in the four age groups and by sex using the Kruskal–Wallis test. If this test showed significant differences at 5% level, pairwise comparison was performed using Wilcoxon two-sample test. The statistical programme SAS version 9.4 was used for the descriptive analyses (SAS Institute, Cary, NC).

Seroincidence estimation

We have previously developed and described a Bayesian mathematical back-calculation model to estimate the incidence rate of *Campylobacter* seroconversion in humans [11]. This model was based on the IgG, IgM and IgA kinetics observed during a longitudinal study of 210 patients with stool culture-confirmed

Figure 2

Measured *Campylobacter* IgG, IgM and IgA optical density values in cross-sectional serum samples in the 1991, 1999 and 2006 studiesa, Denmark (n = 500 per study)



OD: optical density.

The measured OD values are presented on a logarithmic scale. The box shows the interquartile range (IQR), the horizontal line across the box is the median, while the diamond is the mean. Dots above the upper bar represents values that are at least 1.5 times the IQR above the 75th percentile; dots below the lower bar represents values that are at least 1.5 times the IQR below the 25th percentile. Where no such dots are shown, the upper and lower bars represents the maximum and minimum values measured.

[a] Blood samples were taken during the following time periods: (i) February 1991 to May 1992; (ii) March 1999 to January 2001; and (iii) June 2006 to June 2008. These three cross-sectional studies are referred to as the 1991, 1999 and 2006 studies, respectively.

Campylobacter infections in Denmark from 1996 to 1997 [10]. The model incorporates inter-individual variation of peak antibody response and decay rates. By combining the information from the longitudinal study with measurements from the cross-sectional studies analysed here, we obtained estimates of the annual *Campylobacter* seroincidence in the population. These seroincidences were compared pairwise by calculating means and percentiles of the posterior distributions of incidence rate ratios [12].

Based on the mathematical model, a seroincidence calculator tool was developed in the statistical programme R [11] and since March 2015, this tool has been freely available from the European Centre for Disease Prevention and Control (ECDC) [13]. The tool and the underlying model to estimate seroincidences depend on a number of factors, including the level of censoring chosen for the analysis. When we use censoring, we assume that following a campylobacter infection, antibody levels do not decay towards zero but remain elevated above zero, compatible with a baseline *Campylobacter* antibody level a long time after the infection. These baseline antibody levels are reached after 4.5, 2.0 and 2.5 months for IgG, IgM and IgA, respectively. In the tool, censoring means that OD values below the chosen censoring level are not used to calculate the exact time since last infection, but contribute as censored observations where a long time since last infection has occurred. In the study presented here, we applied different censoring levels to illustrate the impact of censoring. In the first analysis, all three antibodies were censored at the OD value 0.25, which is a low censoring level, in particular for IgG and IgM. In the second analysis, IgG, IgM and IgA OD values were censored at 1.0, 0.4 and 0.2, respectively. This set of censoring values was chosen because a clear decrease in OD values following the acute phase of *Campylobacter* infection was still observed in the longitudinal data at these censoring levels [10].

Sources of supporting data

Campylobacter laboratory diagnoses are notifiable in Denmark and all culture-confirmed human cases are entered into the Danish Register of Enteric Pathogens. The number of notified cases per year in individuals aged 30 years and above was extracted from this register and the incidence per 100,000 inhabitants is shown in Figure 1.

The consumption of poultry meat in Denmark per year was obtained from Statistics Denmark [14].

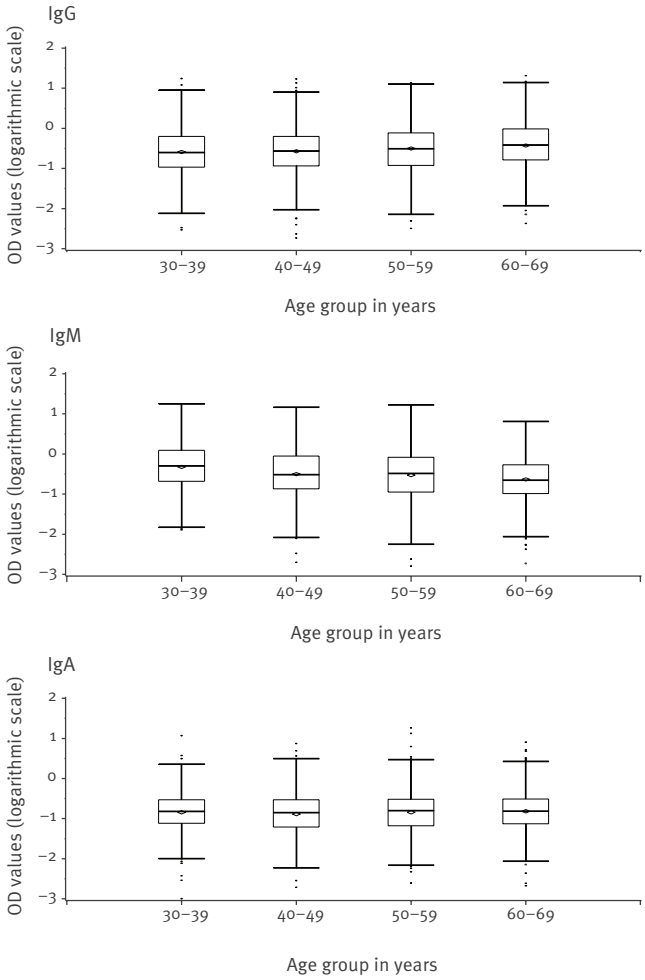
Results

The proportions of men in the 500 individuals selected were 47% (n = 236), 52% (n = 259) and 45% (n = 224) in the 1991, 1999 and 2006 studies, respectively, which were not significantly different from the general population in 1991 and 1999, but the proportion of men was significantly lower in the 2006 study compared with the general population, in which 49.5% (2,685,846/5,427,459; p = 0.036) were men [15].

IgA OD values differed between the three studies (p = 0.039, Kruskal–Wallis test) with a median OD value of 0.48 (interquartile range (IQR): 0.28) in 1999 compared with 0.43 (IQR: 0.25) in 1991 and 0.42 (IQR: 0.29) in 2006. Pairwise comparisons showed significantly higher IgA OD values in 1999 compared with those in 1991 (p = 0.016) and 2006 (p = 0.0486). For IgG, a clear difference was observed between the studies (p = 0.0002, Kruskal-Wallis test), with the highest

Figure 3

Measured *Campylobacter* IgG, IgM and IgA optical density values in cross-sectional serum samples, by age group, in the 1991, 1999 and 2006 studies[a], Denmark (n = 375 per age group)



OD: optical density.

The measured OD values are presented on a logarithmic scale. The box shows the interquartile range (IQR), the horizontal line across the box is the median, while the diamond is the mean. Dots above the upper bar represents values that are at least 1.5 times the IQR above the 75th percentile; dots below the lower bar represents values that are at least 1.5 times the IQR below the 25th percentile. Where no such dots are shown, the upper and lower bars represents the maximum and minimum values measured.

[a] Blood samples were taken during the following time periods: (i) February 1991 to May 1992; (ii) March 1999 to January 2001,; and (iii) June 2006 to June 2008. These three cross-sectional studies are referred to as the 1991, 1999 and 2006 studies, respectively.

median OD value of 0.66 (IQR: 0.59) in 2006 compared with 0.58 (IQR: 0.40) in 1999 and 0.57 (IQR: 0.43) in 1991. Pairwise comparisons showed significantly higher IgG OD values in 2006 compared with those in 1991 ($p<0.001$) and 1999 ($p<0.001$). For IgM, no significant difference in measured OD values was observed between the years ($p=0.416$, Kruskal–Wallis test) (Figure 2).

The distribution of measured campylobacter OD values across the studies and between age groups is shown in Figure 3. IgA OD values did not differ significantly between age groups ($p=0.487$). Kruskal–Wallis test shows significant differences in IgG OD values between the age groups ($p<0.001$), with the lowest median OD value of 0.54 (IQR: 0.44) in those aged 30–39 years and the highest median OD value of 0.66 (IQR: 0.52) observed among those aged 60–69 years. Pairwise comparisons showed significant differences at 5% level between all age groups except between IgG OD values from those aged 30–39 and 40–49 years, as well between the age groups 40–49 and 50–59 years. For IgM OD values, a clear age difference was observed ($p<0.001$), with the highest median OD value of 0.74 (IQR: 0.50) observed among individuals in their 30s compared with those in their 60s, where the median OD value was 0.52 (IQR: 0.41). Pairwise comparisons showed significant differences at 5% level between all age groups except between IgM OD values from those aged 40– 49 and 50–59 years.

Comparing antibody levels by sex in each of the three cross-sectional studies showed that IgG OD values were not significantly different. IgM OD values were significantly higher in women in all three studies while IgA OD values were higher in men in all three studies, but this difference was only significant in the 1991 study.

The estimated *Campylobacter* seroincidences in 1991, 1999 and 2006 using the two different censoring levels described above are shown in Tables 1 and 2. The seroincidence estimates changed when the censoring level changed, resulting in lower estimated seroincidences when censoring levels increased. The annual risk of at least one infection per year per person was estimated to be around 70% when the low censoring level was used and about 50% with the high level. The two different censoring levels produced the same seroincidence patterns over time and both approaches showed no significant differences in seroincidence rate between the 1991, 1999 and 2006 studies. For comparison, the incidence of laboratory-confirmed cases of Campylobacter infection in individuals aged 30 years and above from 1993 to 2011 are presented in Figure 1, showing a clear increase in the number of laboratory-confirmed cases of *Campylobacter* infection from 1993 to 2001.

Discussion

To the best of our knowledge, this is the first study in which serology has been used to estimate *Campylobacter* seroincidence over time and in which this measure has been compared with the number of notified cases during the same period. Although there were minor differences in IgA and IgG values between samples from the three studies, these did not result in significant differences between the seroincidence rates. This is in contrast to the reported number of laboratory-confirmed cases of *Campylobacter* infection, for which a 2.5-fold increase in incidence per 100,000

Table 1

Estimated *Campylobacter* seroincidence in 1991, 1999 and 2006 studies in Denmark, and pairwise comparisons of the estimated incidence using low censoring levels[a]

Study	Sampling period	Estimated seroincidence per person-year	Risk of at least one infection per year per person	Pairwise comparison Incidence rate-ratios (PI)	
		Mean (95% PI)	$1 - e^{-\text{seroincidence} \times 1}$	1991–1992	1999–2001
1991	Feb 1991–May 1992	1.212 (1.131–1.299)	70%	–	–
1999	Mar 1999–Jan 2001	1.170 (1.092–1.253)	69%	1.04 (0.90–1.19)	–
2006	Jun 2006–Jun 2008	1.203 (1.121–1.291)	70%	1.01 (0.88–1.16)	0.97 (0.85–1.12)

PI: prediction interval.

[a] All three antibodies were censored at the optical density value 0.25.

Table 2

Estimated *Campylobacter* seroincidence in 1991, 1999 and 2006 studies in Denmark, and pairwise comparisons of the estimated incidence using high censoring levels[a]

Study	Sampling period	Estimated seroincidence per person-year	Risk of at least one infection per year per person	Pairwise comparison Incidence rate ratios (PI)	
		Mean (95% PI)	$1 - e^{-\text{seroincidence} \times 1}$	1991–1992	1999–2001
1991	Feb 1991–May 1992	0.696 (0.647–0.748)	50%	–	–
1999	Mar 1999–Jan 2001	0.672 (0.625–0.722)	49%	1.04 (0.90–1.20)	–
2006	Jun 2006–Jun 2008	0.737 (0.685–0.792)	52%	0.94 (0.82–1.09)	0.91 (0.79–1.05)

PI: prediction Interval.

[a] IgG, IgM and IgA optical density values were censored at 1.0, 0.4 and 0.2, respectively.

inhabitants aged 30 years or more was observed, from 21.5/100,000 in 1993 (677 cases) to 54.8/100,000 in 1999 (1,821 cases) (Figure 1). Thuesen et al. [16] looked further into who participated in the 2006 study and they found they were older, had a higher educational level and higher income, while non-responders were often living alone, were men, had a higher prevalence of hospitalisation and more days at hospital for any reason. On the other hand, use of prescription drugs and the prevalence of more than one annual contact with general practitioners were higher among responders. Bender et al. [17] found that participants in the 1999 study cohort were older, were more often house owners, wage earners, living with a partner, had a higher education level and higher income. These studies indicate that people who participate in general health examinations have a higher socioeconomic status and they might also be individuals with an interest in a healthy lifestyle. A similar study was not carried out for the 1991 study cohort; however, there is no strong indication that the effect of selection bias will have changed over the years covered in the studies, indicating that the responders were comparable over time.

The seroincidence rates presented in Tables 1 and 2 are very high compared with the number of laboratory-confirmed cases of *Campylobacter* infection (Figure 1). Even when the high levels of censoring were used, the risk of at least one infection per year per person was about 50%, which suggests that the immune system of the study participants aged 30 years and above had been exposed to *Campylobacter* every other year. The seroincidence does not reflect number of clinically ill individuals and it is conceivable that only a fraction of *Campylobacter* infections will lead to clinical illness [18]. Given the fact that *Campylobacter* is ubiquitous in many environmental reservoirs as well as in poultry, it is biologically plausible that the seroincidence is much higher than the number of reported cases, as has been observed for *Salmonella* infections [12,19-21].

In principle, the model may be able to assign a time since infection (and a corresponding distribution) for all cases of *Campylobacter* infection, even when measured antibody levels are low. However, the back-calculation is more precise in a short time period after infection, i.e. within a couple of months, when antibody levels are generally high, rather than several months or years after presumed infection. On this basis, censoring at high OD values that correspond to a short estimated time since infection may be preferable, due to uncertainties in the seroincidence measurement. Other researchers, however, may prefer to include all

available data and present seroincidence rates accordingly, but with larger prediction intervals.

Seroresponse following *Campylobacter* infection may indicate some degree of immunity against infection. In two studies from the United States, students developed gastrointestinal illness following the consumption of unpasteurised milk [22,23]. These outbreaks were caused by *C. jejuni*, and ill students had antibody levels consistent with recent infection. In both studies, there were groups of individuals who had high *C. jejuni* antibody levels who did not become ill. These high antibody levels without illness were correlated with habitual consumption of unpasteurised milk, which indicates the development of immunity [22,23]. In an experimental study of *C. jejuni* infection in humans in the United States, reinfection of volunteers with the same strain did not result in clinical illness, supporting the theory that at least short-term immunity is developed following infection [18]. This underscores that seroincidence rates are not a direct measure of clinical illness but rather a measure of the force of infection at the population level, and that this, to some extent, may be related to immunity.

Studies from Thailand in 1980 and 1987 showed that repeated *Campylobacter* infections are common in early childhood (<24 months-old) and infection rates decrease with age, paralleled by a progressive increase in specific serum antibodies [24,25]. Studies from industrialised countries also indicate the importance of age. A Danish study of the spatial distribution of *Campylobacter* infections found that residence in rural areas and areas with a low population density were both associated with an increased risk of infection, and that this association concerned children aged 0–14 years in particular. This association could explain a third of cases among children in the Danish countryside in 1991 to 2001 [26]. Furthermore, in Wisconsin in the United States, a seroepidemiological study in 1997 to 1999 among rural children showed that increasing age as well as farm residence were associated with increasing *C. jejuni* seropositivity. In the age group 15–18 years, between 85% and 90% of farm-resident children were seropositive, while for children not living on farms, it was between 60% and 65% [27]. Also, in the Netherlands, a high level of *Campylobacter* antibodies and a high seroincidence has been measured from 30 years of age [28]. In the study presented here, all participants were aged 30 years and above and only small differences in measured antibody OD values were observed between the age groups investigated. This corroborates the hypothesis that Danish children and young adults are exposed to *Campylobacter*, after which specific antibodies are produced and the antibody level remains elevated. The age dependency of the IgM response, which reappears less strongly after repeated infections, supports this notion.

In addition to poultry and cattle, major *Campylobacter* transmission routes include animal contact with farm animals, occupations related to farm animals and contact with environment connected to farms [29]. With many different reservoirs harbouring *Campylobacter* and a seasonal variation in the number of *Campylobacter*-infected broiler flocks, with up to 80% of flocks infected during summer, it is likely that many Danes are exposed at regular intervals to *Campylobacter*, followed by seroconversion, although they do not become clinically ill [30,31].

Our group has previously shown that the emergence of *Salmonella* Enteritidis in the 1990s was mirrored by an almost parallel increase in the seroincidence of *Salmonella* [21]. We expected to see a similar pattern for *Campylobacter*, which was considered as an emerging infection in the late 1990s. To our surprise, however, we were unable to demonstrate an increase in the *Campylobacter* seroincidence rates, which raises the hypothesis that at least part of the increased reporting of *Campylobacter* infections may have been due to increased and improved diagnostic activity and awareness, rather than a true increase in incidence. There are, however, some important counter arguments. First of all, the increase in the number of reported culture-confirmed cases during the 1990s in Denmark was similar to the increases seen in other European countries, and these increases have been linked to increased consumption of fresh poultry meat [3,4]. A Danish case–control study from 2000 to 2001 found that the main domestic risk factor for campylobacteriosis was consumption of chicken meat that had been bought fresh and subsequently not frozen [5]. In 2011, source attribution of *Campylobacter* in Denmark indicated that Danish chicken and cattle as well as imported chicken are important sources of infection [29]. The association between poultry and *Campylobacter* infections in humans is corroborated by the fact that during the 1990s, the consumption of poultry meat in Denmark increased from 12.4 kg per resident in 1991 to 19.2 kg in 2000 [14]. In addition, the consumption of fresh poultry meat increased during the same period [32]. As freezing kills *Campylobacter* bacteria, the change in consumer habits towards more poultry meat per inhabitant per year and consumption of fresh poultry meat has most likely increased the *Campylobacter* dose that humans are exposed to [5]. In addition, the proportion of Danish broiler flocks testing positive for *Campylobacter* is highest in August and lowest during winter [30,31], which is the same seasonal pattern as human *Campylobacter* infections [31]. A previous study has shown that the quantity of *Campylobacter*-contaminated food products consumed was directly related to the occurrence and severity of disease [22]. This indicates that the increase in consumption of fresh poultry during the 1990s was a possible explanation why the reported number of human cases of *Campylobacter* infection started to increase without any change in seroincidence rate during the same period, as *Campylobacter* antibody levels were already at a high level. Furthermore, the proportion of reported cases is very small compared

with the estimated seroincidence. We propose that the increase in rates of clinical illness was driven by the increased consumption of fresh poultry, whereas seroincidence is a composite measure reflecting various sources of exposures, of which poultry is merely a fraction. As an example, a number of large outbreaks of *Campylobacter* were recently attributed to contaminated drinking water [33,34].

Use of serology as a tool to measure *Campylobacter* infections in the population is also subject to limitations. The longitudinal study described IgG, IgA and IgM *Campylobacter* antibody decay profiles of 210 patients with stool culture-confirmed campylobacter infections and it is assumed that the seroresponse in the longitudinal study population is similar to that in the cross-sectional populations [11]. In addition, the seroincidence calculator tool can only be used when the cross–sectional samples are analysed using the same ELISA method as that used to analyse the sera from the longitudinal study. Finally, we cannot rule out that we might have observed different seroincidences in the 1991, 1999 and 2006 studies if children and adolescents had been chosen as study populations.

In conclusion, we were unable to associate the emergence of culture-confirmed *Campylobacter* infections with a similar increase in seroincidence at the population level. This suggests that *Campylobacter* was widely present in the Danish population before the increased incidence of poultry-associated clinical human *Campylobacter* infections and that other sources of infection were present. Given our findings, there may be a larger diversity of *Campylobacter* exposure than hitherto thought; however, the development of clinical illness might be related to the *Campylobacter* dose individuals are exposed to.

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Conflict of interest

None declared.

Authors' contributions

Hanne-Dorthe Emborg led the writing of the paper and was responsible for interpretation of the obtained results. All authors provided contributions to the paper and approved the final version. Peter Teunis developed the seroincidence calculator tool used to estimate *Campylobacter* seroincidences. Jacob Simonsen performed the statistical analyses. Kåre Mølbak got the original idea for this study. Karen A. Krogfelt and Charlotte S. Jørgensen were responsible for the laboratory work and provided input to the interpretation of results. Johanna Takkinen provided input to the discussion and interpretations of the obtained results.

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Research article

Comparison of serological assays in human Middle East respiratory syndrome (MERS)-coronavirus infection

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Plaque reduction neutralisation tests (PRNT), microneutralisation (MN), Middle East respiratory syndrome (MERS)-spike pseudoparticle neutralisation (ppNT) and MERS S1-enzyme-linked immunosorbent assay (ELISA) antibody titres were compared using 95 sera from 17 patients with MERS, collected two to 46 days after symptom onset. Neutralisation tests correlated well with each other and moderately well with S1 ELISA. Moreover to compare antigenic similarity of genetically diverse MERS-CoV clades, the response of four sera from two patients sampled at two time periods during the course of illness were tested by 90% PRNT. Genetically diverse MERS-CoV clades were antigenically homogenous.

Introduction

Middle East respiratory syndrome (MERS) poses a major threat to global public health [1]. Validated serological assays are important for diagnosis and for seroepidemiology to define prevalence and risk factors [2,3]. Serological assays for detecting antibody for MERS-coronavirus (CoV) infection include antibody arrays, enzyme-linked immunosorbent assay (ELISA), immune-fluorescence, microneutralisation (MN), plaque reduction neutralisation (PRNT) and MERS-spike pseudoparticle neutralisation tests (ppNT) [2,4-6]. While data from individual case reports exist [6], there are limited comparative data on serological methods for detecting MERS-CoV antibody in humans, because of a lack of well-characterised sera [7]. We used 95 sera from 17 patients with real-time reverse transcription polymerase chain reaction (RT-PCR) confirmed MERS-CoV infection diagnosed during an outbreak of MERS in South Korea [8,9] to compare PRNT antibody titres using 90% ($PRNT_{90}$) and 50% ($PRNT_{50}$) plaque reduction end points, MN, MERS-spike ppNT and S1-ELISA tests. The sera were also used to investigate the antigenic similarity of three genetically diverse strains of MERS-CoVs [10]. We had previously reported that early $PRNT_{50}$ and S1-ELISA antibody responses in this patient-cohort were associated with improved clinical outcome [9].

Methods

Patients

Patients with RT-PCR confirmed MERS-CoV infections admitted to Seoul National University (SNU) Hospital, SNU Boramae Medical Center and SNU Bundang Hospital within the first 14 days after onset of illness during the outbreak of MERS-CoV between May and June 2015 in South Korea were included. Serial serum samples (n = 95 in total) were collected from 17 patients during the first 39 days of illness or up to time of discharge from hospital. This study was approved by the Institutional Review Board of SNU. Clinical data on these patients have been previously reported [9].

Viruses

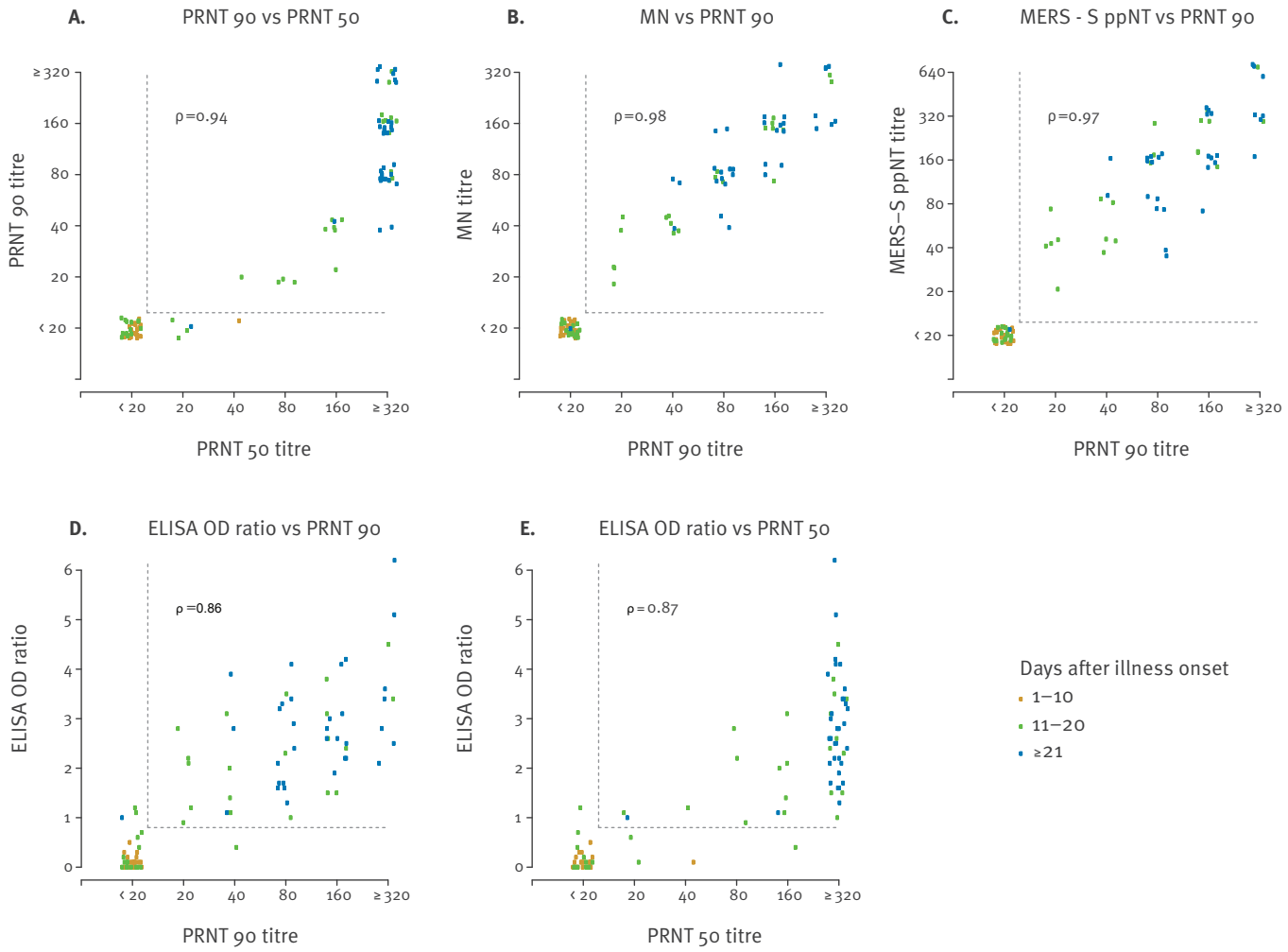
The MERS-CoV strains used in the virus neutralisation assays belonged to clade A (MERS-CoV-strain EMC), clade B (dromedary camel MERS-CoV Al-Hasa FKU-HKU13 2013) as well as a virus from a distinct non A and B clade (dromedary camel Egypt NRCE-HKU 270 2013) as previously described [10].

Serology tests

The sera were heat-inactivated for 30 min at 56 °C before testing. The PRNT assays were performed in a 24-well format in duplicate for each serum dilution. Twofold serum dilutions were incubated with 40 to 60 plaque-forming units of virus for 1 hour at 37 °C. The virus – serum mixture was added onto a Vero cell monolayer and incubated for 1 hr at 37 °C in a 5% CO2 incubator. Then, the supernatant was removed and the cells overlaid with 1% agarose (SeaKem LE Agarose, Lonza,

Figure

Scatter plots comparing antibody titres obtained from different assays in relation to duration (days) after onset of illness due to Middle East respiratory syndrome (MERS)-coronavirus infection



ELISA: enzyme-linked immunosorbent assay; MN: microneutralisation; OD: optical density; PRNT: plaque reduction neutralisation test; ppNT: pseudoparticle neutralisation test.

Spearman correlation for each comparison is denoted in each panel. Sera collected 1–10; 11–20 and ≥ 21 days from onset of illness are denoted in yellow, green and blue, respectively. The MERS-spike ppNT, MN, PRNT50, and PRNT90 titres have been jittered for better presentation. The negative cut-off titres or OD is denoted in a dotted line.

Switzerland) in cell culture medium (Minimum Essential Medium with 2% fetal bovine serum). The plates were fixed and stained after three days incubation. Antibody titres were defined as the highest serum dilutions that resulted at ≥ 50% ($PRNT_{50}$) and ≥ 90% ($PRNT_{90}$) reduction in the number of plaques, respectively.

The ppNT assays were performed as previously described, with triplicate serum dilutions [5,11]. MN tests were carried out to determine the highest serum dilution that suppressed virus cytopathic effect in Vero cells following infection with a virus dose of 100 tissue culture infection $dose_{50}$ mixed with the respective serum dilution [5]. Serum dilutions were done in quadruplicate. Positive and negative controls and virus back-titrations were included in each assay. Antibody titres of ≥ 1:20 were regarded as positive.

The S1 ELISA EI 2604–9601G kit was purchased from EUROIMMUN Luebeck, Germany for detection of human IgG against MERS-CoV. The test was done on single serum samples in duplicate according to the manufacturer's instructions. The assay included a calibrator which defined the upper limit of the reference range in non-infected humans and this value was defined as the cut off. The assay was made semi-quantitative by calculating the ratio of the extinction of the patient sample/extinction of the calibrator. Ratios < 0.8 were considered negative, those ≥ 1.1 as positive and those ≥ 0.8 to < 1.1 regarded as borderline.

Statistical methods

Spearman correlation coefficient was calculated to assess the correlations between the different assays.

Table 1

Proportion of sera that were positive for antibodies to Middle East respiratory syndrome (MERS) in various assays at different times post-disease onset

Time periods in days from onset of illness	Number of serum samples	Number of patients	Proportion of sera with neutralising antibody titres ≥ 1:20 n/N				ELISA positive
			$PRNT_{90}$	$PRNT_{50}$	ppNT	MN	
1–5	7	7	0/7	0/7	0/6[a]	0/7	0/7
6–10	17	11	0/17	0/17	0/17	0/17	0/17
11–15	18	17	7/18	9/18	6/18	6/18	7/18
16–20	19	17	14/19	15/19	14/18[a]	14/19	15/19
≥ 21	34	9	33/34	34/34	31/33[a]	33/34	33/34

ELISA: enzyme-linked immunosorbent assay; MN: microneutralisation; PRNT: plaque reduction neutralisation test; ppNT: pseudoparticle neutralisation test.

[a] One serum in each of these groups could not be tested in the ppNT assay, thus the denominator for the ppNT differed from the others.

Table 2

Antigenic cross-reactivity of human convalescent sera with genetically diverse MERS-CoV in 90% plaque reduction neutralisation tests (PRNT90)

Patient ID	Days of illness	Reciprocal $PRNT_{90}$ antibody titre to MERS-CoV strains representing different MERS-CoV clades		
		Strain EMC (clade A)	Dromedary camel Al-Hasa KFU-HKU13 2013 (clade B)	Dromedary camel Egypt NRCE-HKU 270 2013 (clade non A/B)
B	12	320	160	160
B	39	320	320	640
G	17	40	40	80
G	35	160	80	160

ID: identity; MERS-CoV: Middle East respiratory syndrome (MERS)-coronavirus.

Results

Scatter-plots showing correlation between $PRNT_{90}$, $PRNT_{50}$, ppNT, MN and S1-ELISA assays are shown in Figure A-E. As expected, the $PRNT_{50}$ assay was more sensitive than the $PRNT_{90}$ because it uses the less stringent end-point of 50% reduction in the plaque count (Figure A). There was excellent correlation between the $PRNT_{90}$, MN and MERS-spike ppNT titres with Spearman correlations of 0.97–0.98 (Figure B,C). MERS-CoV S1 ELISA was less strongly correlated with the different neutralisation assays with Spearman correlation of 0.86–0.87 (Figure D and E).

Table 1 shows the proportion of sera that were positive in neutralising tests at titres ≥ 1:20 or in ELISA. None of the patients were seropositive in the first 10 days of illness. At 11–15 days of illness, 50% of sera were positive in $PRNT_{50}$ assays, 39% in $PRNT_{90}$ and S1-ELISA assays and 33% positive in ppNT and MN assays. After 21 days of illness, the majority of patients were seropositive. However, even at day 32 of illness, one patient remained seronegative in $PRNT_{90}$, ppNT and MN assays, borderline positive in the S1 ELISA and was only positive in the $PRNT_{50}$ test at a titre of 1:20. She was aged in her mid-fifties with no underlying diseases, and presented with a relatively mild pneumonic illness (reported more fully in [9]).

Twelve patients seroconverted (fourfold increase in antibody titre) by all five assays and one had static high titres (first serum sample of this patient was at day 13 of illness). The woman in her mid-fifties noted above failed to seroconvert by S1 ELISA, MN and $PRNT_{90}$, and only reached $PRNT_{50}$ antibody titre of 1:20 up to day 32 of illness. Three other patients did not seroconvert in any of the assays, but the latest available sera from them was at day 8, 9 and 16, respectively, too early to conclude whether sera of these patients at a later stage of illness would have shown seroconversion.

In order to compare antigenic similarity of genetically diverse MERS-CoV, we selected four sera from two patients. These sera had been sampled early (day 12, 17) and later (day 35, 39) during the course of illness. The antibody titres of each serum to clade A, clade B and the genetically divergent Egyptian camel viruses were within twofold (Table 2).

Discussion

The different virus neutralisation assays (MN; ppNT $PRNT_{50}$; $PRNT_{90}$) all had excellent correlation among them (Spearman correlation ≥ 0.94) (Figure). The $PRNT_{50}$ antibody test was more sensitive in detecting early antibody responses and had higher antibody titres

throughout, as would be expected, given the less stringent end point of ≥ 50% reduction of plaque numbers, in contrast to the ≥ 90% reduction of plaques needed for the $PRNT_{90}$ antibody titre endpoint (Figure A) (Table 1). In studies of household transmission of MERS-CoV, it was shown that $PRNT_{50}$ can detect some infections undetected by $PRNT_{90}$ tests [12].

Unlike other neutralisation tests that require handling live MERS-CoV in biosafety level (BSL)-3 containment, the MERS-spike ppNT assay does not require BSL-3 containment. It gave good correlation with $PRNT_{90}$ and MN tests. We have previously shown that sera from healthy humans sera from Hong Kong (n = 115), Egypt (n = 100) and Saudi Arabia (n = 237) were negative in the ppNT test [5,13], confirming the specificity of this assay. Although the ppNT assay had been extensively used and validated for seroepidemiology in animals and livestock with good correlation between MERS ppNT and MN assays, [5,11,13], this is the first extensive demonstration of its performance in humans with confirmed MERS-CoV infection and during the first six weeks of infection. ppNTs have proved to be reliable surrogates for neutralisation tests in other infections including avian influenza A(H5N1) [14]. Thus, the MERS-spike ppNT may be usable for large scale seroepidemiology studies to assess extent of MERS-CoV infection in the general population, to assess risk factors of infection in high-risk groups, or when selecting patient sera for plasmapheresis for preparation of convalescent plasma where quantification of neutralising antibody may be important.

The semi-quantitative optical density (OD) ratios of the MERS S1 ELISA had acceptable but lower Spearman correlations (0.86–0.87) with the different neutralisation tests, in terms of the time to becoming positive in patients with MERS (Figure D,E). The S1-ELISA assay was a binding assay detecting IgG alone, rather than a functional neutralising assay and thus the lower correlation with this type of assay was not surprising.

In contrast to viruses such as avian influenza A(H5N1) where there is great antigenic diversity, genetically diverse MERS-CoV remain antigenically homogenous. Similar results had been previously reported using dromedary camel sera [11], and also clade B viruses and MERS-CoV EMC (clade A) were antigenically indistinguishable with human sera [15].

The Korean outbreak was caused by a clade B virus. Limitations of this study are that all the sera tested were from one outbreak and from one ethnic background and that a MERS-CoV isolate from these patients was not available for use in the serology tests. However, we have demonstrated in this, and previous studies, that antibody titres are not affected by the clade of virus used.

In conclusion, the different types of neutralisation or ppNT assays can be used in MERS-CoV diagnosis and seroepidemiology. $PRNT_{50}$ was more sensitive than other assay formats and may be the only assay that can be positive early in the course of infection and in a few patients with poor serologic responses. Genetically diverse MERS-CoV are antigenically homogenous suggesting that future vaccines generated by any MERS-CoV strain will cross-protect against genetically and geographically diverse viruses.

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Conflict of interest

None declared.

Authors' contributions

MP and MDO conceived, planned and coordinated the study, the study; RAPMP carried out the serology assays, RAPMP and LLMP developed the serology assays, WBP, PGC, SJC, JYC, HSO, KHS, JHB, ESK, HBK, SWP, NJK coordinated the clinical studies, EYHL carried out the statistical analysis, and all authors critically reviewed the manuscript.

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