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Ongoing outbreak of an acute muscular *Sarcocystis*-like illness among travellers returning from Tioman Island, Malaysia, 2011-2012

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As of 4 November, 2012, 100 patients with an acute muscular *Sarcocystis*-like illness associated with travel to Tioman Island, Malaysia, have been identified. Thirty-five travelled there mostly during July and August 2011 and 65 mostly during July and August 2012, suggesting an ongoing outbreak. Epidemiological investigations are ongoing. Public health agencies and practicing clinicians should be aware of this rarely-reported disease in humans and consider it as differential diagnosis in travellers returning from Tioman Island.

From 27 July to 4 November 2012, GeoSentinel [1], working with EuroTravNet [2] and TropNet [3], has identified 65 patients with an acute muscular *Sarcocystis*-like illness after recent travel to Tioman Island. All of these patients had traveled to Tioman, located off the east coast of peninsular Malaysia, mostly during July and August, 2012. Reports originated from Germany (n=25), France (n=20), the Netherlands (n=12), Switzerland (n=3), Belgium (n=2), Spain (n=2), and Singapore (n=1). These patients appear to represent the second wave of an outbreak that started in 2011 [4]. An epidemiologic investigation, initiated in November 2011, is ongoing.

Outbreak description

Starting from October 25, 2011, 35 patients with an acute muscular *Sarcocystis*-like illness were identified by early 2012; all traveled to Tioman Island, mostly during July and August of 2011. Following months without reports, new patients have been identified since late July 2012, and 65 have been reported to GeoSentinel by 4 November 2012. Cases presented here with limited, preliminary data, are patients reported to GeoSentinel with suspected acute muscular sarcocystosis, defined as an acute illness characterised by prominent musculoskeletal complaints with or without fever, with unexplained eosinophilia and recent travel to Tioman Island.

Although data collection is incomplete, the clinical presentation appears to be identical to that seen last year: almost all patients have experienced fever and myalgia while fewer have complained of arthralgia, asthenia, headache, cough, and diarrhoea. Only some had pruritic rash or edema of the face or an extremity. At least four patients were hospitalised.

Laboratory investigations showed that in many cases, at the onset of symptoms absolute eosinophil counts and serum creatinine phosphokinase (CPK) levels can be normal but begin to be moderately elevated approximately 30 days or more after departure from Tioman Island. Maximum absolute eosinophil counts and serum CPK levels from patients with data available, have typically been ‹1,000 cells/mm3 (norm: ‹500) and ‹1,500 U/L (norm: ‹200), respectively, but higher values were seen in some patients. To date, all patients with known testing (2011 n=34; 2012 n=8) have been serologically negative for trichinellosis. Eight patients are known to have had a muscle biopsy; for six patients results are available and all revealed myositis (defined as myalgia and inflammation and myocyte degeneration on histopathologic examination of muscle tissue) and in two intramuscular cysts (sarcocysts) were identified.

Among the 65 patients, two asymptomatic persons were identified who, when tested, had elevated eosinophil counts and serum CPK levels. One of them was a child who travelled with several family members, all of whom experienced an acute muscular *Sarcocystis*-like illness. The other person traveled with a companion who developed an acute muscular *Sarcocystis*-like illness and whose muscle biopsy showed inflammation but no intramuscular sarcocysts. The initially asymptomatic person subsequently developed myositis and mild myocarditis and was hospitalised.

Background

Sarcocystis species are intracellular protozoan parasites with an obligatory two-host predator-prey (definitive-intermediate) lifecycle [5]. Humans are the definitive host for *Sarcocystis hominis* and *Sarcocystis suihominis*, which are acquired by eating undercooked sarcocyst-containing beef or pork, respectively. The parasite reproduces sexually in the human intestine, where infection can cause acute gastroenteritis; however, most *S. hominis* and *S. suihominis* infections are likely asymptomatic [6]. Although the specific species have never been identified, humans can become accidental intermediate hosts for one or more of the 130 known *Sarcocystis* species through ingesting oocysts or sporocysts in food or water contaminated with feces from an infected predator animal. Nonspecific symptoms might arise during asexual reproductive and migratory phases of the parasite within the vascular endothelium and this generalised vasculitis has associated with myositis, myocarditis, and perivascular inflammation of the liver, kidneys, and lungs in experimentally and naturally infected animals [7]. The parasite ultimately disseminates to skeletal and cardiac muscle and forms sarcocysts containing large numbers of parasites that, when mature, are infectious for a definitive host. This phase in the lifecycle may or may not be associated with myocyte degeneration and active inflammation, depending on the specific *Sarcocystis* species-host species interaction [7]. Definitive diagnosis of muscular sarcocystosis relies on identifying sarcocysts in a muscle biopsy sample; DNA amplification and PCR testing of muscle tissue and serology is experimental and not widely available. No proven treatment exists for human sarcocystosis.

Fewer than 100 cases of human muscular sarcocystosis have been reported in the literature, with most discovered incidentally in asymptomatic persons [6]. Human sarcocystosis is prevalent in Malaysia; a seroprevalence study found evidence of infection in 20% of 243 Malaysians [8] which is concordant with a 21% prevalence of sarcocysts found in muscle tissue from 100 consecutive autopsies in Malaysia [9]. The largest reported outbreak of apparent acute muscular disease in the past, affected seven of fifteen United States (US) servicemen on maneuvers in a Malaysian jungle [10]. All but one of them had symptoms, four had myositis and one was confirmed to have sarcocysts in his muscle.

Epidemiological investigation

GeoSentinel is coordinating an epidemiological investigation to systematically gather detailed demographic, travel, exposure, and clinical data using structured data collection instruments. Case finding activities have identified 100 persons with suspected *Sarcocystis* species after visiting Tioman Island, mostly during the 2011 and 2012 northern hemisphere summer tourist seasons.

A *Sarcocystis*-specific Western-blot serological assay is under development at the US Centers for Disease Control and Prevention (CDC) to assist in differentiating infected from non-infected persons associated with this outbreak. Histologic examination and DNA amplification is being performed at CDC on existing muscle biopsy specimens from outbreak patients to confirm the diagnosis of muscular sarcocystosis and to identify the responsible *Sarcocystis* species. Updates on the investigation are being provided to public health authorities in Malaysia and countries where patients have been identified.

Discussion and conclusion

This is the largest reported cluster of patients with suspected acute muscular sarcocystosis. With little known about this disease in humans, practicing clinicians' awareness is likely low and many more as yet unrecognised patients may exist. Moreover, in agreement with a previously reported outbreak [10], our findings indicate that asymptomatic infection, and even late presentation, may occur. Since asymptomatic infection may be possible, the true number of persons infected by *Sarcocystis* species while visiting Tioman Island could be underestimated.

So far the data are still incomplete however, most patients analysed experienced acute illness characterised by fever and myositis. Myositis with significant eosinophilia but no serologic evidence of trichinellosis is an unusual clinical entity. Given the consistent pattern of illness among patients identified, the universal history of travel to a single small island in south-east Asia, and the presence of sarcocysts in the muscle biopsies of some of the patients, a *Sarcocystis* species infection with transmission occurring on Tioman Island seems to be the most likely cause of this outbreak. The source of infection for international travellers to Tioman Island remains unknown, but a seasonal pattern of transmission is apparent. This seasonality may reflect peak periods of summer tourist travel to the island, but may also correlate with true seasonally-occurring contamination of food, water, or the environment, perhaps related to specific weather patterns, seasonal food sources, or other seasonal events.

The epidemiology of human muscular sarcocystosis in Malaysia and neighbouring countries is poorly investigated. Detailed ascertainment of risk behaviours and exposures associated with the current cluster of known cases is included in the survey instrument used for the investigation of the outbreak.

Travellers to Tioman Island should be made aware of this potential travel health risk and be reminded about the necessity to practice safe food and water consumption and proper hygiene. Public health officers and practicing clinicians who are aware of persons with prominent musculoskeletal complaints with or without fever, with unexplained eosinophilia and recent travel to Tioman Island, Malaysia, are encouraged to consider sarcocystosis as differential diagnosis. Moreover, they are invited to participate in our investigation and

contact the corresponding author to report patients or receive guidance on how to proceed. Data collection forms and information on how to submit clinical samples (serum for *Sarcocystis*-specific serology and/or tissue for histopathology and *Sarcocystis*-specific PCR testing) are readily available.

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References

1. Freedman DO , Weld LH , Kozarsky PE , Fisk T , Robins R , von Sonnenburg F, et al. Spectrum of disease and relation to place of exposure among ill returned travelers. N Engl J Med. 2006;354(2):119-30.
2. Gautret P, Cramer JP, Field V, Caumes E, Jensenius M, Gkrania-Klotsas E, et al. Infectious diseases among travellers and migrants in Europe, EuroTravNet 2010. Euro Surveill. 2012;17(26):pii=20205. Available from: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20205
3. Bouchaud O, Mühlberger N, Parola P, Calleri G, Matteelli A, Peyerl-Hoffmann G, et al. Therapy of uncomplicated falciparum malaria in Europe: MALTHER – a prospective observational multicentre study. Malar J. 2012;11:212.
4. Centers for Disease Control and Prevention (CDC). Notes from the Field: acute muscular sarcocystosis among returning travelers – Tioman Island, Malaysia, 2011. MMWR Morb Mortal Wkly Rep. 2012;61(2):37-8.
5. Centers for Disease Control and Prevention (CDC) Division of Parasitic Diseases. Diagram of the lifecycle of Sarcocystis spp. Atlanta: CDC. [Accessed 27 Oct 2012]. Available from: http://dpd.cdc.gov/dpdx/html/imagelibrary/s-z/sarcocystosis/body_sarcocystosis_il5.htm
6. Fayer R. Sarcocystis spp. in human infections. Clin Microbiol Rev. 2004;17(4):894–902.
7. 7.Dubey JP, Speer CA, Fayer R. Sarcocystosis of animals and man. Boca Raton, Florida: CRC Press; 1989.
8. Thomas V, Dissanaike AS. Antibodies to Sarcocystis in Malaysians. Trans R Soc Trop Med Hyg. 1978;72(3):303–6.
9. Wong KT, Pathmanathan R. High prevalence of human skeletal muscle sarcocystosis in south-east Asia. Trans R Soc Trop Med Hyg. 1992;86(6):631–2.
10. Arness MK, Brown JD, Dubey JP, Neafie RC, Granstrom DE. An outbreak of acute eosinophilic myositis attributed to human Sarcocystis parasitism. Am J Trop Med Hyg. 1999;61(4):548–53.

Recurrent pyelonephritis due to NDM-1 metallo-beta-lactamase producing *Pseudomonas aeruginosa* in a patient returning from Serbia, France, 2012

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We describe the first isolation in France of a New-Delhi metallo-beta-lactamase-1 (NDM-1) producing *Pseudomonas aeruginosa*. In March 2012, a patient with history of prior hospitalisation in Serbia was diagnosed in France with acute pyelonephritis due to NDM-1 producing *P. aeruginosa*. Clinical and microbiological cure was obtained under appropriate antibiotic treatment. Two months later, she presented with a recurrence due to the same bacteria, with a favourable evolution. During both hospitalisations, contact isolation precautions were implemented and no cross-transmission was observed.

In March 2012, a patient with a history of prior hospitalisation in Serbia was diagnosed in France with acute pyelonephritis due to New Delhi metallo-beta-lactamase-1 (NDM-1) producing *Pseudomonas aeruginosa*.

Case report

In February 2012, a woman in her early 60s was referred to the Infectious Diseases Department of the Military Hospital Bégin (Saint-Mandé, France) for an acute pyelonephritis. She reported having undergone a surgical intervention in Serbia in November 2011. She stayed one month in hospital, with urinary catheterisation of undetermined duration but less than one month. The medical records reported a first treatment with cefuroxime and streptomycin just after surgery, and a history of fever, drowsiness and inflammatory syndrome two weeks after surgery, treated with ceftriaxone and streptomycine. Laboratory data were unavailable.

Since her return to France, in early February 2012, she complained of urinary frequency, dysuria and urinary incontinence. In late February, she presented to her general practitioner (GP) with fever (38.7 °C), vomiting, diarrhoea and diffuse abdominal pain and she was referred to our hospital. White blood cell count was 4,470/mm^3 (norm: 4,000-10,000/mm^3), C-reactive protein 52 mg/L (norm: < 5 mg/L), creatinine 36 µmol/L (norm: 62-106 µmol/L).

The urinalysis recovered 29,106 leukocytes/mL and 10^6 CFU/ml *P. aeruginosa* serotype 011 (HIABP11). The rectal swab, performed for multidrug resistant bacteria screening according to the French recommendations for patients with a history of hospitalisation abroad in the previous year [1], was also positive for *P. aeruginosa*. Blood cultures remained negative.

Kidney ultrasonography was normal, kidney CT-scan showed a left pyelonephritis without abscess or urinary obstruction. The patient underwent urinary catheterisation and three weeks antibiotic treatment with aztreonam (2g TID) and colistin (2 million units TID).

Antimicrobial sensitivity testing and molecular diagnostics

For the isolates from urine and rectal swabs, antimicrobial drug susceptibility testing was performed by the disk diffusion method on Mueller-Hinton agar (I2A Laboratories, Perols, France) and interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations [2]. It demonstrated resistance to imipenem, meropenem, doripenem, to all antipseudomonal cephalosporins, aminoglycosides, fluoroquinolones, rifampicine and tigecycline. Both isolates remained susceptible to piperacillin-tazobactam and intermediate to aztreonam (Table 1). Minimal inhibitory concentrations (MICs) of selected antimicrobials determined by microdilution and Etest (Biomérieux, Marcy l'Etoile, France) confirmed the results and showed susceptibility to colistin. The metallo-betalactamase-production screening in the meropenem-dipicolinic acid combined disk test (*Klebsiella pneumoniae* carbapenemas (KPC) + metallo-beta-lactamase (MBL) Confirm ID Pack, Rosco Diagnostica, Taastrup, Denmark) and in E-test with

Table 1

Antibiotic susceptibility of *Pseudomonas aeruginosa* serotype 011 (HIABP11) strain isolated in a patient with history of previous hospitalisation in Serbia, France 2012

Antibiotic	MIC (µg/mL)	Susceptibility
Ticarcillin	› 256	R
Ticarcillin/Clavulanic acid	›256	R
Piperacillin	12	S
Piperacillin/Tazobactam	12	S
Ceftazidime	›256	R
Cefepime	›256	R
Aztreonam	3	I
Imipeneme	› 32	R
Meropeneme	› 32	R
Doripenem	› 32	R
Tobramycin	›256	R
Gentamicin	›256	R
Amikacin	192	R
Ciprofloxacin	›32	R
Colistin	2	S
Doxycycline	32	R
Tigecycline	128	R

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

imipenem alone or combined with EDTA (Biomérieux, Marcy l'Etoile, France) was positive.

PCR for carbapenemases, including MBL bla_{IMP}, blaVIM, bla_{NDM} revealed that HIABP11 harboured bla_{NDM}. Sequencing showed 100% homology with bla_{NDM-1}. PCR for detection of other beta-lactamase genes, plasmid-borne quinolone resistance genes and methylases were negative.

Monitoring of plasmatic and urinary concentrations of antibiotics

Aztreonam plasmatic peak and residual concentrations were 40 times and 4 times the MIC respectively for a dosage of 2g TID, while urine concentration was 166 times the MIC. Colistin plasmatic residual concentration (12 hours after injection) was under the MIC (0.7 mg/L, while the expected residual concentration is 2 mg/L, eight hours after injection); urine concentration was 10 times the MIC (Table 2).

Fever and abdominal pain resolved within 48 hours after administration of antibiotics while urinary incontinence persisted, requiring prolonged urinary catheterisation. Urinalysis 72 hours after the beginning of antibiotic treatment was normal and did not show any bacteria growth. The patient was discharged from hospital 21 days after admission.

During the stay at our hospital, a squamous-cell carcinoma of the oropharynx had been discovered and the patient underwent a first chemotherapy course at the beginning of May and was discharged from our hospital. One week later, she experienced dysuria and abdominal pain, without fever or flank pain. She was treated with ofloxacin (10 days) and prednisolone (seven days) by her GP. The urinalysis showed $42x10^4$ leukocytes/mL (significant leukocytes count $›10^4$/mL) and *P. aeruginosa* 10^3 CFU/mL. *P. aeruginosa* with the same antibiotic susceptibility pattern as previously was isolated and PCR for bla_{NDM-1} was again positive. Kidney ultrasound was normal.

The patient received piperacillin-tazobactam (4g TID) for three weeks, and her second chemotherapy course, without complication. The urinalysis after 72 hours of treatment was negative and the patient was discharged for at-home hospitalisation.

During both hospitalisations, contact isolation precautions with dedicated healthcare personnel were implemented. All patients hospitalised in the same ward were screened weekly with a total of 111 rectal swabs performed in 52 patients and no transmission of NDM-1 producing *P. aeruginosa* occurred.

Discussion and conclusion

Since the first description in 2008, of an NDM-1 carbapenemase in single isolates of *Klebsiella pneumoniae* and *Escherichia coli* [3], NDM-1-producing *Enterobacteriaceae* have been reported worldwide, mostly in patients with an epidemiological link to India or Pakistan [4,5]. However, among 77 patients infected or colonised by NDM-1 producing *Enterobacteriaceae* reported in Europe from 2008 to 2010, five had been hospitalised previously in the Balkan region [6]. Clinical isolates of NDM-1-producing *A. baumanii* are also increasingly reported in Europe [7] and importation of NDM-1 -producing *A. baumannii* from Serbia has been reported in 2011 [8].

We report here the first case of infection due to NDM-1-producing *P. aeruginosa* in France. To date,

Table 2

Monitoring of plasmatic and urinary concentrations of antibiotics in patient with pyelonephritis caused by *Pseudomonas aeruginosa* serotype 011 (HIABP11) and history of previous hospitalisation in Serbia, France 2012

	Aztreonam	Colistin
Plasmatic peak concentration (mg/L)	127.6	NA
Plasmatic residual concentration (mg/L)	14.3	0.7
Urinary concentration (mg/L)	› 500	22.3

NA: not available.

only two other cases of colonisation or infection by NDM-1-producing *P. aeruginosa* have been reported worldwide, occurring in two patients hospitalised in Belgrade,Serbia. Both had undergone invasive surgical interventions and none of them had travelled outside Serbia. No epidemiological connection was evidenced between them [9, 10].

NDM-1 producing bacteria are undoubtly challenging: firstly, they are usually multiresistant to antibiotics because bla_{NDM-1} encoding plasmids co-harbor multiple resistance determinants. *P. aeruginosa* shows a high level of intrinsic resistance to antimicrobial agents. Its ability to acquire and combine different resistance determinants represents a major threat, compromising therapeutic options. The acquisition of MBL-carbapenemase (Verona integron-encoded metallo-beta-lactamase (VIM), imipenemase (IMP), Sao Paulo Metallo-beta-lactamase (SPM), Australia imipenemase (AIM), German imipenemase (GIM), Dutch IMipenemase (DIM), NDM, led to emergence of multidrug-resistant (MDR) or extensively drug-resistant (XDR) *P. aeruginosa*. The case presented highlights the difficulties of therapeutic management, with only three antibiotics categorised as susceptible or intermediate (colistin, aztreonam, piperacillin-tazobactam). Due to the low MIC recommended for the inferior breakpoint for aztreonam by EUCAST, wild type *P. aeruginosa* are reported as 'intermediate'. However MBL-carbapenemases do not hydrolyse the monobactam aztreonam and high dose therapy can be useful for patients infected with MBL-producing *P. aeruginosa* [11,12].

Secondly, NDM-1 producers have a potential for spread through the transfer of the plasmid-borne bla_{NDM1} gene [5]. In *P. aeruginosa*, there is no complete documentation for plasmid-borne or chromosomal localisation for bla_{NDM1} gene yet. However, many outbreaks including carbapenemase-producing *P. aeruginosa* and spread of MDR *P. aeruginosa* clones have been recently reported, underlining that cross-transmission plays a major role in the spread of MDR *P. aeruginosa* in hospital settings [13, 14]. These considerations combined with the emerging character of our isolate in France are reason why all members of the medical and paramedical staff agreed to set up a dedicated team to care for the patient and a weekly screening of all contemporary patients on the same ward.

This strategy is recommended in France for carbapenemase-producing *Enterobacteriae* [1] without any mention of carbapenemase-producing Acinetobacter or Pseudomonas. However, in this particular case of a first isolation of NDM-1 producing *P. aeruginosa* in France, this strategy allowed us to assess the absence of cross-transmission for this isolate.

This observation highlights the emergence of NDM-1 not only in Enterobacteriaceae, but also in *P. aeruginosa* in Balkan area and France. In our view, NDM screening should be performed when a carbapenemase-producing *Pseudomonadaceae* clinical isolate is identified.

References

1. Haut Conseil de la Santé Publique (HCSP). Maîtrise de la diffusion des bactéries multirésistantes aux antibiotiques importées en France par des patients rapatriés ou ayant des antécédents d'hospitalisation à l'étranger, 2ème version. [Control of the spread of multiresistant bacteria imported in France by repatriated patients or patients previously hospitalised abroad, 2nd version]. Paris: HCSP. Nov 2010. Available from: http://www.hcsp.fr/explore.cgi/avisrapportsdomaine?clefr=201
2. European Committee on Antimicrobial Susceptibility Testing (EUCAST). EUCAST breakpoint tables for interpretation of MICs and zone diameters, Version 2.0] [Accessed 29 Oct 2012]. Available from: http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/Breakpoint_table_v_2.0_120221.pdf
3. Yong D, Toleman MA, Giske CG, Cho HS, Sundman K, Lee K, et al. Characterization of a new metallo-beta-lactamase gene, bla(NDM-1), and a novel erythromycin esterase gene carried on a unique genetic structure in Klebsiella pneumoniae sequence type 14 from India. Antimicrob Agents Chemother. 2009;53(12):5046-54.
4. Kumarasamy KK, Toleman MA, Walsh TR, Bagaria J, Butt F, Balakrishnan R, et al. Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. Lancet Infect Dis. 2010 Sep;10(9):597-602.
5. Nordmann P, Poirel L, Walsh TR, Livermore DM. The emerging NDM carbapenemases. Trends in microbiology. 2011;19(12):588-95.
6. Struelens MJ, Monnet DL, Magiorakos AP, Santos O'Connor F, Giesecke J, the European NDM-1 Survey Participants. New Delhi metallo-beta-lactamase 1–producing Enterobacteriaceae: emergence and response in Europe. Euro Surveill. 2010;15(46):pii=19716. Available from: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19716
7. Bonnin RA, Poirel L, Naas T, Pirs M, Seme K, Schrenzel J, et al. Dissemination of New Delhi metallo-beta-lactamase-1-producing Acinetobacter baumannii in Europe. Clin Microbiol Infect. 2012;18(9):E362-5.
8. Pfeifer Y, Wilharm G, Zander E, Wichelhaus TA, Gottig S, Hunfeld KP, et al. Molecular characterization of blaNDM-1 in an Acinetobacter baumannii strain isolated in Germany in 2007. J Antimicrob Chemother. 2011;66(9):1998-2001.
9. Livermore DM, Walsh TR, Toleman M, Woodford N. Balkan NDM-1: escape or transplant? Lancet Infect Dis. 2011;11(3):164.
10. 10.Jovcic B, Lepsanovic Z, Suljagic V, Rackov G, Begovic J, Topisirovic L, et al. Emergence of NDM-1 metallo-beta-lactamase in Pseudomonas aeruginosa clinical isolates from Serbia. Antimicrob Agents Chemother. 2011;55(8):3929-31.
11. Moriyama B, Henning SA, Childs R, Holland SM, Anderson VL, Morris JC, et al. High-dose continuous infusion beta-lactam antibiotics for the treatment of resistant Pseudomonas aeruginosa infections in immunocompromised patients. Ann Pharmacother. 2010;44(5):929-35.
12. Prevotat A, Leroy S, Perez T, Wallet F, Wallaert B. [Tolerance and efficacy of ceftazidime in combination with aztreonam for exacerbations of cystic fibrosis]. Rev Mal Respir. 2010;27(5):449-56.
13. Cholley P, Thouverez M, Hocquet D, van der Mee-Marquet N, Talon D, Bertrand X. Most multidrug-resistant Pseudomonas aeruginosa isolates from hospitals in eastern France belong to a few clonal types. J Clin Microbiol. 2011;49(7):2578-83.
14. Garcia-Castillo M, Del Campo R, Morosini MI, Riera E, Cabot G, Willems R, et al. Wide dispersion of ST175 clone despite high genetic diversity of carbapenem-nonsusceptible Pseudomonas aeruginosa clinical strains in 16 Spanish hospitals. J Clin Microbiol. 2011;49(8):2905-10.

Hospitalisation associated with the 2009 H1N1 pandemic and seasonal influenza in Hong Kong, 2005 to 2010

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Reliable estimates of the morbidity burden caused by the 2009 pandemic influenza (pH1N1) are important for assessing the severity of the pandemic. Poisson regression models were fitted to weekly numbers of cause-specific hospitalisation in Hong Kong from 2005 to 2010. Excess hospitalisation associated with the 2009 pandemic and seasonal influenza was derived from the model by incorporating the proxy variables of weekly proportions of specimens positive for the pandemic influenza A(H1N1)pdm09, seasonal influenza A (subtypes H3N2 and H1N1) and B viruses. Compared with seasonal influenza, pH1N1 influenza was associated with higher hospitalisation rates for acute respiratory disease (ARD) among children younger than 18 years and adults aged between 18 and 64 years, but among the elderly aged 65 years and older the hospitalisation rates were lower for pH1N1 than for seasonal H3N2 and H1N1 influenza. Hospitalisation rates for chronic diseases associated with pH1N1 influenza were generally higher than those associated with seasonal influenza. The reported hospitalised cases with laboratory-confirmed pandemic infections accounted for only 16% of pH1N1 influenza-associated hospitalisations for ARD in the age group 75 years and older, and 5–66% of hospitalisations for chronic diseases in those older than 40 years. The 2009 H1N1 influenza pandemic was associated with a dramatically increased risk of hospitalisation among children and young adults. The morbidity burden of pandemic was underreported in old people and in those with chronic conditions.

Introduction

In April 2009 a novel influenza A(H1N1) virus of swine-origin (referred to as pH1N1 hereafter) emerged and quickly spread worldwide [1]. Previous studies have reported that annual hospitalisation rates with laboratory-confirmed pandemic infections ranged from 3 to 110 per 100,000 person-years [2-4]. However, it is likely that these rates seriously underestimated the true burden of the pandemic, as many cases were not tested for pandemic infections due to limited laboratory capacity [5]. Furthermore, the tests could have been biased towards children and young adults in whom the pH1N1 infection rates were reported to be higher, but severe outcomes after infections were more likely to occur in the elderly aged 65 years or older [6,7]. Previous studies have further found that few pandemic-associated fatalities in persons older than 65 years were captured by intensive laboratory tests [8,9]. Given the potential underreporting of pandemic cases, it is necessary to compare excess hospitalisation associated with seasonal and pandemic influenza to assess the severity of 2009 H1N1 pandemic. We and others have demonstrated that excess hospitalisation estimated from Poisson models can provide reliable estimates for true morbidity burden of seasonal influenza [10,11].

Two subtypes of seasonal influenza A(H3N2) and (H1N1) (referred to as sH1N1 hereafter), as well as influenza B viruses, have been circulating in humans for many years [12]. There is evidence that these viruses are distinct in terms of transmission efficiency and risk of hospitalisation or mortality [13,14]. However, few studies have compared disease burden associated with different types or subtypes of seasonal influenza and pandemic viruses. In this study, we aimed to comprehensively assess the age-specific hospitalisation burden associated with each type (or subtype) in subtropical city Hong Kong which had a population of 7.07 million in 2011 [15].

Methods

Data

Hospitalisation records from 2005 to 2010 were obtained from the electronic health record system of the Hospital Authority (HA), which manages all 41 public hospitals and covers 78% of hospital-bed days in the entire territory of Hong Kong [16]. We aggregated the records into weekly hospitalisation numbers by all discharge diagnoses (up to 15 diagnoses) that were coded in the International Classification of Diseases, Ninth Revision, Clinical Modification (ICD9-CM) [17].

We considered the following disease categories: acute respiratory disease (ARD; ICD9 460–466, 480–487), pneumonia and influenza (P&I; ICD9 480–487) for the age groups of 0–5, 6–17, 18–39, 40–64, 65–74, and ≥75 years, and cardiovascular disease (CVD; ICD9 390–459), diabetes (ICD9 250), ischaemic heart disease (IHD; ICD9 410–414), and stroke (ICD9 430–438) for the age groups of 40–64, 65–74, and ≥75 years. Mid–year population by age groups was obtained from the Census and Statistics Department for the study period.

We obtained the weekly numbers of detected influenza viruses reported by the Public Health Laboratory Centre, Hong Kong from the weekly report 'Flu Express' available on the website of the Centre for Health Protection at the Department of Health (DH) (http://www.chp.gov.hk/en/guideline1_year/441/304.html). The numbers of specimens were estimated from the summary tables of the Centre's Sentinel Surveillance website (http://www.chp.gov.hk/en/sentinel/26/44/292.html). The respiratory specimens were collected from hospitalised patients and outpatients from the public and private healthcare sectors in Hong Kong and were tested by viral culture and subtyped by haemagglutination inhibition tests. As the online reports for other respiratory viruses were not publicly available from the DH during the study period, we also obtained the virology surveillance data for respiratory syncytial virus (RSV), adenovirus and parainfluenza viruses from the microbiology laboratory of Queen Mary Hospital.* This single laboratory covered 21% of all specimens for the DH surveillance network during the study period, and the influenza virology data from these two sources were highly correlated (r=0.81) during that period. The seasonal patterns of these viruses were also consistent with those from the DH [18]. Meteorological data for temperature and relative humidity were obtained from the Hong Kong Observatory.

Statistical analysis

We fitted the following quasiPoisson regression models to weekly numbers of hospitalisation from 2005 to 2010 for each age–disease category.

$$\begin{cases} Y_t \sim quasiPoisson(\mu_t, \phi\mu_t) \\ \log(\mu_t) = \beta_0 + \beta_1 SH1_t + \beta_2 H3_t + \beta_3 B_t + \beta_4 PH1_t \\ \quad + \beta_5 RSV_t + \beta_6 Adeno_t + \beta_7 P1_t + \beta_8 P2_t + \beta_9 P3_t \\ \quad + s(t) + s(Temp_t) + s(Hum_t) + \beta_{10} D_t + \beta_{11} D_t \times PH1_t \end{cases}$$

where Yt denotes the number of hospitalisations in week t (t=1,2,…,312) which was assumed to follow a quasiPoisson distribution with an over-dispersion parameter ϕ. $SH1_t$, $H3_t$, B_t and $PH1_t$ denote the weekly proportions of specimens tested positive for sH1N1, H3N2, influenza B and pH1N1, respectively. These proxy variables were added to quantify the effects of each type/subtype. RSV_t, $Adeno_t$, $P1_t$, $P2_t$ and $P3_t$ were proportions of specimens positive for RSV, adenovirus and three types of parainfluenza viruses. As nearly all annual total numbers of cause-specific hospitalisation almost doubled during the study period, we adjusted for the long-term and seasonal trends of hospitalisation data by adding a natural cubic spline $s(t)$ into the model. Because the time series of acute respiratory diseases (ARD and P&I) tend to show greater seasonal variations than those of chronic diseases, we decided to use 3 degrees of freedom (*df*) per year (totally 18 *df* during the whole study period) in ARD and P&I, but to use a minimum of 2 or 3 *df* in total for the whole period for CVD, IHD, stroke and diabetes. $s(Temp_t)$ and $s(Hum_t)$ were the natural cubic spline of temperature and relative humidity with *df*=3 to adjust for the variation of meteorological factors. During the containment phase of the 2009 pandemic (26 April to 27 June 2009), every pH1N1 case was admitted regardless of the disease severity. To adjust for the change in hospitalisation admission thresholds during this period, we added the dummy variable D_t for the period of this containment phase, and its product term with the pandemic virus proxy D_tx$PH1_t$, into the model to allow a different hospitalisation risk during the containment phase.

Morbidity burden of influenza was measured by excess hospitalisation rate associated with each influenza type or subtype. We first defined the baseline hospitalisation number as the number of expected hospitalisations when the proxy variables for pH1N1, sH1N1, H3N2 and B viruses were set to zero. Excess hospitalisation number was then calculated as the difference between the baseline and predicted hospitalisation numbers with all variables taking the observed values. The 95% confidence intervals (CI) were calculated by bootstrapping the weekly excess hospitalisation numbers and subsequently refitting the same models 1,000 times. We also calculated the annual excess rate by dividing the average annual total numbers of excess hospitalisation by the annual age-specific population sizes derived from the 2001, 2006 and 2011 censuses [15,19,20] through linear interpolation.

Model validation

On 1 May 2009 the HA established an electronic reporting system 'e-flu' in response to the influenza A(H1N1) pandemic. Patients with acute respiratory symptoms were routinely tested for influenza A(H1N1) by RT-PCR. The e-flu data recorded outpatient visits, hospitalisation and deaths with laboratory-confirmed influenza A(H1N1) infections [21]. Given the intensive laboratory screening for influenza A(H1N1)pdm09 infections during the pandemic, the hospitalised pH1N1 cases reported by the e-flu system could be regarded as the low boundary of true burden. Hence we calculated the disease-specific laboratory-confirmed pandemic hospitalisation according to any-listed discharge diagnosis. As the e-flu surveillance was relaxed after the first wave, we compared these numbers with our estimates of age- and cause-specific excess numbers during

Table 1

Virology surveillance data and annual hospitalisation rate (per 100,000 person-years) in Hong Kong, 2005–2010

	2005	2006	2007	2008	2009	2010
Virology data n (% of total influenza-positive specimens)						
Total specimens	39,580	37,108	40,258	44,171	12,5100	69,680
Total influenza-positive specimens	6,204	4,196	6,422	4,786	30,576	8,773
Seasonal influenza A(H1N1)	413 (6.7)	3,047 (72.6)	133 (2.1)	1,490 (31.1)	2,821 (9.2)	1 (0.0)
Influenza A(H3N2)	4,751 (76.6)	225 (5.4)	4,845 (75.4)	2,039 (42.6)	8,165 (26.7)	4,484 (51.1)
Influenza A(H1N1)pdm09	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	19,279 (63.1)	2,022 (23.0)
Influenza B	1,040 (16.8)	924 (22.0)	1,444 (22.5)	1,257 (26.3)	311 (1.0)	2,266 (25.8)
Hospitalisations						
ARD	1,023.5	903.9	986.0	1,247.0	1,417.5	1,606.8
P&I	682.6	616.7	665.8	905.2	1,072.2	1,217.0
CVD	1,963.2	1,940.3	2,012.9	2,813.2	3,864.4	4,810.9
IHD	507.1	496.5	502.4	683.8	935.4	1,167.3
Stroke	427.6	411.5	427.7	666.1	914.8	1,115.2
Diabetes	534.1	513.5	550.6	844.6	1,408.2	1,772.6

ARD: acute respiratory disease; CVD: cardiovascular disease; IHD: ischaemic heart disease; P&I: pneumonia and influenza.

The annual figures in this Table were obtained by cumulating weekly data from week 1 to 52 (or 53) of each year.*

the first wave of the pandemic from 1 May 2009 to 2 January 2010.

The ethical approval for this study was obtained from the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster (UV11-264). All analyses were conducted using R package version 2.12.2 (R Development Core Team).

Results

Descriptive statistics

Annual total numbers of specimens collected by the DH ranged from 37,108 to 125,100 during the study period (Table 1). Influenza A(H3N2) was dominant in 2005 and 2007, while in 2006 and 2008, seasonal influenza A(H1N1) and B viruses were isolated more. The pandemic pH1N1 virus first emerged in May 2009, reached a peak around September and remained at a low level after November. A few specimens positive for seasonal influenza were found during the pandemic period from May to November 2009. Influenza B became dominant after March 2010, followed later by influenza A(H3N2) followed. During the study period from 2005 to 2010, the average annual hospitalisation rate was 1,197.4, 859.9, 2,900.8, 715.4, 660.5 and 937.3 per 100,000 person-years, for ARD, P&I, CVD, IHD, stroke and diabetes, respectively (Table 1). Persons aged 75 years and older had the highest hospitalisation rate for all the disease categories.

Annual excess hospitalisation associated with influenza

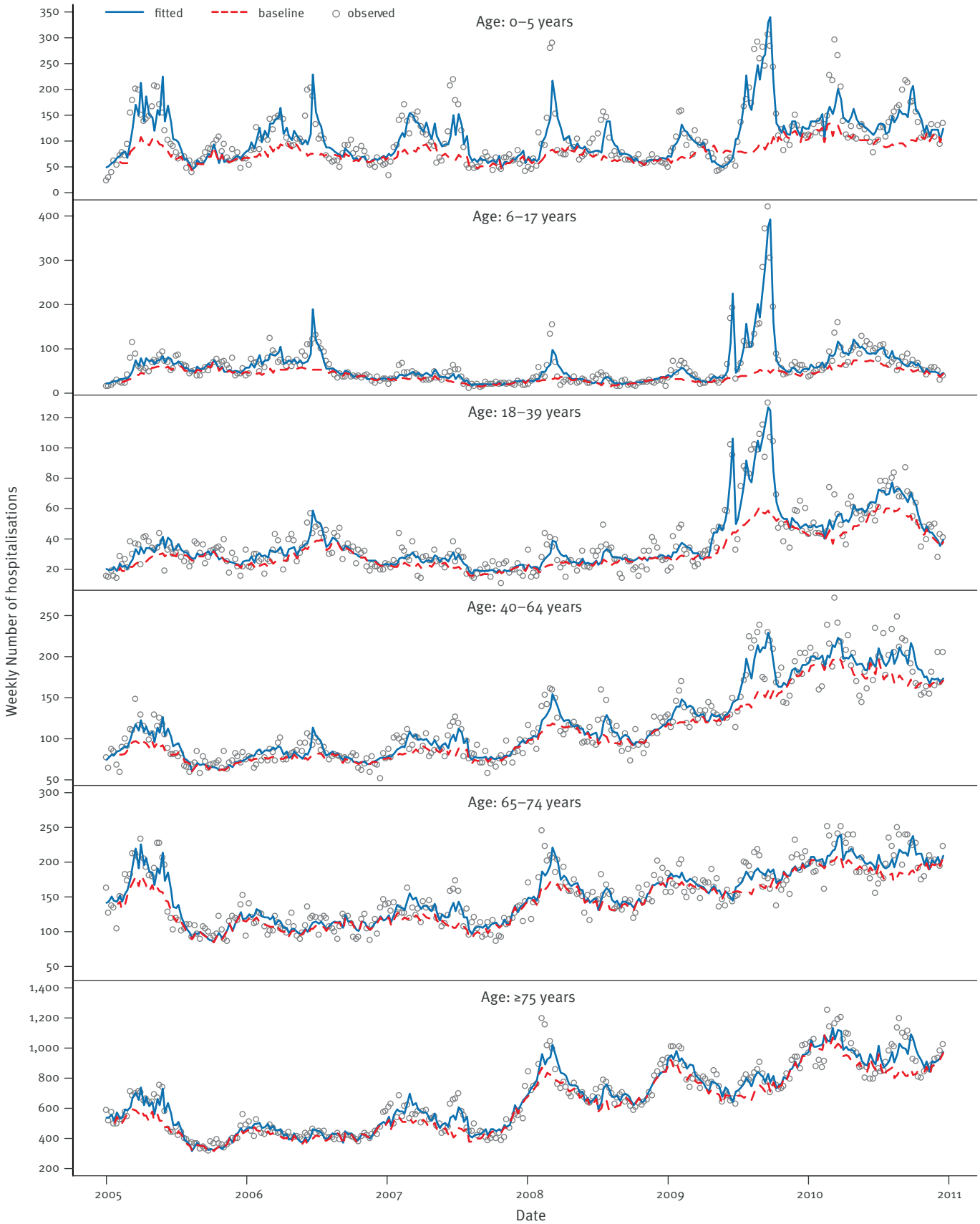
An example of model fit by weekly observed and fitted numbers of P&I hospitalisation is shown in Figure 1. Significant association of influenza virus types (or subtypes) with hospitalisation was found in most age–disease categories studied ($p<0.05$). Annual age-specific excess hospitalisation rates showed great year-to-year variations in all the disease categories. Across the six-year study period, the highest annual excess rate for ARD, P&I and IHD was observed in 2009, and for CVD, stroke and diabetes in 2010, after adjustment for age structure by age standardisation (Table 2). During the study period, the highest annual excess rates of ARD and P&I hospitalisation were found in the pandemic year 2009 for children and adults aged 18–64 years. For old people, excess rates of hospitalisation associated with influenza markedly increased during the post-pandemic year of 2010 for most disease categories, whereas the rates in 2009 remained at the level similar to or slightly higher than those in the preceding interpandemic years (data now shown).

Excess hospitalisation associated with the pandemic

During the whole pandemic period from May 2009 to July 2010, a total of 10,377 hospitalisations with ARD and 7,204 with P&I were associated with pH1N1 influenza. Of these, 60% occurred in children and 13% in the elderly (Table 3). For chronic diseases, the total numbers of pH1N1-associated hospitalisation were 1,676, 848, 359 and 1,550, for CVD, IHD, stroke and diabetes, respectively. More than 80% of these hospitalisations

Figure 1

Weekly observed (squares) and fitted (solid blue line) hospitalisations with any-listed diagnosis of pneumonia and influenza Hong Kong, 2005–2010



Broken red line indicates the baseline when all the influenza proxies were assumed zero.

Table 2

Age-standardised annual excess rate per 100,000 person-years of hospitalisation associated with influenza, with the 2006 population as standard, Hong Kong, 2005–2010

Disease	2005	2006	2007	2008	2009	2010
	ER (95% CI)	ER (95% CI)	ER (95% CI)	ER (95% CI)	ER (95% CI)	ER (95% CI)
ARD	115.9 (70.2; 157.0)	80.5 (39.8; 120.3)	116.1 (70.3; 158.8)	96.7 (51.7; 138.1)	166.8 (109.5; 221.7)	152.7 (96.3; 204.9)
P&I	76.2 (44.3; 105.1)	56.6 (27.9; 84.4)	75.0 (44.1; 104.0)	64.9 (33.7; 94.1)	130.9 (88.0; 170.3)	109.5 (67.5; 147.6)
CVD[a]	30.4 (-24.9; 84.2)	51.1 (10.2; 90.3)	31.5 (-10.7; 71.9)	48.3 (-2.3; 99.5)	63.3 (-3.9; 125.9)	102.3 (6.6; 192.4)
IHD[a]	3.7 (-15.5; 21.9)	8.8 (-6.2; 23.4)	3.3 (-11.8; 17.7)	6.0 (-11.1; 21.9)	19.4 (-2.6; 39.7)	12.6 (-21.0; 44.4)
Stroke[a]	8.5 (-6.0; 21.5)	12.0 (1.6; 22.2)	8.1 (-2.9; 18.6)	12.3 (-1.7; 25.4)	18.9 (0.5; 35.5)	19.4 (-6.5; 43.1)
Diabetes[a]	9.2 (-9.7, 27.9)	16.5 (1.6; 30.6)	9.3 (-6.3; 24.0)	16.7 (-2.9; 36.6)	36.7 (7.9; 63.6)	50.6 (8.2; 91.4)

ARD: acute respiratory disease; CI: confidence interval; CVD: cardiovascular disease; ER: excess rate; IHD: ischaemic heart disease; P&I: pneumonia and influenza.

[a] Excess rates are assumed zero in the age groups younger than 40 years.

occurred in people aged 65 years and older. In the group including all ages, around 80% of pH1N1-associated hospitalisations for all the disease categories studied occurred during the first wave of the pandemic from May 2009 to January 2010.

To compare our estimates with the total numbers of laboratory-confirmed pH1N1 cases requiring hospitalisation, we calculated the ratio of the admission numbers reported by the e-flu system to excess hospitalisation associated with pH1N1 influenza in the same age–disease groups during the first wave. The ratio ranged from 0.72 to 0.92 for ARD and from 1.05 to 1.68 for P&I in the age groups younger than 75 years (Table 3). In persons 75 years and older, the confirmed hospitalised cases reported by e-flu for ARD and 45% for P&I in the same age group only accounted for 16% of excess hospitalisations that were estimated from the statistical models to be attributable to pH1N1 influenza. For the chronic diseases CVD, IHD, stroke and diabetes, the total numbers of confirmed pH1N1 hospitalisation accounted for only 25–66%, 11–28%, 5–15% of pH1N1-associated excess hospitalisation, for the age groups of 40–64, 65–74 and ≥75 years, respectively.

Comparison of type- or subtype-specific excess hospitalisation

In the group including all ages, pH1N1 influenza was associated with the highest annual excess rate of ARD hospitalisation (95.9 per 100,000 person-years; 95% CI: 66.9–122.1), followed by influenza A(H3N2) (49.4; 95% CI: 36.7–61.5), B (38.3; 95% CI: 21.7–54.2) and sH1N1 (18.3; 95% CI: 10.4–26.2). A similar pattern was also observed for P&I hospitalisation. Across different age groups, excess rates of ARD and P&I associated with pH1N1, sH1N1 or B influenza were higher among children under the age of five years, whereas the rates associated with H3N2 influenza were higher among persons older than 75 years (Figure 2). Excess rates associated with pH1N1 influenza were 2–5 times as high as those with seasonal influenza in children younger than five years, 5–12 times as high in children aged 6–17 years, but 50% and 70% lower than the rates of seasonal influenza A(H3N2) in persons aged 65–74 and ≥75 years.

Of three seasonal influenza viruses, H3N2 accounted for a higher proportion of ARD and P&I hospitalisation than the other two viruses in all the age groups except the groups aged 6–17 and 18–39 years, in which influenza B had higher rates. For chronic diseases, pH1N1 was associated with higher excess rates of diabetes, CVD and IHD, but the rates were similar to those of other seasonal viruses for stroke. The fractions of all-ages excess hospitalisation in the total numbers of corresponding cause-specific hospitalisation ranged from 0.4% to 9.4% for pH1N1 and from 1.7% to 8.8% for seasonal influenza A and B combined (Table 4). Compared with the age-specific proportions of seasonal influenza A and B combined, the proportions of pH1N1 were markedly higher in the age group of 6–17 years for ARD and in the age groups 6–17 and 18–39 years for P&I, but lower in the age groups older than 40 years for all the disease categories studied except diabetes.

Table 3

Age- and cause-specific excess hospitalisation associated with the 2009 influenza A(H1N1) pandemic, Hong Kong, 2005–2010

Diseases	Age group (years)	Whole pandemic period[a]				First wave[b]				e-flu[c]		Ratio[d]
		Excess number		Excess rate		Excess number (95% CI)		Excess rate		Number	Rate	
ARD	0-5	2,829	(1,875; 3,819)	763.7	(505.9; 1,030.8)	2,330	(1,496; 3,183)	1,027.5	(659.8; 1,404.1)	2,153	949.6	0.92
	6-17	3,408	(3,050; 3,836)	315.4	(282.2; 355.0)	3,003	(2,678; 3,369)	454.2	(405.1; 509.4)	2,506	379.0	0.83
	18-39	1,585	(1,213; 1,930)	54.1	(41.4; 65.9)	1,515	(1,181; 1,823)	84.5	(65.9; 101.7)	1,096	61.2	0.72
	40-64	1,134	(639; 1,658)	32.8	(18.5; 47.9)	1,008	(580; 1459)	47.6	(27.4; 68.9)	902	42.6	0.89
	65-74	233	(-295; 744)	38.8	(-49.0; 123.7)	192	(-270; 648)	52.1	(-73.2; 176.1)	171	46.5	0.89
	≥75	1,186	(-642; 2,942)	209.7	(-113.4; 519.9)	1,009	(-515; 2,525)	291.4	(-148.8; 729.4)	159	45.9	0.16
	All ages	**10,377**	**(5,840; 14,930)**	**133.3**	**(97.2; 168.4)**	**9,057**	**(5,150; 13,007)**	**187.9**	**(135.3; 238.5)**	**6,987**	**126.8**	**0.77**
P&I	0-5	1,916	(1,394; 2,450)	517.2	(376.1; 661.2)	1,921	(1,512; 2,349)	847.4	(666.8; 1,036.1)	2,074	914.8	1.08
	6-17	2,707	(2,439; 3,001)	250.5	(225.7; 277.7)	2,334	(2,093; 2,594)	353.0	(316.5; 392.3)	2,454	371.1	1.05
	18-39	834	(647; 1,014)	28.5	(22.1; 34.6)	631	(472; 773)	35.2	(26.3; 43.1)	1,062	59.3	1.68
	40-64	777	(384; 1,177)	22.5	(11.1; 34.0)	642	(344; 935)	30.3	(16.2; 44.2)	883	41.7	1.38
	65-74	174	(-276; 606)	28.9	(-45.9; 100.7)	154	(-159; 478)	41.9	(-43.1; 129.9)	169	45.9	1.10
	≥75	796	(-812; 2,390)	140.7	(-143.6; 422.4)	354	(-968; 1,502)	102.3	(-279.7; 433.9)	158	45.6	0.45
	All ages	**7,204**	**(3,775; 10,637)**	**102.6**	**(74.2; 130.9)**	**6,036**	**(3,293; 8,632)**	**134.3**	**(98.2; 167.2)**	**6,800**	**123.4**	**1.13**
CVD	40-64	337	(-958; 1,512)	9.7	(-27.7; 43.7)	314	(-749; 1,315)	14.9	(-35.4; 62.1)	206	9.7	0.66
	65-74	483	(-634; 1,428)	80.3	(-105.4; 237.5)	407	(-532; 1,205)	110.5	(-144.5; 327.4)	87	23.6	0.21
	≥75	857	(-1,581; 3,248)	151.4	(-279.5; 574.0)	711	(-1,315; 2,703)	205.5	(-379.7; 780.8)	82	23.7	0.12
	All ages	**1,676**	**(-3,174; 6,188)**	**17.0**	**(-33.6; 66.2)**	**1,432**	**(-2,595; 5,224)**	**23.3**	**(-45.9; 90.6)**	**375**	**6.8**	**0.26**
IHD	40-64	181	(-320; 673)	5.2	(-9.2; 19.5)	115	(-262; 485)	5.4	(-12.4; 22.9)	29	1.4	0.25
	65-74	164	(-225; 537)	27.2	(-37.4; 89.3)	138	(-189; 454)	37.5	(-51.3; 123.5)	38	10.3	0.28
	≥75	503	(-269; 1347)	88.9	(-47.5; 238.0)	415	(-221; 1,112)	119.9	(-63.8; 321.1)	20	5.8	0.05
	All ages	**848**	**(-813; 2,557)**	**1.6**	**(-15.9; 17.6)**	**668**	**(-672; 2,051)**	**2.2**	**(-21.7; 24.1)**	**87**	**1.6**	**0.13**
Stroke	40-64	33	(-258; 312)	1.0	(-7.4; 9.0)	31	(-212; 269)	1.5	(-10.0; 12.7)	20	0.9	0.65
	65-74	129	(-204; 400)	21.4	(-34.0; 66.6)	109	(-172; 339)	29.6	(-46.8; 92.2)	17	4.6	0.16
	≥75	197	(-512; 909)	34.8	(-90.5; 160.6)	164	(-427; 758)	47.4	(-123.4; 218.9)	24	6.9	0.15
	All ages	**359**	**(-974; 1,621)**	**1.8**	**(-10.5; 13.8)**	**304**	**(-811; 1,367)**	**2.4**	**(-14.4; 19.0)**	**61**	**1.1**	**0.20**
Diabetes	40-64	365	(-228; 916)	10.6	(-6.6; 26.5)	307	(-192; 773)	14.5	(-9.1; 36.5)	101	4.8	0.33
	65-74	449	(-128; 949)	74.7	(-21.2; 157.8)	376	(-107; 794)	102.1	(-29.0; 215.8)	41	11.1	0.11
	≥75	736	(-150; 1,635)	130.1	(-26.4; 288.9)	611	(-124; 1,359)	176.4	(-35.7; 392.5)	39	11.3	0.06
	All ages	**1,550**	**(-505; 3,499)**	**17.7**	**(-3.4; 38.1)**	**1,294**	**(-422; 2,926)**	**24.2**	**(-4.6; 52.2)**	**181**	**3.3**	**0.14**

ARD: acute respiratory disease; CI: confidence interval; CVD: cardiovascular disease; IHD: ischaemic heart disease; P&I: pneumonia and influenza.

[a] May 2009 to July 2010.

[b] May 2009 to January 2010.

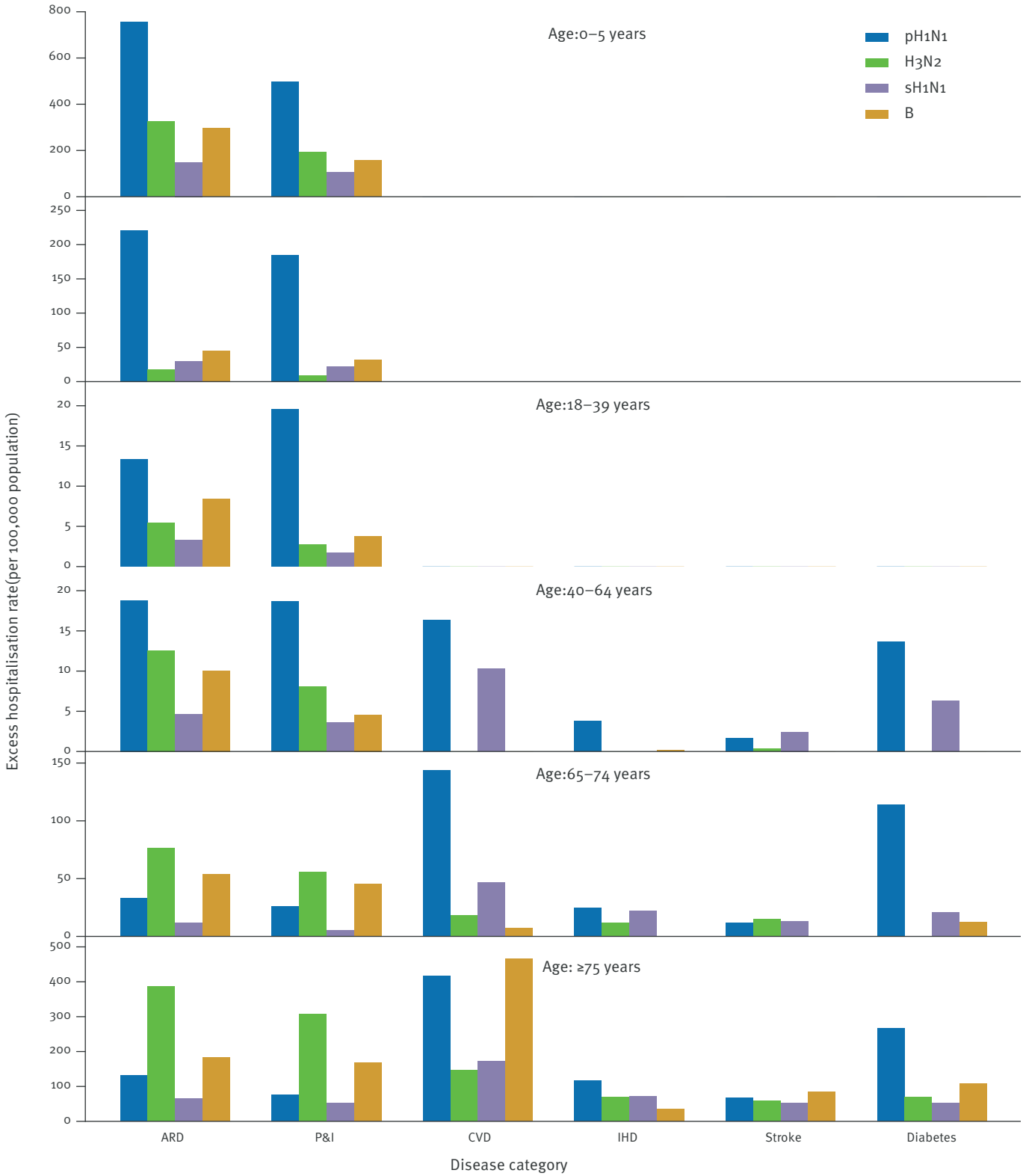
[c] The total numbers of laboratory-confirmed pandemic hospitalisation during the first wave were obtained from the e-flu database managed by the Hospital Authority. The cause-specific cases were grouped by any-listed diagnoses.

[d] Ratio=numbers of hospitalised cases with laboratory confirmed pH1N1 infection/excess numbers of hospitalisation associated with influenza A(H1N1)pdm09 during the first wave.

Note: The total numbers of all ages may not be equal to the sum of each age group due to roundup

Figure 2

Annual age- and cause-specific excess rate per 100,000 person-years of hospitalisation associated with A(H1N1)pdm09, seasonal influenza A(H3N2), A(H1N1) and B, Hong Kong, 2005–2010



ARD: acute respiratory disease; CVD: cardiovascular disease; IHD: ischaemic heart disease; P&I: pneumonia and influenza

Table 4

Percentage of excess hospitalisation in total cause-specific hospitalisation, Hong Kong, 2005–2010

Disease	2005–2010						1996–2000[a]
	Age group (years)	Seasonal influenza A(H1N1)	Influenza A(H3N2)	Influenza B	Influenza A(H1N1)pdm09	Seasonal influenza A+B	Seasonal influenza A+B
ARD	0-5	2.5	5.4	4.9	12.5	12.8	9.3[b]
	6-17	4.9	3.1	7.5	36.7	15.5	9.3[b]
	18-39	2.0	3.3	5.1	8.0	10.4	7.2[c]
	40-64	1.4	3.8	3.1	5.7	8.3	11.0
	65-74	0.6	3.8	2.7	1.7	7.1	11.5
	≥75	0.7	4.1	1.9	1.4	6.7	8.7
	All ages	**1.5**	**4.1**	**3.2**	**8.0**	**8.8**	**10.9**
P&I	0-5	5.4	9.7	7.9	25.0	23.0	14.7[b]
	6-17	6.3	2.8	9.2	53.3	18.3	14.7[b]
	18-39	2.0	3.1	4.4	22.6	9.5	10.5[c]
	40-64	1.5	3.4	1.9	7.8	6.8	8.7
	65-74	0.3	3.3	2.7	1.5	6.3	11.0
	≥75	0.6	3.6	2.0	0.9	6.2	7.1
	All ages	**1.6**	**4.0**	**2.9**	**9.4**	**8.5**	**11.6**
CVD	40-64	0.5	NA[d]	NA[d]	0.9	NA[d]	NA
	65-74	0.5	0.2	0.1	1.6	0.8	NA
	≥75	0.7	0.6	1.9	1.7	3.2	NA
	All ages	**0.6**	**0.3**	**0.8**	**1.3**	**1.7**	**NA**
IHD	40-64	NA[d]	NA[d]	0.0	0.8	NA[d]	2.0
	65-74	0.8	0.4	NA[d]	1.0	NA[d]	0.8
	>75	1.2	1.2	0.6	1.9	3.0	3.2
	All ages	**0.9**	**0.6**	**NA[d]**	**0.4**	**NA[d]**	**1.8**
Stroke	40-64	0.7	0.1	NA[d]	0.5	NA[d]	0.8
	65-74	0.6	0.7	NA[d]	0.5	NA[d]	1.4
	≥75	0.8	0.9	1.3	1.0	3.0	2.4
	All ages	**0.7**	**0.7**	**0.6**	**0.4**	**2.0**	**1.5**
Diabetes	40-64	0.9	0.0	NA[d]	2.0	NA[d]	3.5
	65-74	0.6	0.0	0.4	3.3	1.0	3.0
	≥75	0.7	1.0	1.6	3.8	3.3	5.4
	All ages	**0.8**	**0.3**	**0.6**	**3.1**	**1.7**	**3.5**

ARD: acute respiratory disease; CVD: cardiovascular disease; IHD: ischaemic heart disease; NA: not available; P&I: pneumonia and influenza.

[a] Adapted from reference [34].

[b] Age group 0–14.

[c] Age group 15–39.

[d] Not estimated because of negative excess risk estimates.

The fraction of ARD and P&I excess hospitalisation in the group of 0–5 year-olds was comparable between pH1N1 and seasonal influenza.

Discussion

There are lots of obstacles in obtaining the estimates on hospitalisation due to influenza simply based on surveillance of symptomatic cases, because many suspected cases were not tested in a timely manner due to limited laboratory capacity in most countries. Presanis and colleagues estimated that only 20–30% of hospitalised pH1N1 cases in the United Kingdom confirmed by the laboratory surveillance during the 2009 pandemic [22]. In the present study we used a Poisson regression model, which has been widely adopted for estimation of the influenza disease burden [23-25], to assess excess hospitalisation associated with seasonal influenza. We estimated that during the pandemic period of May 2009 to February 2010, 7,856 admissions for ARD in children and adults younger than 65 years were attributable to pH1N1 infections. This estimate was close to the total number of 6,657 laboratory-confirmed pH1N1 cases hospitalised for ARD in Hong Kong. The close match between model-derived and laboratory-confirmed cases in young age groups is not surprising as they were immediately identified as high risk groups at the beginning and underwent intensive laboratory screening for pH1N1 infections throughout the pandemic period. We found that the estimated in old population groups, excess hospitalisations associated with pH1N1 were dramatically higher than the numbers of confirmed hospitalised cases, for both acute and chronic diseases. This is in agreement with our previous findings that the mortality burden of pH1N1 in children and young adults was fully captured by intensive screening, but seriously underreported in the elderly [8]. We speculated that the neglected burden in the old population could be due to their low antibody and viral titres after infection, or the time lag between primary infection and hospital admission for exacerbated underlying conditions. To capture these cases, it is necessary to enhance the surveillance at community level so as not to miss the time when the symptoms of primary infection are evident.

A shift in the age of severe and fatal cases towards younger age groups has been observed in historical influenza pandemics. Consistent with many other studies [26-28],our estimates demonstrated that for ARD and P&I, the influenza A(pH1N1)pdm09 virus was associated with dramatically elevated excess hospitalisation in children and young adults compared with the seasonal viruses, whereas its impact on the elderly was less than that of influenza A(H3N2), although comparable to sH1N1 and influenza B. A study in Denmark also found significantly higher hospitalisation rates for pH1N1 among persons younger than 65 years, but the rates among the elderly were similar for the pandemic and seasonal viruses [29]. That old people are less affected by pH1N1 infection could be explained by their preexisting immunity against pH1N1 [30]. Despite a low attack rate of pH1N1 in elderly people in Hong Kong living in the community, a large proportion of the hospitalised and fatal cases still occurred in old people, who had markedly higher case–intensive care unit and case–fatality rates according to data from the e-flu database [31].

H3N2 viruses were believed to have more frequent antigenic drift than sH1N1 and influenza B [32]. Previous studies have demonstrated that influenza was associated with markedly more morbidity and mortality during the H3N2-dominated years, than during the years dominated by sH1N1 or influenza B [13]. By simultaneously adding the different virus proxies, regression models allow the separate assessment of the disease burden caused by different influenza types or subtypes. Zhou and colleagues fitted a negative binomial regression model, which is similar to Poisson regression with adjustment for over-dispersion, on weekly hospitalisation data of respiratory and cardiovascular diseases in the United States (US) from 1993 to 2008 [33]. They reported much lower excess rates of hospitalisation associated with sH1N1 and influenza B (1.9 and 17.5 per 100,000 person-years) than with influenza A(H3N2) virus (44.4 per 100,000). Interestingly, our estimates showed that among the three seasonal influenza viruses, H3N2 influenza contributed to higher hospitalisation rates for ARD and P&I. For chronic diseases, however, the hospitalisation rates associated with H3N2 influenza were lower for CVD and diabetes; the rates associated with sH1N1 and H3N2 influenza were similar for IHD and stroke, but those associated with influenza B were slightly lower (Figure 2). Further studies are needed from more countries to compare the virulence of different influenza viruses.

We estimated that during 2005–08, the annual excess rate associated with seasonal influenza ranged from 80.5 to 116.1 per 100,000 person-years for ARD, and from 30.4 to 51.1 for CVD. These rates were higher than the excess hospitalisation rates of 32.6–82.7 for respiratory and circulatory diseases which were estimated from the US study for the same study period [33], although these two sets of estimates may not be directly comparable since we defined disease categories by any-listed diagnosis. Our estimates were the double or triple of the previous estimates using the similar model for the period of 1996–2000 [34] (Table 2). The increased morbidity burden in recent years may not be solely due to more virulent virus strains, as we did not observe any obvious increase in excess mortality associated with influenza in Hong Kong since 1998, except in 2007 when a novel H3N2 strain A/Brisbane/59/2007-like emerged [8]. Instead, this increase is more likely the result of expanding health service capacities and lowering admission criteria due to the aging population of Hong Kong [16]. In fact, overall the proportions of excess hospitalisation in total numbers of cause-specific hospitalisation are quite similar between our present and previous studies, suggesting that the morbidity burden of seasonal

influenza remained comparable during the interpandemic years (Table 4). Interestingly, the percentage of excess hospitalisation associated with seasonal influenza among the total hospitalisations for ARD and P&I among children and young adults was higher during the study period of 2005–2010 than those during 1996–2000. However, it was lower among the elderly, which probably reflects changing health-seeking behaviour of parents and young people, particularly after the outbreak of severe acute respiratory syndrome in 2003 that significantly awakened the public awareness of respiratory viruses.

Surprisingly, we found a heavy morbidity burden of influenza in 2010 in Hong Kong with a similar magnitude as in the pandemic year 2009. However, the majority of influenza isolates since August 2010 have been an H3N2 strain which is an antigenic match to the 2010–2011 vaccine composite strain A/Perth/16/2009. Similar observations were also reported in several European countries [35]. We noticed that in 2010, the summer peak of influenza did not appear until August–September, the months when influenza viruses usually become dormant after the normal peak months of June and July [36]. Most of excess hospital admissions in 2010 were associated with influenza A(H3N2), not pH1N1 that was the dominant strain in the winter peak 2009/10 (data not shown). The potential explanation for altered influenza seasonality after the pandemic could be depletion of the pool of susceptibles by the preceding pandemic, interference of pandemic viruses, or relaxation of the control measures implemented during the pandemic period (border control, designated influenza clinics, etc), all of which certainly warrant further investigations.

There are several limitations to our study. Firstly, our virology data for pH1N1 only covered a period shorter than two years, which might have compromised the power of the Poisson model, as reflected by the non-significant estimates in most age–disease categories. Secondly, other than disease severities there are many unmeasured factors that could have affected hospital admissions, such as influenza vaccination status, health seeking behaviour and admission criteria. Our assessment of cause-specific hospitalisation could be further complicated by varying coding practice of frontline medical practitioners. Although we adjusted for long-term and seasonal trends of hospitalisation in our models to take into account changes in admission threshold and coding practice, this may not be adequate for all the disease categories, as evidenced by some negative estimates and underestimation of P&I excess hospitalisation during the pandemic. Nevertheless, our previous validation study demonstrated that excess hospitalisation derived from the Poisson regression models closely matched the true incidence rates of laboratory-confirmed influenza among a cohort of paediatric patients [10]. Moreover, our estimates were overall larger than the laboratory-confirmed cases requiring hospitalisation, although the difference was not as big as we expected. Therefore, our estimates of excess hospitalisation rates are likely conservative, not exaggerating the true burden of seasonal and pandemic influenza.

In this study we comprehensively assessed the impact of seasonal and pandemic influenza by deriving the age- and cause-specific estimates of excess hospitalisation associated with different types or subtypes of influenza viruses. Compared with seasonal influenza viruses, the pH1N1 virus was associated with dramatically increased hospitalisation risk among children and young adults. Although the attack rate of the pandemic was relatively low in the elderly, their risk of hospitalisation was comparable to the interpandemic years and seriously underreported by the clinical surveillance. This highlights the need to enhance the community surveillance on pandemic outbreaks among old people. Our study provides an approach for reliable assessment of the pandemic severity and also a piece of critical evidence for proper evaluation of control measures implemented during the pandemic.

*Author's correction

On request of the authors, the first three sentences of the second paragraph of the Methods section were replaced and a footnote was added to Table 1. These corrections were made on 14 November 2012.

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References

1. Miller MA, Viboud C, Balinska M, Simonsen L. The signature features of influenza pandemics--implications for policy. N Engl J Med. 2009;360(25):2595-8.
2. Echevarria-Zuno S, Mejia-Arangure JM, Mar-Obeso AJ, Grajales-Muñiz C, Robles-Pérez E, González-León M, et al. Infection and death from influenza A H1N1 virus in Mexico: a retrospective analysis. Lancet. 2009;374(9707):2072-9.
3. Mølbak K, Widgren K, Jensen KS, Ethelberg S, Andersen PH, Christiansen AH, et al. Burden of illness of the 2009 pandemic of influenza A (H1N1) in Denmark. Vaccine. 2011;29(Suppl 2):B63-9.
4. Bolotin S, Pebody R, White PJ, McMenamin J, Perera L, Nguyen-Van-Tam JS, et al. A new sentinel surveillance system for severe influenza in England shows a shift in age distribution of hospitalised cases in the post-pandemic period. PLoS One. 2012;7(1):e30279.
5. Campbell CN, Mytton OT, McLean EM, Rutter PD, Pebody RG, Sachedina N, et al. Hospitalisation in two waves of pandemic influenza A(H1N1) in England. Epidemiol Infect. 2011;139(10):1560-9.
6. Chowell G, Echevarria-Zuno S, Viboud C, Simonsen L, Tamerius J, Miller MA, et al. Characterizing the epidemiology of the 2009 influenza A/H1N1 pandemic in Mexico. PLoS Med. 2011;8(5):e1000436.
7. Jhung MA, Swerdlow D, Olsen SJ, Jernigan D, Biggerstaff M, Kamimoto L, et al. Epidemiology of 2009 pandemic influenza A (H1N1) in the United States. Clin Infect Dis. 2011;52 (Suppl 1):S13-26
8. Yang L, Chan KP, Cowling BJ, Chiu SS, Chan KH, Peiris JS, et al. Excess mortality associated with 2009 pandemic H1N1 in Hong Kong. Epidemiol Infect. 2012;140(9):1542-50.
9. Castilla J, Etxeberria J, Ardanaz E, Floristán Y, López Escudero R, Guevara M. Estimating the impact of the 2009 influenza A(H1N1) pandemic on mortality in the elderly in Navarre, Spain. Euro Surveill. 2010;15(5):pii:=19481. Available from: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19481
10. Yang L, Chiu SS, Chan KP, Chan KH, Wong WH, Peiris JS, et al. Validation of statistical models for estimating hospitalization associated with influenza and other respiratory viruses. PLoS One. 2011;6(3):e17882.
11. Gilca R, De Serres G, Skowronski D, Boivin G, Buckeridge DL. The need for validation of statistical methods for estimating respiratory virus-attributable hospitalisation. Am J Epidemiol. 2009;170(7):925-36.
12. Cox NJ, Subbarao K. Global epidemiology of influenza: past and present. Annu Rev Med. 2000;51:407-21.
13. Simonsen L, Fukuda K, Schonberger LB, Cox NJ. The impact of influenza epidemics on hospitalisations. J Infect Dis. 2000;181(3):831-7.
14. Thompson WW, Shay DK, Weintraub E, Brammer L, Cox N, Anderson LJ, et al. Mortality associated with influenza and respiratory syncytial virus in the United States. JAMA. 2003;289(2):179-86.
15. Hong Kong Statistics. 2011 Population Census. Hong Kong: Census and Statistics Department. [Accessed 17 March 2012]. Available from: http://www.census2011.gov.hk/en/census-result.html
16. Hospital Authority Statistical Report 2009–2010. Hong Kong: Hospital Authority;2011. Available from : http://www.ha.org.hk/upload/publication_15/321.pdf
17. Centers of Disease Control and Prevention (CDC). International classification of diseases, ninth revision, clinical modification (ICD-9-CM). Atlanta: CDC; 2010. Available from: http://www.cdc.gov/nchs/icd/icd9cm.htm
18. Mak GC, Wong AH, Ho WY, Lim W. The impact of pandemic influenza A (H1N1) 2009 on the circulation of respiratory viruses 2009-2011. Influenza Other Respi Viruses. 2012;6(3):e6-10.
19. Hong Kong Statistics. 2006 Population By-census. Hong Kong: Census and Statistics Department. [Accessed 17 March 2012]. Available from: http://www.censtatd.gov.hk/hkstat/sub/so440.jsp
20. Hong Kong Statistics. 2001 Population Census. Hong Kong: Census and Statistics Department. [Accessed 17 March 2012]. Available from: http://www.censtatd.gov.hk/major_projects/2001_population_census/index.jsp
21. Riley S, Kwok KO, Wu KM, Ning DY, Cowling BJ, Wu JT, et al. Epidemiological characteristics of 2009 pandemic influenza based on paired sera from a prospective community cohort. PLoS Med. 2011;8(6):e1000442.
22. Presanis AM, Pebody RG, Paterson BJ, Tom BD, Birrell PJ, Charlett A, et al. Changes in severity of 2009 pandemic A/H1N1 influenza in England: a Bayesian evidence synthesis. BMJ. 2011;343:d5408.
23. Warren-Gash C, Bhaskaran K, Hayward A, Leung GM, Lo SV, Wong CM, et al. Circulating influenza virus, climatic factors, and acute myocardial infarction: a time series study in England and wales and Hong Kong. J Infect Dis. 2011;203(12):1710-8.
24. Gran JM, Iversen B, Hungnes O, Aalen OO. Estimating influenza-related excess mortality and reproduction numbers for seasonal influenza in Norway, 1975-2004. Epidemiol Infect. 2010;138(11):1559-68.
25. de Blasio BF, Xue Y, Iversen B, Gran JM. Estimating influenza-related sick leave in Norway: Was work absenteeism higher during the 2009 A(H1N1) pandemic compared to seasonal epidemics? Euro Surveill. 2012;17(33):pii=20246. Available from: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20246
26. Karageorgopoulos DE, Vouloumanou EK, Korbila IP, Kapaskelis A, Falagas ME. Age distribution of cases of 2009 (H1N1) pandemic influenza in comparison with seasonal influenza. PLoS One. 2011;6(7):e21690
27. Writing Committee of the WHO Consultation on Clinical Aspects of Pandemic (H1N1) 2009 Influenza, Bautista E, Chotpitayasunondh T, Gao Z, Harper SA, Shaw M,et al. Clinical aspects of pandemic 2009 influenza A (H1N1) virus infection. N Engl J Med. 2010;362(18):1708-19.
28. Jacks A, Ollgren J, Ziegler T, Lyytikainen O. Influenza-associated hospitalisations in Finland from 1996 to 2010: unexpected age-specific burden during the influenza A(H1N1)pdm09 pandemic from 2009 to 2010. Euro Surveill. 2012;17(38):pii=20276. Available from: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20276
29. Widgren K, Nielsen J and Mølbak K. Registry-based surveillance of influenza-associated hospitalisations during the 2009 influenza pandemic in Denmark: the hidden burden on the young. PLoS One. 2010;5(11):e13939.
30. Steens A, Waaijenborg S, Teunis PF, , Reimerink JH, Meijer A, van der Lubben M, et al. Age-dependent patterns of infection and severity explaining the low impact of 2009 influenza A (H1N1): evidence from serial serologic surveys in the Netherlands. Am J Epidemiol. 2011;174(11):1307-15.
31. Wu JT, Ma ES, Lee CK, Chu DK, Ho PL, Shen AL, et al. The Infection attack rate and severity of 2009 pandemic H1N1 influenza in Hong Kong. Clin Infect Dis. 2010;51(10):1184-91.
32. Rambaut A, Pybus OG, Nelson MI, Viboud C, Taubenberger JK, Holmes EC. The genomic and epidemiological dynamics of human influenza A virus. Nature. 2008;453(7195):615-9.
33. Zhou H, Thompson WW, Viboud CG, Ringholz CM, Cheng PY, Steiner C, et al. Hospitalisations Associated With Influenza and Respiratory Syncytial Virus in the United States, 1993-2008. Clin Infect Dis. 2012;54(10):1427-36.
34. Wong CM, Yang L, Chan KP, Leung GM, Chan KH, Guan Y, et al. Influenza-associated hospitalisation in a subtropical city. PLoS Med. 2006;3(4):e121.
35. Beaute J, Broberg E, Plata F, Bonmarin I, O'Donnell J, Delgado C, et al. Overrepresentation of influenza A(H1N1)pdm09 virus among severe influenza cases in the 2011/12 season in four European countries. Euro Surveill. 2012;17(9):pii=20105. Available from: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20105
36. Yang L, Wong CM, Lau EHY, Chan KP, Ou CQ, Peiris JS. Synchrony of Clinical and Laboratory Surveillance for Influenza in Hong Kong. PLoS One. 2008;3(1):e1399.

Assessment of the bio-preparedness and of the training of the French hospital laboratories in the event of biological threat

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A national laboratory network 'Biotox-Piratox' was created in 2003 in France with the purpose of detecting, confirming and reporting potential biological and chemical threat agents. This network is divided into three levels: Level 1 is dedicated to the evaluation of risks (biological, chemical, radiological), to sampling and packing. Level 2 consists of university and military hospitals, who deal with biological specimens, and of environmental and veterinary laboratories, who deal with environmental and animal samples. Level 3 comprises national reference laboratories and the Jean Mérieux biosafety level (BSL)-4 laboratory in Lyon. This report presents the results of four bio-preparedness exercises to check critical points in the processing of samples. These exercises took place in 2007, 2009, 2010 and 2011. Each of them consisted of two parts. The first part was the identification of an unknown bacterial strain and its susceptibility to antibiotics used as a default in case of a bioterrorist event. The second part was the detection of Class III microorganisms, mainly by molecular techniques. The main lesson learnt in these exercises was that the key to successful detection of biological agents in case of a biological threat was standardisation and validation of the methods implemented by all the laboratories belonging to the network.

Introduction

A national laboratory network 'Biotox-Piratox' was created in France in 2003 with the purpose of detecting, confirming and reporting potential biological and chemical threat agents [1,2]. This network is an interministerial laboratory response network organised in three levels (Figure), similar to the surveillance laboratory network in the United States (US) [3-6]. A scientific advisory committee of the Biotox-Piratox network was appointed and entrusted with the three following missions: coordination of the network, training of the laboratories, and monitoring diagnostic tests in development. Since 2007, the committee has organised four inter-laboratory exercises to check critical points in the processing of samples: transport and reception, traceability, the diagnostic tests implemented, the reactivity and involvement of laboratories. The aim of this report is to present the results of the four biological exercises and the lessons learnt.

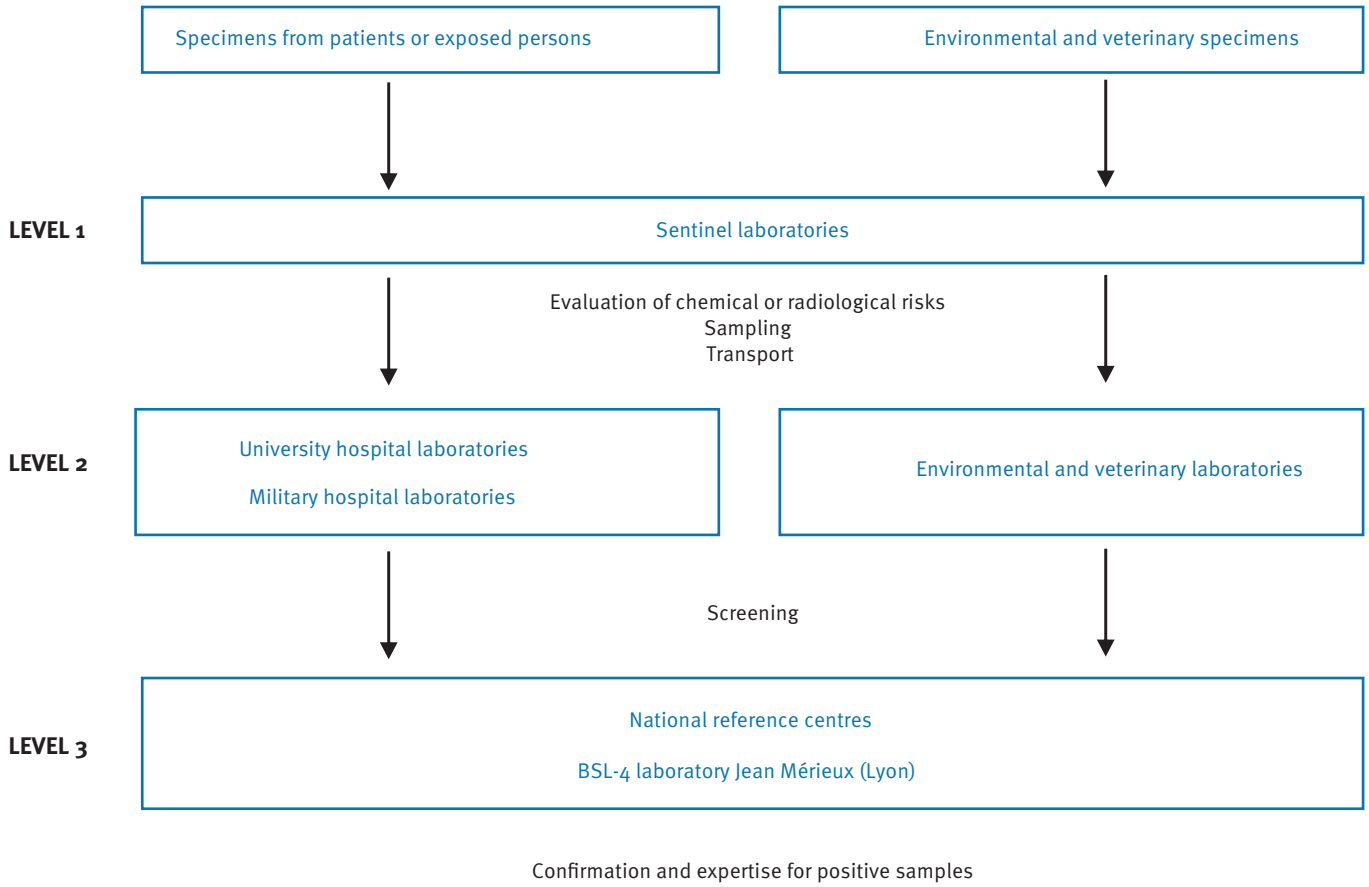
Methods

The exercises, which took place in 2007, 2009, 2010 and 2011, were essentially directed at the second level laboratories. The number of participating labs in these years varied from 27 to 28. Sixteen laboratories used the same diagnostic equipment and techniques: nine university hospitals, six military hospitals and one military research centre (Institut de Recherche Biomédicale des Armées, IRBA). The other participating centres dedicated to processing environmental samples used equipment and techniques which were more heterogeneous.

The authorisations for transporting and holding samples were obtained from the French national agency for the security of medicines and health products (Agence nationale de sécurité du médicament et des produits de santé; ANSM) for all exercises. All samples were dispatched to the participating centres using a specialised carrier according the French and European regulations. The second exercise was conducted in partnership with the Robert Koch Institute (RKI, Berlin, Germany), and the authorisations were obtained according to the German and French regulations for the transport of the biological material from Berlin (Germany) to Paris (France). For each sample, a scenario describing a realistic situation

Figure

The national Biotox-Piratox laboratory network in France



and the conditions of sampling were provided to the laboratories to guide their analytic strategy. All results were collected in a coordinating centre and were analysed by members of the Scientific Advisory Committee of the Biotox-Piratox laboratory network.

First exercise (2007)

The first exercise focused on the transport of specimens, the turnaround time for detection as well as the specificity and the sensitivity of the techniques implemented. The biological agents that were to be detected were a *Bacillus thuringiensis* strain resistant to ciprofloxacin (identification and susceptibility testing) and *B. anthracis* DNA cloned into an *Escherichia coli* strain (detection by PCR) [7].

Second exercise (2009)

The main objectives of the second exercise were to create relationships with other European countries, here with the Robert Koch Institute (Germany), to cope with administrative and logistic problems (transport or import), to list the various diagnostic techniques available, to evaluate the performance of diagnostic methods and to motivate participating centres to continue the training of technicians and biologists. The laboratories had to detect different concentrations of the following agents: *Francisella tularensis* subsp. *holartica,* irradiation-inactivated monkeypox virus (Mpx) and live vaccinia virus (Vac). The bacterial and viral samples were prepared and quantified by the Centre for Biological Safety at the RKI. It was confirmed that *F. tularensis* samples were viable for at least two weeks if stored at +4 °C in phosphate-buffered saline (PBS). The stability of the viral samples was controlled after four weeks at +4°C. The samples were transported by plane from Berlin to Paris in a single parcel, and then picked up by the French gendarmerie and taken to a reference laboratory, where the samples were divided into aliquots and dispatched by the police force to all participating centres.

Third exercise (2010)

The goal of the third exercise was to evaluate the performance of DNA-based techniques and the involvement of a national reference centre at the expertise level (Level 3) for positive samples detected at Level 2. This exercise consisted of a strain of *Yersinia pseudotuberculosis* (identification and susceptibility testing) and a DNA extract of *Y. pestis* EV76 (detection by PCR) [8]. At the end of the exercise, the DNA extracts were sent back to the IRBA laboratory to evaluate the performance of the DNA extraction method used by the participating laboratories. Ct (cycle threshold) values were obtained from real-time PCR amplifications

performed three times. The *caf1* gene was the criterion for this evaluation. In addition, positive samples were sent for confirmation to the Plague National Reference Laboratory (Dr. E. Carniel, Institut Pasteur, Paris).

Fourth exercise (2011)

At the annual seminar in 2011, it was decided to make the fourth exercise harder. Two Class III microorganisms were mixed together in the same medium: an *E. coli* strain modified to carry the plasmid pXO1 (gene *pag*) of *B. anthracis*, and the vaccine strain EV76 of *Y. pestis* modified to *pgm*-, pYV+, pFra+, pPst+, in which the *caf* and *pla* genes had been replaced by the gene conferring resistance to kanamycin and zeocin. The second part of the exercise consisted in the identification and susceptibility testing of a strain of *Burkholderia thailandensis*.

Results

Overall, 22, 26, 28 and 27 laboratories took part in the four exercises, respectively. The results were compared with the expected results, defined as a correct analytical result associated with a correct interpretation.

In all four exercises, parcels were transported and delivered without major problems, except for two cases. The first was a zip code unknown to the carrier. The second was the lack of authorisation of the carrier to transport Class III microorganisms to La Réunion. The delivery time was considered as acceptable (less than 24 hours) in metropolitan France.

At the end of every exercise, samples and waste were destroyed by autoclaving at 134 °C for 18 min, or at 121 °C for one hour, or by complete immersion in 2.5% chlorine when an autoclave was not available (e.g. in field laboratories).

First exercise (2007)

The results of the first exercise are summarised in Table 1. The first sample contained a strain of *B. thuringiensis* (*Bacillus* of the *cereus* group genetically close to B. anthracis) resistant to ciprofloxacin. Results were submitted from 22 centres; they took between 28 hours and 15 days to complete the exercise. Phenotypic identification was achieved by all 22 laboratories except for two. The identification systems used were API 20 and API 50CHB (bioMérieux, France; n=18 laboratories), Vitek-2 (bioMérieux, France; n=2 laboratories) and Phoenix (Becton-Dickinson, US; n=1 laboratory; one laboratory used more than one method). B. *thuringiensis* was correctly identified in all except one laboratory which falsely identified it as *B. anthracis*. Antibiotic susceptibility testing was carried out by 17 laboratories, of which 16 detected a resistance to ciprofloxacin and 14 confirmed a susceptibility to doxycycline.

The second part of the first exercise consisted of *B. anthracis* DNA (strain Sterne) cloned into an *E. coli* strain. *B. anthracis* strain Sterne is peculiar in that it harbours the gene *pagA* (encoding protective antigen) but not the gene *capA* (encoding capsule). This analysis was carried out by 19 laboratories with satisfactory results (Table 1). The results of this second part were returned after a period of three hours to 15 days. Two laboratories also looked for the presence of a chromosomal *Bacillus* gene (*ba*813). Only six laboratories drew the accurate conclusion that the strain was the vaccine strain Sterne based on its genotypic characteristics.

Table 1

Results of the first exercise assessing the performance of the Biotox-Piratox laboratory network: identification of *Bacillus* strains, France, 2007

First sample	*Bacillus thuringiensis* resistant to ciprofloxacin
Participants	22
Correct analytical result	20
False interpretation	1 *B. anthracis*
Interpretation not done	1
Results for antibiogram	16/17
Second sample	**DNA *Bacillus anthracis* Sterne**
Participants	19
Correct analytical results by PCR	19

Second exercise (2009)

For the second exercise, the results were variable both for bacterial and viral samples (Table 2). For the identification of *Francisella tularensis* subsp. *holarctica*, several techniques were implemented and associated: culture (16 laboratories), PCR (21 laboratories), sequencing (four laboratories), immunochromatography (eight laboratories). Susceptibility testing for bacteria was performed by 18 of 25 participants.

Of the 21 laboratories participating in the exercise for viral samples, 20 used a genotypic method, two used culture on Vero cells, and three used sequencing or pyrosequencing. No false positive result was obtained for viral samples, but a lack of sensitivity of the real-time PCR was observed for the lowest viral loads (Table 2).

Third exercise (2010)

For the third exercise, 28 laboratories participated. The samples were delivered in less than 24 hours except for the transport to one laboratory which was overseas (La Réunion). The delays in obtaining the results have varied between 6.5 hours and eight days. Most of the laboratories (n=20) delivered the results in less than three days. The detection of *Y. pestis* in four samples with different DNA concentrations was performed accurately by only 19 of 28 laboratories (Table 3). Two of the three laboratories using 16S DNA sequencing gave a wrong identification. To determine the performance of DNA extraction methods, 28 extracts were sent to the IRBA laboratory for detection of the *caf1* gene (Table 4). A maximal difference of 12.72 Ct between

Table 2

Results of the second exercise assessing the performance of the Biotox-Piratox laboratory network: determination of bacterial and viral concentrations, France, 2009

Bacteriology	*Francisella tularensis* LVS				
Dilution	B1	B2	B3	B4	B5
Cell count/mL	$5x10^6$	$5x10^6$	$5x10^3$	0	$5x10^6$
Participants	25	26	26	26	26
Correct analytical result	24	26	25	23	25
False interpretation	0	0	0	0	0
Virology	**Monkeypox inactivated by irradiation**				
Dilution	V1	V2	V4	V5	
pfu/mL	$1,4x10^4$	0	$2,8x10^7$	$4,1x10^6$	
Participants	21	21	21	21	
Correct analytical result	15	21	20	20	
False interpretation	2	0	4	4	
	Vaccinia virus in PBS, stable and infectious				
Dilution	V2	V3	V6	V7	
pfu/mL	0	$<10^2$	$2,3x10^3$	$4x10^5$	
Participants	21	21	21	21	
Correct analytical result	21	5	16	20	
False interpretation	0	0	2	4	

LVS: live vaccine strain; PBS: phosphate-buffered saline; pfu: plaque forming units.

the laboratories was observed. This highlights the importance of the extraction step including an optional pretreatment step (lysis), the sample volume used for extraction, the extraction method chosen (manual or automated) and its intrinsic performance for yield and purity of nucleic acid isolation, as well as the eluate volume. Detection of the *caf1* gene was possible in 26 extracts from samples with high concentrations, but only in 18 extracts from samples with low concentrations. It should be noted that in some cases IRBA detected *caf1* in extracts that had tested negative in the laboratories. These observations highlight the differences in the performance of the amplification step depending on the protocol used (detection threshold), but could also reflect differences in technical training.

Table 3

Results of the third exercise assessing the performance of the Biotox-Piratox laboratory network: detection of *Yersinia pestis* EV76 DNA, France, 2010

Bacteriology	*Yersinia pestis* EV76				
	A	B	C	D	All
Cell count/mL	10^2	0	10^5	10^3	-
Participants	28	28	28	28	28
Correct analytical results	26	27	24	22	19

The second part of the exercise was the identification of *Y. pseudotuberculosis*. This microorganism was accurately identified by all laboratories. Identification methods were either manual (API strips, bioMérieux, France) or automated (Vitek-2, bioMérieux, France). Two laboratories used mass spectrometry to identify the strain [9]. Ten laboratories confirmed their result by DNA sequencing. However, sequencing of 16S rRNA did not allow distinguishing *Y. pestis* from *Y. pseudotuberculosis*. Antibiotic susceptibility testing was performed either with discs, E-test strips (bioMérieux, France) or Vitek-2 cards (bioMérieux, France).

Fourth exercise (2011)

This exercise consisted of the identification and susceptibility testing of a strain of *B. thailandensis* (Table 5). A small majority of laboratories identified this isolate correctly at the species level. Depending on the biochemical identification method, *B. thailandensis* could easily be misidentified as *B. pseudomallei*. Only the arabinose test can distinguish these two species. Various methods of identification were employed: phenotypic methods alone or in association with either mass spectrometry or PCR and DNA sequencing. Mass spectrometry was used by four laboratories and gave an accurate identification in all four cases.

As expected, the second part of this exercise was more difficult. Only one third of the laboratories gave a correct result (Table 6). These nine laboratories identified both the plasmid pXO1 cloned in a strain of *E. coli* and the modified vaccine strain of *Y. pestis*. The results of

Table 4

Results of the third exercise assessing the performance of the Biotox-Piratox laboratory network: comparison of *Yersinia pestis* DNA extracts by detection of the *caf1* gene, France, 2010

Ct values	10^5	10^3	10^2
Ct values with the reference procedure (3 tests)	25.03–25.18	31.62–32.74	35.23–36.49
Mean Ct values	25.79	33.54	34.99
Lowest Ct values	19.99	27.08	30.04
Highest Ct values	30.54	39.81	41.19
Laboratories with expected result	26	22	18

Ct: cycle threshold.

the other laboratories were incomplete or wrong. Once it is grown, the strain of *Y. pestis* can be identified either by a phenotypic method, by a molecular method or by mass spectrometry. Among the phenotypic methods, the rhamnose test allowed to differentiate this modified *Y. pestis* isolate from *Y. pseudotuberculosis*. As in the third exercise, it was not possible to distinguish the two *Yersinia* species, neither by molecular methods, nor by mass spectrometry or sequencing.

Discussion

The main challenge of these exercises, as also performed by the Laboratory Response Netwok (LRN) in the US [10], was to assess the capacity and competence of Level 2 laboratories through relevant exercises involving unknown samples within the framework of the national regulation applied to microorganisms of Class III [11]. Several lessons can be learnt concerning the organisation and the implementation of the exercises, and the methods and techniques that should be used by Level 2 laboratories.

For each exercise, getting the authorisations for transportation and delivery to all laboratories was a long process which required continuous liaison between the Ministry of Health, the French regulatory authority (ANSM) and the organisers. Although all participants agreed that the regulations need to be strict, they were of the opinion that the administrative procedures should be simplified for such exercises. As laboratory exercises on biological threat agents are important with a view to biodefense, exercises and proficiency tests should be integrated in the national regulations. Moreover, as no other European country runs such exercises, it would be worth implementing them under the auspices of European governments.

Since 2002, participating university hospitals have used the same molecular methods with the same primers and probes, the same PCR material, equipment and positive controls. Equipment of military hospital laboratories was different from that used in university hospitals, but all military hospitals used the same equipment, the same primers and probes, and followed similar methodologies which made it possible to ensure coherent results, while non-hospital laboratories (mainly those dedicated to environmental and veterinary samples) showed a larger disparity in the methods and, therefore, in the results. However, the general disparity in the extraction methods (type of protocols and commercial extraction kits, manual or automated methods) has to be underlined.

All methods used to identify bacteria were appropriate for identification at genus and species level, but identification of the species is not sufficient to the government or health authorities to make a decision. Antibiotic susceptibility testing results are important to validate the treatment and implement a public health strategy for antimicrobial treatment and prophylaxis management. Two classes of antibiotics have to be tested because they are recommended as first line therapy or prophylaxis in the event of a biological threat: fluoroquinolones and doxycycline. The minimal inhibitory concentration can easily be determined by the E-test method [12-13]. During the exercises, antibiotic susceptibility results were not obtained systematically from the participants. Consequently, the Scientific Advisory Committee of the Biotox-Piratox laboratory network recommends determining minimal inhibitory concentrations for field and environmental laboratories. If this technique is not available in some

Table 5

Results of the fourth exercise assessing the performance of the Biotox-Piratox laboratory network: identification of *Burkholderia thailandensis*, France, 2011

First sample	*Burkholderia thailandensis*
Participants	27
Correct analytical result	16
False identifications	1 *Aneurinibacillus aneurinilyticus* 1 *B. pseudomallei, Pseudomonadaceae* 3 *B. cepacia* 1 *Moraxella lacunata* 1 *Yersinia pestis* PCR-positive 1 *Y. pestis* PCR-negative 3 no identification
Antibiogram performed	10/27

Table 6

Results of the fourth exercise assessing the performance of the Biotox-Piratox laboratory network: detection of *Yersinia pestis* (vaccine strain modified) and pXO1 in *Escherichia coli* in a mix of microorganisms, France, 2011

Second sample	*Yersinia pestis* + *Escherichia coli* (pXO1)
Participants	27
Correct analytical results with interpretation	9
Other results	1 *Y. pestis* 1 *Bacillus spp.* 2 *Y. pseudotuberculosis* 1 *E. coli* O157:H7 4 *E. coli* 1 *Clostridium botulinum* toxin B 1 staphylococcal enterotoxin not detected 5 *pagA* only detected 2 *Y. pestis* only detected

environmental laboratories, attention is drawn on the importance of rapidly sending the isolate to a hospital laboratory.

Molecular methods were widely used for the identification of bacteria and viruses. The third exercise underlined that the limitations of the PCR method were mainly related to the yield and quality of nucleic acid extraction, to contamination during the PCR and to the sensitivity of the method. As expected, the best results were achieved for the samples with the highest nucleic acid concentrations, especially for viral samples. The worst results were associated with the lowest viral loads, especially for the vaccinia virus sample (V3) containing only traces of vaccine DNA (‹10^2 pfu/mL). The DNA extraction step must be optimised to improve sensitivity. Another problem observed for some centres was the lack of interpretation or unsuitable interpretation accompanying the results. Indeed, the results are intended to be used by the government or health authorities, and must be interpreted and clearly explained so that non-specialists can understand them. This is critical to making accurate decisions.

The organisers set some traps, especially in the fourth exercise. The first trap was the combination of two important pathogens in the same sample, which could happen in case of a bioterrorist attack. The second trap was the modified vaccine strain which required primers different from those used in the previous exercises. The last trap was the presence of a plasmid cloned into an *E. coli* strain, which was overlooked by a number of laboratories.

The conclusion that can be drawn from these exercises is that the key to successful detection of biological agents in case of a biological threat is the training of the laboratory personnel (microbiologists and technicians) dealing with class III organisms as well as the standardisation and validation of methods implemented by all laboratories of the network. This last point was exemplified during the third exercise for molecular methods. However, the French Biotox-Piratox laboratory network is a network of networks. Thus it is not possible or realistic to force all laboratories to use identical equipment or techniques. Nevertheless, it is important to provide specific guidelines to all laboratories involved in the network. The publication of such guidelines for biological specimens is in progress. Moreover, administrative procedures should be adapted to accommodate for national exercises.

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References

1. Binder P, Brucker G, Josseran L. [From alert to laboratory: a coherent network designed to deal with to naturally occurring infectious disease outbreaks and bioterrorism.] Bull Acad Natl Med. 2007;191(6):1005-18. French.
2. La coordination départementale et zonale. [Departmental and zonal coordination]. Bordeaux: Gestion des risques exceptionnels et NRBC. [Management of exceptional and CBRN risks]. [Accessed 6 Nov 2012]. French. Available from: http://.nrbc-sudest.org/public/public_manuscrite.aspx?PAGE_CODE=PUBLIC_CONTEXTE_COORD
3. Gilchrist MJ. A national laboratory network for bioterrorism: evolution from a prototype network of laboratories performing routine surveillance. Mil Med. 2000;165(7 Supple 2):28-31.
4. Peterson LR, Hamilton JD, Baron EJ, Tompkins LS, Miller JM, Wilfert CM, et al. Role of clinical microbiology laboratories in the management and control of infectious diseases and the delivery of health care. Clin Infect Dis. 2001;32(4):605-11.
5. Pien BC, Saah JR, Miller SE, Woods CW. Use of sentinel laboratories by clinicians to evaluate potential bioterrorism and emerging infections. Clin Infect Dis. 2006.42(9):1311-24.
6. Kalish BT, Gaydos, CA, Hsieh YH, Christensen BE, Carroll KC, Cannons A, et al. National survey of laboratory response network sentinel laboratory preparedness. Disaster Med Public Health Prep. 2009;3(2 Suppl):S17-23.
7. Mock M, Fouet A. Anthrax. Annu Rev Microbiol. 2001;55:647-71.
8. Loïez C, Herwegh S, Wallet F, Armand S, Guinet F, Courcol RJ. Detection of Yersinia pestis in sputum by real time PCR. J Clin Microbiol. 2003;41(10):4873-5.
9. Blondiaux N, Gaillot O, Courcol RJ. MALDI-TOF mass spectrometry to identify clinical bacterial isolates: evaluation in a teaching hospital in Lille. Pathol Biol. 2010;58(1):55-7.
10. Wagar EA, Mitchell MJ, Carroll KC, Beavis KG, Petti CA, Schlaberg R, et al. A review of sentinel laboratory performance: identification and notification of bioterrorism agents. Arch Pathol Med. 2010;134(10):1490-503.
11. Arrêté du 30 Avril 2012 fixant la liste des micro-organismes et toxines prévue à l'article L.5139-1 du code de la santé publique. [Decree of 30 April 2012 establishing the list of micro-organisms and toxins in Article L. 5139-1 Code of Public Health]. Official Journal No. 0109 from 10 May 2012; p. 8788. Text No. 111. French. Available from: http://www.legifrance.gouv.fr/affichTexte.do?cidTexte=JORFTEXT000025837146&dateTexte=&categorieLien=id
12. Merens A, Vaissaire J, Cavallo J-D, Le Doujet C, Gros C, Bigaillon C, et al. E-test for antibiotic susceptibility testing of Bacillus anthracis, Bacillus cereus and Bacillus thuringiensis: evaluation of a French collection. Intern J Antimicrob Agents. 2008;31(5):490-2.
13. Valade E, Vaissaire J, Merens A, Hernandez E, Gros C, Le Doujet C, et al. Susceptibility of 71 French isolates of Francisella tularensis subsp. holarctica to eight antibiotics and accuracy of E-test® method. J Antimicrob Chemother. 2008;62(1):208-10.