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Letters to the Editor

Chronic-relapsing varicella zoster meningitis – Successfully treated with varicella vaccine

Dear Editor,

We note with interest recent reports on the problems of diagnosing central nervous system (CNS) infections,[1] particularly the study comparing the presence of varicella zoster virus (VZV) DNA in blood and cerebrospinal fluid (CSF) in patients with VZV-related neurological symptoms.[2] Here we report a case of chronic-relapsing VZV meningitis, where the episodes occurred with frequent and debilitating regularity.

A 62-year-old man, who worked as a General Practitioner at an asylum centre, presented with a 2-year history of headache, fatigue, diffuse myalgia, a low-grade fever, night sweats and mood disturbances. His symptoms waxed and waned every 1–3 weeks, without ever fully resolving, but never included seizures or focal neurological symptoms. He had lost 9 kg during this period. His work routinely exposed him to patients with various exotic and chronic infections, including a varicella outbreak 2 years previously.

General physical examination was unremarkable, apart from some limb myalgia. Specifically, neither neck stiffness nor any other focal neurological signs were detected. Other investigations (chest X-ray, echocardiography, abdominal ultrasound) were all normal. An MRI-brain showed some non-specific lesions in the deep white matter, compatible with Virchow-Robin spaces.

Full blood counts with differentiation, ferritin, lipid profile, thyroid stimulating hormone, CA19-9, liver function, serum glucose, urea and electrolytes and erythrocyte sedimentation rate were all normal, apart from a mild non-specific anaemia. Serological testing for HIV, cytomegalovirus (CMV), Epstein-Barr virus, herpes simplex virus (HSV), *Brucella, Borrelia, Yersinia, Toxoplasma*, and *Mycoplasma pneumoniae* were either negative or just indicated past infection.

Cerebrospinal fluid examination revealed a slightly raised total protein (0.66 g/L) and a low glucose (1.9 mmol/L) with a glucose ratio of 0.44. No erythrocytes were seen, but 95×10^6/L leucocytes were observed (primarily lymphocytes and monocytes). Polymerase chain reaction testing for CSF HSV and VZV was negative. Further CSF investigations using immuno-blotting showed intense IgG bands in the CSF, with fewer, weaker bands in a contemporaneous serum sample (Fig. 1): CSF intrathecal VZV IgG titre was 2500, with a VZV-IgG index of 98.52, indicating intrathecal VZV-IgG synthesis.

A diagnosis of chronic-relapsing VZV meningitis was made, and a 2-week course of intravenous acyclovir (1000 mg TD) was given. The patient improved on treatment, but the meningitis symptoms recurred every 2 weeks once treatment had stopped.

Further, repeated CSF examinations, during symptomatic and asymptomatic periods, showed similar profiles to the above. Contemporaneous serum VZV DNA PCR was mostly negative except once when it was positive at a very low level (63 copies/ml of VZV DNA). Repeat isoelectric focusing and immune-blotting revealed an intrathecal oligoclonal IgG response, together with a VZV-specific monoclonal IgG response (Fig. 2).

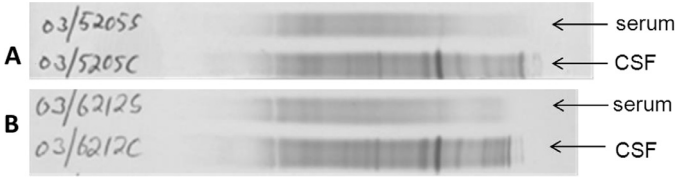

Fig. 1. Isoelectric focusing for VZV IgG. Isoelectric focusing of paired CSF and serum (**A** and **B**) stained for IgG in CSF, showing prominent oligoclonal bands in CSF, but absent from serum (consistent with local or intrathecal synthesis). The patterns have been aligned against the prominent band in the centre of the pattern on CSF to allow easy comparison. Although the distribution of bands appears slightly different between CSF samples this just reflects inter-assay variability in the analytical system (each sample pair was run on a different gel). The anode (+) electrode is on the left. The paired CSF and serum in images **A** and **B** are from samples taken approximately two months apart during acute illness episodes.

Further treatment with oral valacyclovir (1000 mg TDS for 7 days) followed by a suppressive dose (valacyclovir, 500 mg BD) for three months was then tried. However, the patient continued to experience meningitis symptoms on this medication. Treatment was continued with oral valacyclovir (1000 mg BD), but now in combination with monthly VZV hyper-immune globulin (VZIG, IM, 400 units). Although there was some improvement, the meningitis symptoms recurred every 3 weeks.

Several years later, we received an update from the patient. He had been on holiday in South Africa, during which he had mistakenly received 3 doses of the paediatric varicella vaccine instead of the regular VZIG. His symptoms improved for 4–5 weeks. On returning to The Netherlands, on this basis, his neurologist suggested zoster vaccine (which contains at least 19,400 PFU – plaque forming units – of VZV, per dose) for longer-term symptom relief. As this was not yet available, he was given 18 doses of paediatric varicella vaccine (which contains at least 1350 PFU, per dose) instead. This resolved his symptoms. He then received a depot corticosteroid injection for shoulder pain, after which his meningitis symptoms returned 3 weeks later. After a further 18-dose course of paediatric varicella vaccine, he has remained symptom-free.

Varicella-zoster virus remains latent in the neurons of cranial nerves and dorsal root ganglia for the entire life of the host after primary infection (chickenpox).[3] Herpes zoster or shingles is the most common form of VZV reactivation and is characterised by a dermatomal rash and radicular pain. Rarely, VZV spreads to the spinal cord and brain. When this occurs, CNS complications may develop without concomitant zoster rash.[4,5] Aseptic meningitis due

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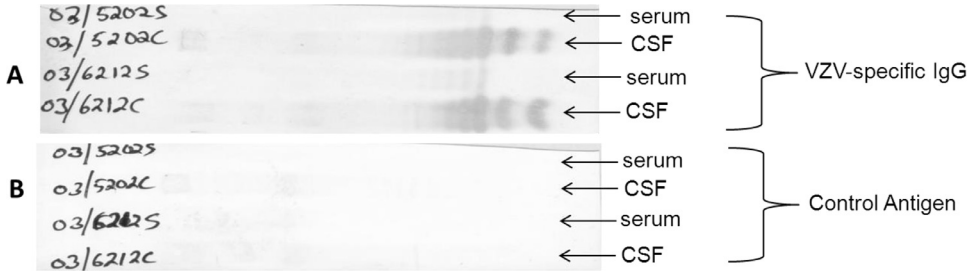

Fig. 2. VZV-specific IgG. VZV-specific IgG showing a ladder-pattern of bands, predominantly in the CSF (**A**). This pattern is also just visible in the serum samples. The ladder-pattern is characteristic of a monoclonal antibody. Due to the strong staining, the anodic (left-most) bands have merged. The absence of any bands in the control immunoblot (using a cell-line without VZV, **B**) shows that the IgG in the patient's CSF and serum is highly VZV-specific.

to VZV is seen in both immunocompetent as well as immunocompromised patients.[6] The diagnosis is usually confirmed by a positive CSF VZV DNA PCR result, or less commonly, by the intrathecal detection of VZV-specific IgG.[7]

The repeated failure to detect CSF VZV DNA in this case during acute episodes may be due to the chronic course of the disease. A similar decrease in the percentage of CSF HSV PCR-positive cases can be seen within 2 weeks for HSV encephalitis,[8] which may also be true for VZV. The onset of symptoms in this patient was 2 years before the first CSF VZV PCR was performed. His symptoms were also limited to viral meningitis rather evolving into encephalitis, which may have also explained the negative VZV PCR results.[9] However, the extensive intrathecal VZV IgG production confirmed the diagnosis of VZV aseptic viral meningitis.

The finding of a distinctly monoclonal intrathecal VZV-specific IgG is unusual and atypical (Fig. 2). Normally, an oligoclonal response is required to clear the virus efficiently. Therefore, the patient may have a defect in his VZV-specific antibody affinity maturation, i.e. a partial 'immunological scotoma' to VZV, explaining his inability to clear (i.e. 'neutralise') the virus efficiently, allowing recurrent episodes of meningitis. Note that this case does not meet Bruyn's diagnostic criteria for Mollaret's meningitis,[10] as the patient was not completely symptom-free between episodes.

To our knowledge, such chronic-relapsing varicella zoster viral meningitis of such periodicity has not been previously described. The remarkable response to multiple doses of paediatric varicella vaccine was both fortunate and serendipitous. This patient likely suffers from a VZV-specific antibody maturation deficit that appears to have been overcome by the massive immune stimulation provided by multiple doses of paediatric varicella vaccine. Whilst such cases are rare, this report may assist in their management.

Conflict of interest

None of the authors have any conflicts of interest to declare.

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We thank the patient for his cooperation in the reporting of this case, as well as other members of the NHNN team that have been involved in his management over the years, particularly Geoff Keir who produced the images shown in Figs. 1 and 2.

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Incidence rate of depression as an adverse effect of multidrug-resistant tuberculosis treatment

To the editors,

We would like to take the opportunity to comment on the systematic review by Alene et al.[1] The authors pooled data of prevalence in patients with multidrug-resistant tuberculosis (MDR-TB). However, we found that prevalence differed extremely among studies, ranged from 3% to 79%, leading to a high statistical heterogeneity (I^2=98%).

Table 1
Checked data extraction for the pooled prevalence of depression in the review by Alene et al.

Study	Incidence/ prevalence	number of events	Total number
Yew 2000	Incidence	3	25
Furin 2001	Incidence	11	60
Vega 2004	Incidence	10	75
Nathanson 2004	Incidence	51	818
Shin 2007	Incidence	21	244
Seung 2009	Incidence	13	76
Karagoz 2009	Incidence	–[a]	142
Palacios 2009[b]	Prevalence	–	36
Isaakidis 2011	Incidence	2	58
Brust 2013	Incidence	20	91
Das 2014	Prevalence	–[c]	45
Kaukab 2015	Prevalence	–[c]	70
Merassa 2015	Incidence	69	612
Chandra 2016	Prevalence	–[c]	100
Javaid 2017	Prevalence	253	289

[a] Only the total number of psychosis and depression was reported (n = 33).
[b] Only baseline information before treatment initiation was reported.
[c] For diverse tools were applied in prevalence estimate of depression and diagnosis standard was not uniformed, we did not extract some event numbers in this Table.

After checking, we surprisingly found that only four studies reported the prevalence of depression after MDR-TB treatment, one study reported the prevalence at baseline, while the others reported the incidence of depression as an adverse effect of MDR-TB treatment (Table 1). The authors may not carefully distinguish the conception of prevalence and incidence. In addition, for those studies adopting different scales and diagnosis criteria, the prevalence rate may change according to the criteria. It is not recommended to pool these data into meta-analysis. We hope future studies can adopt a unified standard of depression in the research, to better estimate the prevalence of depression after MDR-TB treatment.

The we conducted a meta-analysis by STATA 15.1, to calculate the incidence of depression as an adverse effect of MDR-TB treatment. The results showed an incidence rate of 11% (95% Confidence Intervals: 8–14%, I^2=77.5%) (Fig. 1). We consider that this result may provide more reference to clinical doctors.

The authors have discussed that the cause of psychosis may be cycloserine.[1] In addition, several studies mentioned that depression might also be an adverse effect of cycloserine.[2,3]

To summarize, we appreciate the authors' hard work, but there were some mistakes in data pooling. We estimated the incidence rate of depression as an adverse effect of MDR-TB treatment by performing a meta-analysis. Future studies are needed to estimate the prevalence.

Declarations of interest

None.

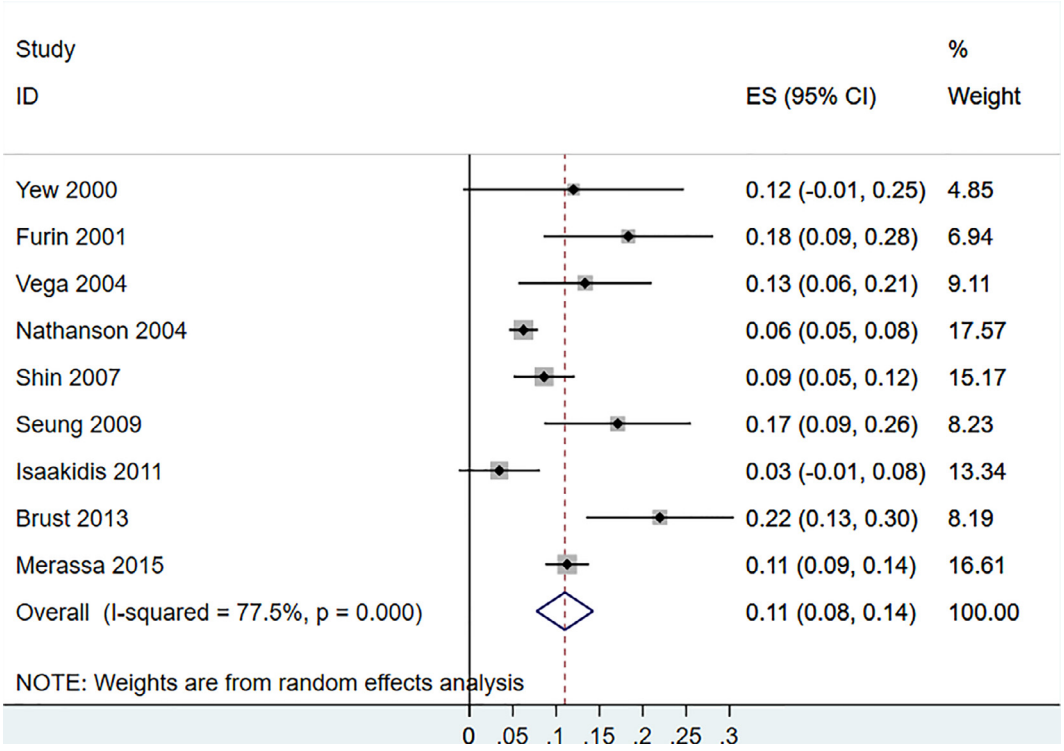

Fig. 1. Meta-analysis for the incidence of depression as an adverse effect of MDR-TB treatment.

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Community based serosurvey of naïve population indicate no local circulation of Zika virus in an hyper endemic area of China 2016

Dear editor,

We previously reported in this Journal that,[1] in early 2016, the public authority of China enforced the detection of imported zika cases in international airports regarding to the continuing global transmission of Zika virus (ZIKV). During February to September, 2016, 28 imported ZIKV infection cases were detected in China, of which 15 were imported from Venezuela, Suriname and Guatemala to Enping County, Guangdong, China (Fig. 1) where is the hometown of more than 450,000 Guangdong residents work and live in South America and South Pacifica countries. Most of these residents maintain their permanent home and travel frequently between Enping County and ZIKV endemic countries in South America or South Pacifica. Therefore, Enping was considered as the most threaten area by ZIKV in Guangdong, China due to high incidence of importation of zika cases. Additionally, Guangdong Province is a hyper-endemic areas of arboviruses located in Southern China, where both *Aedes albopictus* and *aegypti* mosquitoes are encountered simultaneously. Dengue occurred annually. Chikungunya were also occasionally imported and caused an local outbreak in 2010.[2,3] However, whether there were previously importation of ZIKV within returned ex-patriot Chinese in Enping, Guangdong, China and result in unware local transmission before 2016 in naïve population is still remained unknown.

During February to October 2016, a community based serological surveillance study of returned ex-patriot Chinese and naïve population was conducted in Enping County, Guangdong Province, China to address whether those populations have been exposed to ZIKV before the importation in 2016. The 227 ex-patriot Chinese were randomly selected from 1161 Guangdong residents work and live in South America and South Pacifica countries returned to Enping County. The information of returned ex-patriot Chinese (travelers in total, 227; male, 129; female, 98; range 1–76 years old) were informed by entry-exit inspection and quarantine bureau of Guangdong Province. All these travelers showed no symptoms of ZIKV infection, either fever (>38°C), rash, arthralgia or conjunctivitis. Local Center for Disease Control and Prevention (CDC) staff contacted these travelers at the time of their arrival in Enping County by phone or household visits. Blood sample were taken at the local hospital upon their arrival, and transferred to the Guangdong Provincial CDC for serum separation and ZIKV RNA testing. The naïve population were choosed from the communities in Enping County where the 15 imported zika cases detected, and in a neighbors Heshan County (fifty kilometers away from Enping) where most of the oversea Chinese live in non ZIKV endemic areas of Southeast Asia (Fig. 1). The naïve population was recruit from the local residents who have no travel history to ZIKV endemic areas of South America and South Pacifica countries, and without any contact history with imported zika cases. Finally, a total of 665 naïve residents (male, 278; female, 387; range 1–95 years old) were recruit, of which 330 and 335 came from Enping and Heshan County, respectively. The blood samples of these 665 residents were taken from each of them, and transferred to the local CDC. A total of 892 serum samples were collected and transferred to Guangdong Provincial CDC for further ZIKV RNA and neutrality antibody detection.

The quantity of ZIKV RNA Envelope gene was determined by quantitative real-time reverse transcription PCR (qRT-PCR) as we previously reported.[4] No ZIKV RNA were detected among all the 892 serum samples which indicated no acute stage infection of ZIKV. Although cross-reactive binding antibodies are commonly detected in flavivirus-immune serum, neutralization assays are more specific and can distinguish the antibodies response to various flaviviruses exposure previously,[5] thus, serum antibodies against to ZIKV in both 227 returned and 665 naïve residents were determined to make sure whether those persons exposed to ZIKV or not by a Vero cell based micro-neutralization assay against to ZIKV, CHIKV, DENV 1, and DENV 2. The results indicated that ZIKV could be neutralized by serum samples of 32 (No. 1-32) (14.1%, 32/227) returned persons (IC_{50} range from 1:8 to 1:1024) who had travel history in South America and South Pacific areas (Table 1). Twenty one of these 32 ZIKV antibody positive serum samples showed across-neutrality effects against to DENV-2 but not DENV-1. Four of these 32 samples showed minor cross-neutrality effects against to CHIKV (IC_{50} range from 1:8 to 1:32). Moreover, 18 serum samples from returned persons showed minor cross-neutralization against to CHIKV (No. 33-50, IC_{50} range from 1:8 to 1:32) but not ZIKV or DENV. Surprisingly, one serum samples (No. 51) from one naïve resident in Enping County showed significantly neutrality ability against to ZIKV (IC_{50} = 1:1024) and CHIKV (IC_{50} = 1:1024), while the remained 664 of 665 naïve residents samples showed no any neutrality effects against to ZIKV, CHIKV, as well as DENV.

Transmission of infection through international travel is a growing health issue, and the frequency of imported infection is increasing in China. The recently studies report the profile of travel-related infections among arriving travelers in mainland China, and highlight to increase public awareness of the potential risk of imported infections.[6] Indeed, the global transmission of ZIKV has been suspected through international travelers between endemic areas and local countries, and addressed by recently phylodynamic analysis.[7,8] In Guangdong, China, the airport active screening system found 18 imported zika cases which indicate

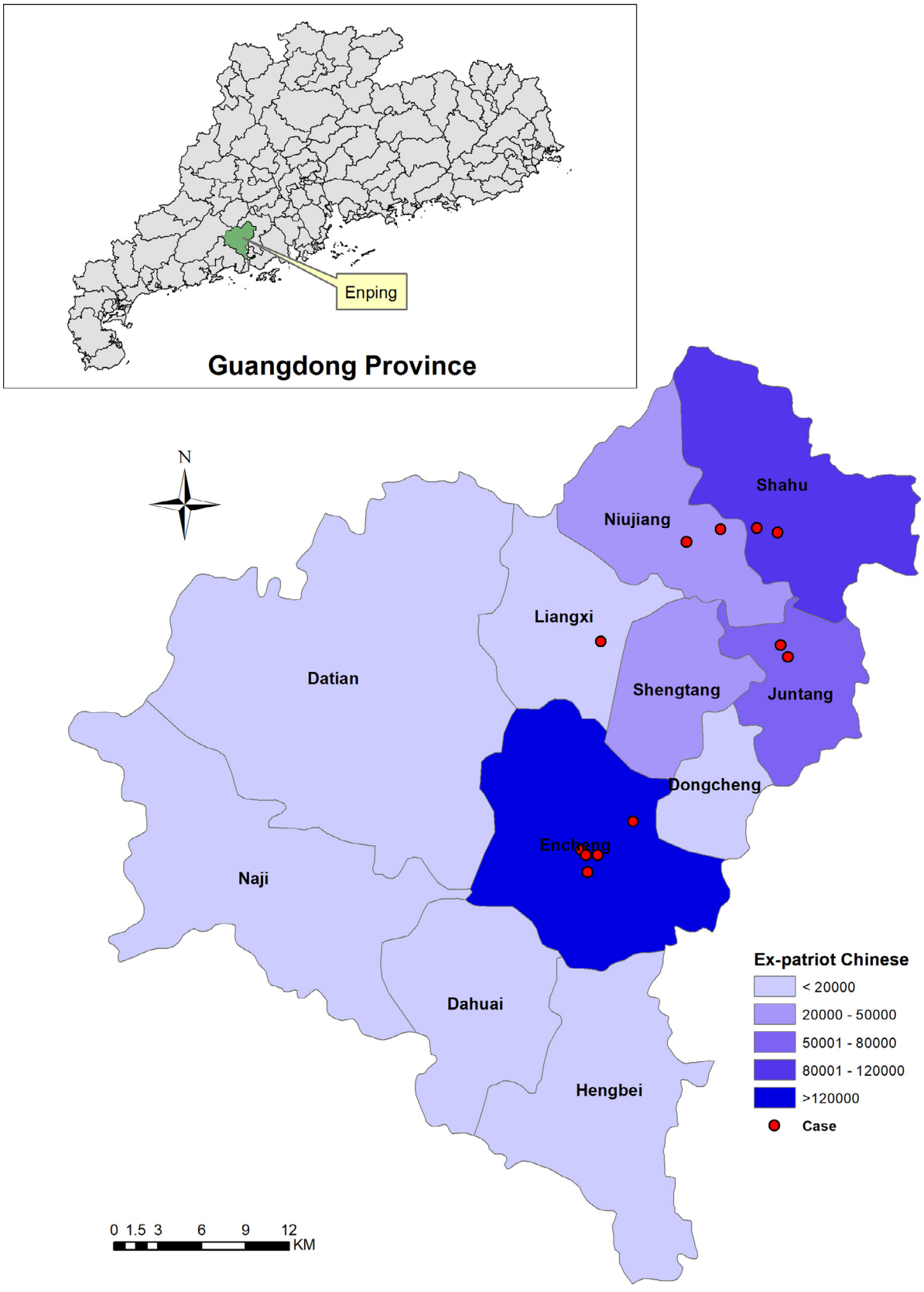

Fig. 1. Geographical location of ex-patriot Chinese and the distribution of imported zika cases in Enping County, Guangdong, China 2016. The ex-patriot Chinese (more than 450,000): Venezuela, ~180,000; Costa Rica, ~50,000; Mexico, ~40,000; Dominica, ~30,000; Hongkong, ~120,000; other countries, ~30,000.

Table 1
DENV, CHIKV, or ZIKV neutralization profiles (IC50) for persons with travel history to ZIKV endemic areas[a].

	ZIKV	CHIKV	DENV-1	DENV-2
1	1024	–	–	8
2	**512**	–	–	64
3	**512**	–	–	32
4	256	16	–	32
5	256	–	–	32
6	256	–	–	16
7	128	–	–	8
8	128	–	–	–
9	64	–	–	64
10	64	–	–	–
11	32	8	–	64
12	32	–	–	64
13	32	–	–	32
14	32	–	–	16
15	32	–	–	–
16	16	8	–	16
17	16	–	–	32
18	16	–	–	8
19	8	16	–	–
20	8	–	–	512
21	8	–	–	32
22	8	–	–	16
23	8	–	–	16
24	8	–	–	8
25	8	–	–	8
26	8	–	–	–
27	8	–	–	–
28	8	–	–	–
29	8	–	–	–
30	8	–	–	–
31	8	–	–	–
32	8	–	–	–
33	–	32	–	–
34	–	16	–	–
35	–	16	–	–
36	–	16	–	–
37	–	16	–	–
38	–	16	–	–
39	–	8	–	–
40	–	8	–	–
41	–	8	–	–
42	–	8	–	–
43	–	8	–	–
44	–	8	–	–
45	–	8	–	–
46	–	8	–	–
47	–	8	–	–
48	–	8	–	–
49	–	8	–	–
50	–	8	–	–
51	1024	16	–	–

[a] Sample No. 1-32 ($n = 32$) were ZIKV antibody positive samples of 227 returning Chinese from Venezuela during 2016 in Jiangmen, Guangdong, China; sample No. 33-50 ($n = 18$) were ZIKV antibody negative, while CHIKV antibody positive samples of 227 returning Chinese from Venezuela during 2016 in Jiangmen, Guangdong, China; sample 51 ($n = 1$) was a unique ZIKV antibody positive patient detected during serological survey of 665 naïve population in Jiangmen, Guangdong, China. ZIKV, Zika virus; DENV, dengue virus; CHIKV, chikungunya virus; IC50, 50% inhibitory concentration.

Guangdong is under a high risk of zika transmission due to wide distribution of competent vector of ZIKV, *Aedes albopictus* and *aegypti*. Although the present community based follow-up serological survey found 14.10% (32/227) of ex-patriot Chinese returned from ZIKV endemic countries were ever exposed to ZIKV, none of them were ZIKV cases at acute stage according to the results of ZIKV RNA detection. In contrast, we found most of naïve residents were not exposure to ZIKV. In addition, ZIKV antibody positive serum samples from returned population showed minor cross-neutrality effects against to DENV-2 and CHIKV but not DENV-1. It is not surprising because of continuously circulation of DENV-2 and CHIKV in South American countries,[9,10] therefore, those travelers may have been infected by DENV-2 or CHIKV before they returned to China. However, we did not observed any cross-neutrality effects against to ZIKV, CHIKV and DENV among the 665 naïve residents except for the unique suspect asymptomatic ZIKV and CHIKV case who do not want declare his travel history.

In conclusion, this report indicated that although numbers of zika case had been imported to a hyper endemic area of arboviruses in Guangdong, China, the community based surveillance of naïve populations showed ZIKV was only tentatively imported to Guangdong, whereas no local transmission has been established in Guangdong, China before 2016. It highlight the control strategies taken by public health authorities in Guangdong 2016 probably prevent the onward transmission of ZIKV in these areas.

Acknowledgments

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Conflict of interest

No conflict of interest were reported.

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Characterization of three clade 2.3.4.4 H5 highly pathogenic avian influenza viruses isolated from wild birds

Dear Editor,

In recent years, pathogenicity and transmissibility as well as increasing potential human infection ability of H5N6 avian influenza viruses (AIVs) have been documented in this journal.[1,2] Since 2003, H5N1 highly pathogenic avian influenza viruses (HPAIVs) have become enzootic in many countries and pose a serious challenge to the poultry industry and public health.[3] As of 2013, H5N6 HPAIVs have emerged and circulated in poultry, causing sporadic human infections in China.[4] Since 2014, H5N8 HPAIVs have been circulating in Asia, North America, and Europe.[5] Wild birds are considered a natural reservoir for AIVs and play an important role in viral transmission.[6] In this study, we have presented a brief characterization of three H5 HPAIVs with different NA subtypes (H5N1, H5N6, and H5N8) isolated from wild birds.

During AIV surveillance in wild birds in 2015–2016, we isolated three strains of H5 HPAIVs, designated A/Anser cygnoides/Jiangxi/P126/2015(H5N1) (P126/H5N1), A/Anser fabalis/Jiangxi/P560/2015 (H5N6) (P560/H5N6), and A/swan/Shanxi/ST/2016(H5N8) (ST/H5N8), from Poyang Lake of Jiangxi Province and Shengtian Lake of Shanxi Province. To clarify their genetic characteristics, we sequenced all gene segments of the three viruses and compared them with influenza virus sequences in GenBank. The pathogenicities of the three isolates were additionally determined in chickens, ducks and mice.

For virus isolation, feces samples were resuspended in PBS solution with antibiotics and subsequently inoculated into specific pathogen-free (SPF) embryonated chicken eggs as described previously.[7] RNA was extracted using the Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The full genomes of the viruses were sequenced and deposited in GenBank under accession nos. MK616066–MK616089.

To characterize the three isolates, we determined clade distribution based on the HA gene sequences. P126/H5N1, P560/H5N6, and ST/H5N8 isolates belonged to clade 2.3.4.4 (Fig. 1). Sequence analyses further showed high nucleotide identities of HA genes of the three H5 viruses with A/chicken/Tong Hai/302/2014(H5N1), A/oriental magpie robin/HK/6154/2015(H5N6), and A/chicken/Uganda/17RS115-15/2017(H5N8), which are currently circulating in Asia (Fig. 1).

To assess the risk of likelihood of transmission from avian to mammalian species, genotypic markers were evaluated based on their genome sequences. The receptor binding sites in the HA gene of three H5 HPAIVs contained Q226 and G228 (H3 numbering), suggesting binding preference for avian-like receptors.[8] The H5 viruses shared four to six amino acid changes within the receptor binding site (RBS) of HA, specifically, S133L (ST/H5N8), and S137A in the 130-loop, D187N, K193N, and Q196K in the 190-helix, and S227R (ST/H5N8) in the 220-loop (Table S1). Remarkably, HA genes of the three H5 isolates lacked an N-linked glycosylation site at position 158, whose absence is considered to promote receptor binding affinity in guinea pig transmission.[9] Mutations within the RBS and loss of the glycosylation site at residue 158 suggested that these H5 isolates confer receptor preference changes.

Mutations related to pathogenicity in mammals,[10] such as E627K and D701N in PB2, were not detected in the three avian isolates. However, substitutions in internal genes were identified, including nine in PB2 (T63I, L89V, G309D, T339K, Q368R, H447Q, R477G, I495V, and A676T), eight in PB1 (A3V, L13P, R207K, K328N, S375N, H436Y, L473V, and M677T), two in PA (H266R and S/A515T), three in MP (V15I, N30D, and T215A), and six in NS (A/P42S, T/D92E, L98F, I101M, V149A, and N200S), that potentially enhanced polymerase activity and increased virulence in mammals. These mutations appeared to confer not only increased receptor binding properties but also greater virulence of H5 HPAIVs, thus posing an increased threat to public health.

To evaluate the pathogenicity of the three H5 isolates in chickens, groups of ten 6-week-old SPF chickens were intravenously inoculated with 0.1 mL (1/10 dilution) of the allantoic fluid of each virus and mortality observed over a 10-day period. The characteristics of P126/H5N1, P560/H5N6, and ST/H5N8 viruses, which were highly pathogenic in chickens, are presented in Table S2.

To evaluate the pathogenicity of the H5 isolates in ducks, we inoculated groups of 10 3-week-old SPF ducks intranasally with 10^6 EID_{50} of each virus and observed mortality over a 10-day period. The characteristics of P126/H5N1, P560/H5N6, and ST/H5N8 viruses are shown in Table S2. Our data clearly indicated high pathogenicity of these viruses in ducks.

To determine the pathogenicity of the viruses in mammalian hosts, we inoculated BALB/c mice intranasally with 10^6 EID_{50} of each virus in a total volume of 50 μL. Five inoculated mice were euthanized on day 5 post-inoculation (p.i.) for virus titration in the lungs, nasal turbinate, liver and brain. The remaining 10 mice were monitored daily for weight loss and mortality for a total of 2 weeks. After intranasal administration of P126/H5N1 and P560/H5N6, high virus titers were detected in lung, nasal turbinate and liver, but not brain, while the virus was identified in lung and nasal turbinate, but not liver or brain of the ST/H5N8 group. Mice showed signs of illness. Over the 14-day observation period, all mice in groups inoculated with P126/H5N1 and P560/H5N6 isolates died (Fig. 2). However, no mortality was observed in the ST/H5N8 group (Fig. 2), suggesting that P126/H5N1 and P560/H5N6, but not the ST/H5N8 strain, were highly pathogenic in mice.

In summary, we characterized three H5 HPAI viruses with different NA subtypes isolated from apparently healthy wild birds in 2015–2016. All three isolates were highly pathogenic in chickens and ducks while the H5N1 and H5N6 subtypes showed high pathogenicity in mice. Many species of wild birds have long-distance dispersal and migration capacity. This behavior increases the likelihood of HPAIV spread, which presents a serious threat to poultry and human health.

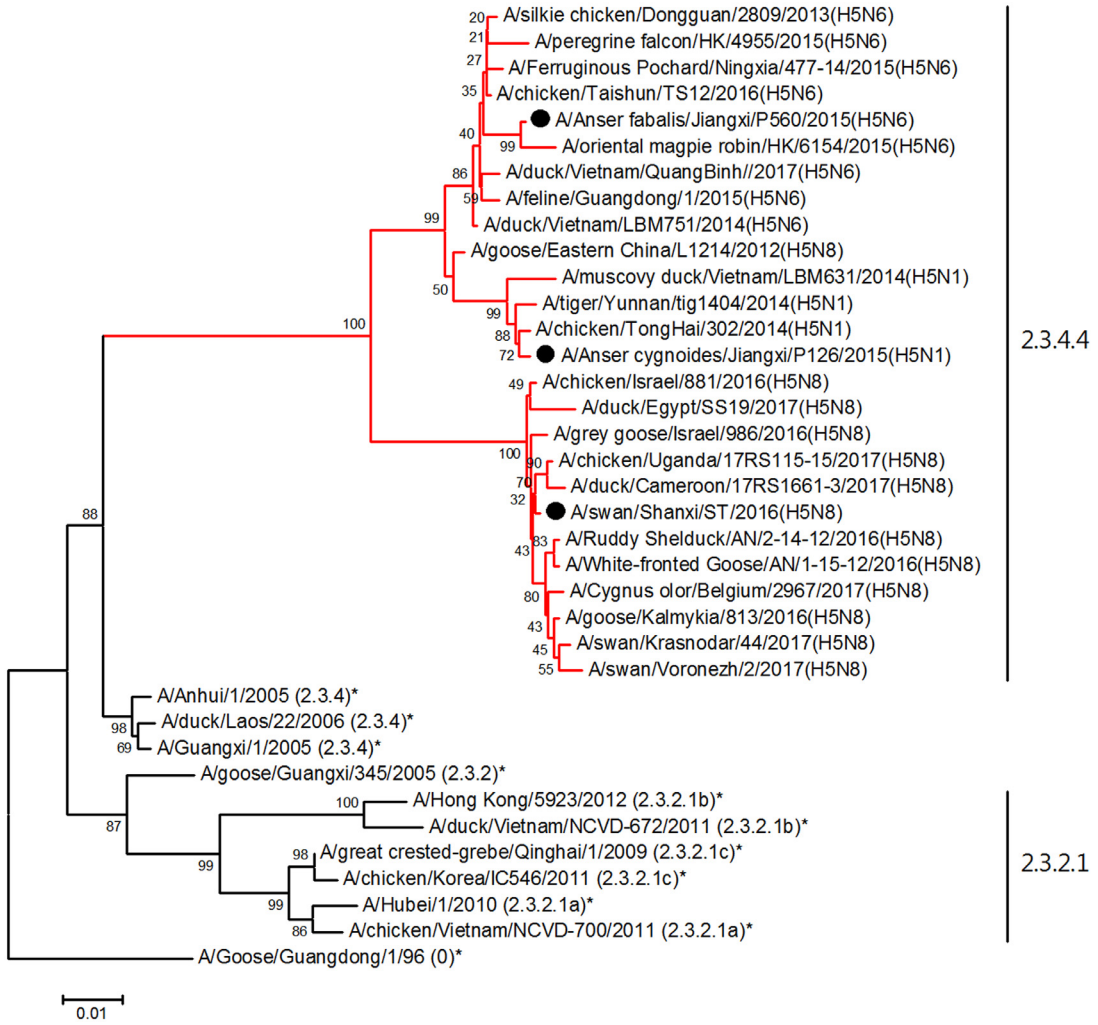

Figure 1. Phylogenetic trees of hemagglutinin genes of H5 subtype influenza viruses. Clade numbers are indicated on the right in panel. Trees were constructed with MEGA5 software using the neighbor-joining method. Bootstrap analysis was performed with 1000 replications. "•" is for viruses obtained in this study. Scale bars indicate nucleotide substitutions per site.

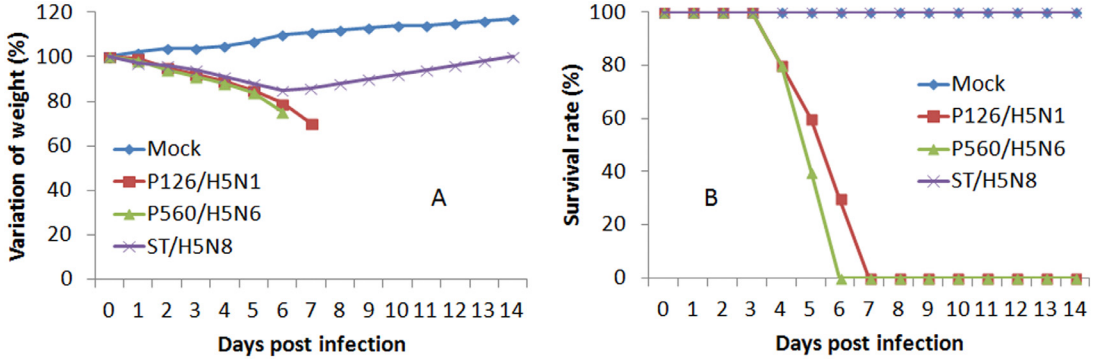

Figure 2. Virulence of the viruses isolated from wild birds in mice. Body weight changes (A) and mortality (B) of BALB/c mice were monitored daily for 14 days.

Conflicts of interest

There are no potential conflicts of interest.

Acknowledgments

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Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jinf.2019.03.011.

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Continuous adaptation of the HA and NA gene of H3N2 subtypes of avian influenza virus in South China, 2017–2018

Dear Editor,

Recently, we read with interest a letter in the *Journal of Infection*, which confirmed avian influenza viruses has posed a new challenge for public health.[1] The H3N2 subtype influenza virus has resulted in global pandemics since 1968,[2] and H3 has a wide host range from birds to various mammalian species, including humans, pigs, dogs, and horses.[3,4] Previous studies have shown that avian influenza viruses (AIVs) such as H5N1 and H7N9 could adapt to mammals and gain the ability to infect humans. Song reported cross-species transmission of an intact avian influenza virus (H3N2) to dogs in South Korea that caused acute respiratory disease.[5] In addition, seasonal influenza viruses of strain H3N2 have circulated in the human population every year, and sequence analysis has shown that the novel H3N2 contains genes from H7N3 and H7N7 that viruses are closely related to the H7N9 virus, suggesting that reassortment occurred between H3N2 and other influenza virus subtypes.[6] Thus, evolution of H3N2 AIVs was demonstrated in this study.

Two influenza A(H3N2) virus-positive isolates, A/duck/Guangdong/F138/2017(H3N2) and A/duck/Guangdong/F352/2018 (H3N2) (hereafter F138, F352), were collected from live poultry markets in Guangdong province during November 2017–March 2018. Molecular characterization of the two H3N2 isolates confirmed an amino acid HA-226Q, HA-228 G (H3 numbering), NA-292R (N2 numbering) and motif PEKQTR↓GL at its HA-cleavage site, which was the characteristic of low-pathogenic AIVs. Moreover, these strains were susceptible for NA inhibitors (oseltamivir and zanamivir). The detailed information of two H3N2 isolates were shown in Supplementary Table 1.

Available reference sequences, including those of avian-origin, canine-origin, swine and human-origin of the H3N2 subtype used for phylogenetic analysis, were acquired from GenBank (http://ncbi.nlm.nih.gov/genbank/) and GISAID (http://platform.gisaid.org/), and phylogenetic trees of all sequences were prepared by the Bayesian method using MrBayes version 3.2.6. Preliminary analyses were conducted to estimate the best-fit nucleotide substitution model using MrModeltest version 2.3.[7] Phylogenetic analysis confirmed that the HA and NA genes of F138 and F352 belonged to the avian-Eurasion lineage and were not related to canine, swine or human influenza viruses (Fig. 1A and B). The HA segments of F138 and F352 showed the closest relationship with A/duck/Hubei/ZYSYF2/2015(H3N6), A/duck/Fujian/SD063/2017(H3N3) and A/chicken/Guangxi/165C7/2014(H3N2) (Fig. 1a). Moreover, the HA gene of F138 showed the highest similarity to A/duck/Hubei/ZYSYF2/2015(H3N6) between nucleotide positions 101 and 1621, except around position 823, where it was similar to A/duck/Hubei/ZYSYF2/2015(H3N6) and A/duck/Fujian/SD063/2017(H3N3) based on **Sim**ilarity **Plot**ting (Simplot) (Fig. 2A). These findings suggested the HA gene of F138 was mainly derived from the A/duck/Hubei/ZYSYF2/2015(H3N6) but reassorted with A/duck/Fujian/SD063/2017(H3N3). F352 showed the highest similarity to A/chicken/Guangxi/165C7/2014 (H3N2) between nucleotides 101 and 1621, indicating that the HA gene of F352 was mainly derived from A/chicken/Guangxi/165C7/2014(H3N2) (Fig. 2B).

The NA segments of F138 and F352 were most closely related to A/duck/Zhejiang/727042/2014(H6N2), A/duck/Hunan/S2046/2011(H4N2) and A/muscovy_duck/Vietnam/LBM201/2012(H3N2) based on phylogenetic trees (Fig. 1b). Simplot analyses showed that the NA gene of F138 was most similar to A/duck/Zhejiang/727042/2014(H6N2), A/duck/Hunan/S2046/2011(H4N2) and A/muscovy_duck/Vietnam/LBM201/2012(H3N2) between nucleotides 101 and 262, 262 and 382, and 382 and 502, respectively. The greatest similarity was with A/duck/Hunan/S2046/2011 (H4N2) between nucleotides 502 and 1063; however, positions 1063–1301 showed the highest similarity with A/duck/Zhejiang/727042/2014(H6N2) (Fig. 2C). These findings indicate the NA gene of F138 mainly originated from the N2 segments of A/duck/Zhejiang/727042/2014 (H6N2), A/duck/Hunan/S2046/2011 (H4N2) and A/muscovy_duck/Vietnam/LBM201/2012 (H3N2).

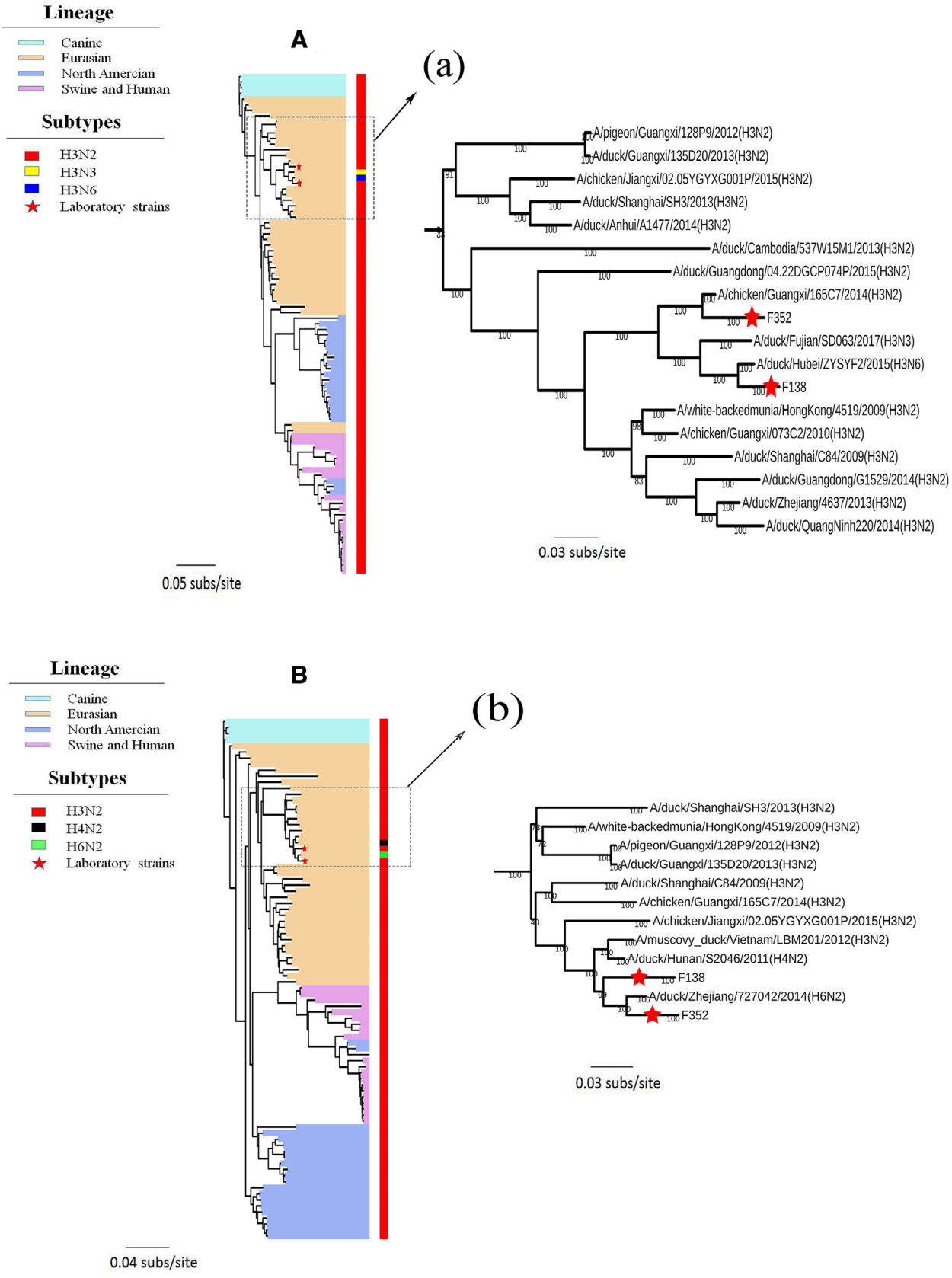

Fig. 1. Evolutionary history of influenza A (H3N2) virus. (A) Phylogenetic tree of H3N2 hemagglutinin (HA) gene. Trees were constructed by the Bayesian method using MrBayes version 3.2.6, and the best-fit nucleotide substitution model was General Time Reversible, Gamma distributed with Invariant sites (GTR+G+I). Isolated viruses belonging to different lineages and subtypes are distinguished by colors. The red stars represent H3N2 AIVs in our study. The scale bar represents the number of nucleotide substitutions per site (subs/site). (a) Enlargements of (A). (B) Phylogenetic tree of H3N2 neuraminidase (NA) gene. Isolated viruses belonging to different lineages and subtypes are distinguished by colors. (b) Enlargements of (B).

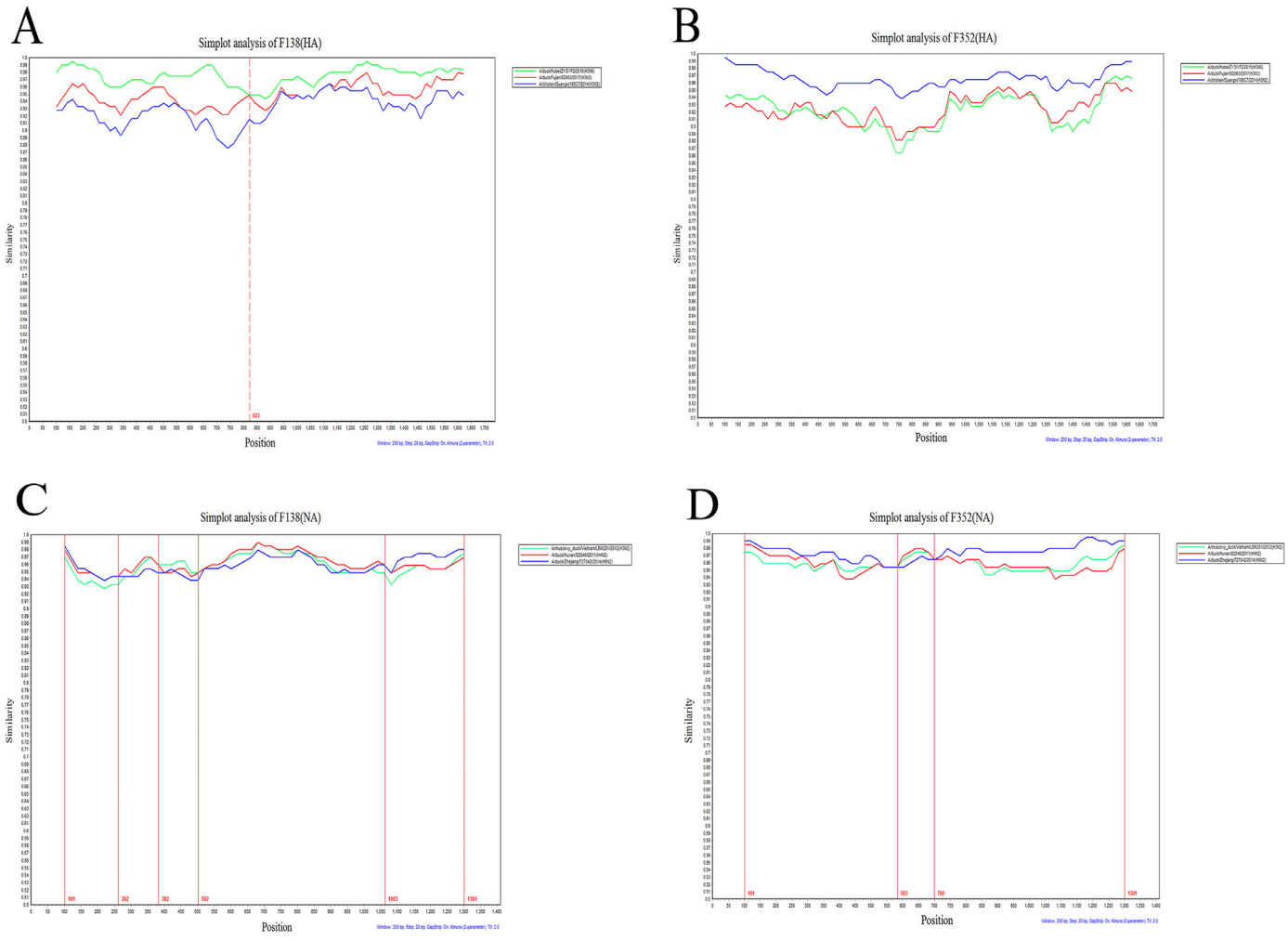

Fig. 2. Similarity plotting analysis of H3N2 subtypes of avian influenza virus. Bootscanning of F138 and F352 sequences. Bootscan evidence of recombination was based on the pairwise distance model with a window size of 200, step size of 20, and 1000 bootstrap replicates generated by the Simplot program. The *x*-axis represents the nucleotide position, and the *y*-axis represents similarity of sequences. Red vertical lines divide the part of the highest nucleotide similarity into different subtypes. Different subtype strains used for Simplot analysis with F138, F352 are distinguished by colors. (A) Similarity Plotting analysis of HA gene of F138. (B) Similarity Plotting analysis of HA gene of F352. (C) Similarity Plotting analysis of NA gene of F138. (D) Similarity Plotting analysis of NA gene of F352.

The NA gene of F352 showed the highest similarity to A/duck/Zhejiang/727042/2014 (H6N2) between nucleotide positions 101 and 583 and 700 and 1301, except for 583–700, which was most similar to A/duck/Hunan/S2046/2011 (H4N2) and A/muscovy_duck/Vietnam/LBM201/2012 (H3N2). These findings indicated that the NA gene of F352 was mainly derived from the N2 segments of the major parent A/duck/Zhejiang/727042/2014 (H6N2), but underwent reassortment with A/duck/Hunan/S2046/2011 (H4N2) and A/muscovy_duck/Vietnam/LBM201/2012 (H3N2) (Fig. 2D).

To estimate the rates of nucleotide or codon substitutions, we gathered sequences of currently available H3N2 subtype AIVs from NCBI and GISAID from January 2015 to March 2018 and divided them into two groups (isolates of 2015–2016 and 2017–018) for analysis by the uncorrelated relax-clock Bayesian Markov chain Monte Carlo method using BEAST v1.8.4.[8] BEAST analysis showed that the mean rates of HA and NA gene nucleotide substitutions during 2017–2018 were decreased when compared with 2015–2016 (Table 1). In addition, the mutation rates of second nucleotide of each codon within the open reading frame (ORF) of the HA gene was 0.188 during 2015–2016, but the rates were 0.454 during 2017–2018, which confirmed that the mutation rates had doubled (Table 1). Interestingly, most of the H3N2 subtypes of AIVs belonged to the Eurasion lineage and contained six potential N-glycosylation sites, including positions ^{24}NST^{26}, ^{38}NGT^{40},

Table 1
Rates of nucleotides substitution.

Gene	Mean rate of nucleotide substitution (substitution/per/year)			
	2015–2016	95% HPD*(E^{-3})	2017–2018	95% HPD*(E^{-3})
HA	5.048E^{-3}	[3.727–6.482]	3.644 E^{-3}	[2.004–5.676]
NA	3.666 E^{-3}	[3.044–4.324]	1.054 E^{-3}	[6.765E^{-4}–1.44E^{-3}]
	Mutation rate of codon nucleotide			
	Position*	2015–2016	2017–2018	
HA	1st	0.355	0.237	
	2nd	0.188	0.454	
	3rd	2.459	2.312	
NA	1st	0.39	0.272	
	2nd	0.22	0.223	
	3rd	2.393	2.507	

*HPD: highest probability density.
*Position: the three nucleotides of each codon.
E: scientific notation.

^{54}NAT^{56}, ^{181}NVT^{183}, ^{301}NGS^{303}, and ^{499}NGT^{501} in the HA protein; however, mutation of amino acids at the position 24N→S resulted in ^{24}NST^{26} N-glycosylation sites of some strains disappearing and a new ^{22}NDS^{24} site appearing in the HA1 protein since 2010. Strikingly, the codon of amino acids at position 24 had a one nucleotide (AAC) difference from the nucleotide (AGC) that leads to the mutation of 24N→S. This might have been related to the mutation rates of second nucleotide of each codon doubling with time, thereby changing the N-glycosylation sites of F138 and F352, which indicated viral replication and adaptation of H3N2 subtypes AIVs might have evolved substantially in the hosts.

In conclusion, AIVs with the H3N2 subtype will be ignored because of their low pathogenic and isolation rates. Our findings revealed that the HA and NA gene of F138 and F352 reassortant with the H3N2, H3N3, H4N2, H6N2 subtypes AIVs and the mean rates of second nucleotide of each codon of the HA gene had doubled. However, although the mean rates of the HA and NA gene nucleotide substitutions decreased, the reassortant process might have been affected by the frequent changes in antigenicity of other prevalent AIV strains so that better conditions are needed for the reassortant process in H3N2 subtypes of AIVs to proceed. In addition, consistent with Gao' research,[9] the binding affinity for the α(2–3)-linked sialic acid receptor of H7N9 subtype AIVs in 2013 was decreased, mainly due to the lack of N-glycosylation sites at amino acid position 150 in the HA protein. The H3N2 influenza virus has a wide host range and new N-glycosylation sites of F138 and F352 have been found, indicating that the H3N2 subtype AIVs might have attempted to adapt to different hosts. Importantly, H5N1 and H7N9 subtype AIVs have adapted to mammals and now pose a public health concern to mammals and humans. Hence, ongoing surveillance of H3N2 subtypes of AIVs in birds is warranted.

Conflicts of interest

The author declare no competing financial interests.

Acknowledgments

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Authors' contributions

Jingkai Hu, Yunfeng Zha and Xuanjiang Jin contributed equally to this work. Jingkai Hu, Xuanjiang Jin, Ming Liao and Weixin Jia conceived and designed experiments. Yunfeng Zha and Xiaohui Wen played an important role in sample collection. Jingkai Hu, Xuanjiang Jin, Zhixian Li, Xiao Wang, Yixue Dai, Xiao Li, Ming Liao, Weixin Jia performed the experiments. Jingkai Hu, Xuanjiang Jin Ming Liao and Weixin Jia conducted the phylogenetic and data analysis. Zhixian Li, Xiao Wang, Yixue Dai, Xiao Li, Ming Liao, Weixin Jia prepared and revised the figures. Jingkai Hu, Xuanjiang Jin, Zhixian Li, Xiao Wang, Yixue Dai, Xiao Li, Ming Liao, Weixin Jia wrote and modified the manuscript.

Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jinf.2019.01.009.

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Next-generation sequencing of the BALF in the diagnosis of community-acquired pneumonia in immunocompromised patients

Dear Editor,

We read with great interest the recent publication by Xie et al., addressing the issue of next-generation sequencing (NGS) for diagnosis of severe pneumonia in China.[1] They suggested that NGS might lead to more rapid and accurate diagnosis with better clinical prognosis than conventional detection methods in severe pneumonia in ICU. NGS is a novel method to DNA/RNA sequencing. Due to its high-throughput capacity and fast turnover time, NGS technology has been widely applied to the field of medical microbiology in recent years. NGS has been reported in the application for pathogen detection from blood, cerebrospinal fluid, tissue and intraoperative samples.[2–4] However, no studies have been reported from bronchoalveolar lavage fluid (BALF) samples in community-acquired pneumonia (CAP) patients with immunosuppression. Compared with the immunocompetent host, CAP can be potentially fatal and present significant challenges in immunocompromised host (ICH). In addition to common pathogens, some unusual organisms can cause severe infection in ICH. Besides, multiple concurrent infections are also common in ICH.[5] These characteristics pose a severe challenge to establish a specific diagnosis to guide therapy. A diagnostic tool should be pursued aggressively for favorable outcomes in this population. We recently explored the utility of NGS of bronchoalveolar lavage on the diagnosis of pneumonia in immunocompromised patients.

Thirteen immunocompromised patients with a diagnosis of communty-acquired pneumonia (CAP) were recruited from critical care department of Shanghai Ruijin Hospital. The diagnosis of CAP is based on the presence of select clinical features (e.g., cough, fever, sputum production, and pleuritic chest pain) and is supported by imaging of the lung, usually by chest radiography. Immunosuppression, defined as chemotherapy or neutropenia < 1000 μL during the past 28 days; treatment ≥ 20 mg corticosteroids daily for ≥ 14 days; human immunodeficiency virus infection; immunosuppressive therapy after organ or bone marrow transplantation; and active tuberculosis. Fiberoptic bronchoscopy was performed under local anesthesia with topical lidocaine for patients. BALF specimens using three aliquots of 20 mL of 0.9% saline were obtained. The first was discarded as recommended. The others were pooled and separated into two aliquots. One aliquot was sent to the microbiology lab for direct examination with Gram stain and culture. The other one was sent for DNA extraction and sequencing.

Standard procedures (SP) of BALF microbiologic testing were performed in all patients, which included quantitative cultures for bacteria, mycobacteria and fungi. In addition, multiplex PCR for influenza A/B, parainfluenza 1/2/3/4, coronavirus OC43, coronavirus 229E/NL63, respiratory syncytial virus A/B, adenovirus, human metapneumovirus, human rhinovirus, enterovirus and Human bocavirus was performed on BALF and nasopharyngeal. CMV, EBV, HSV-I, and HSV-II serology for IgG and IgM antibodies were measured.

Clinical characteristics including sex, age, APACHE II score at the time of diagnosis of CAP were demonstrated in Table 1. All patients were considered to be immunocompromised due to corticosteroids therapy or other immunosuppressive drugs.[6]

SP identified pathogens in 6 patients (6/13), four of which were bacteria, one fungi and one virus detected by clinical respiratory viral PCR panels (Table 2). It is worth noting that two samples positive for *Acinetobacter baumannii* by SP were considered co-infected

Table 1
Clinical characteristics of patients included.

Characteristics	13 patients
Age (years)	52 (46.5–71)
Female/male (n)	5/8
APACHEII score	23 (19–30.5)
Laboratory findings	
White blood cells (10^9 cells/L)	8.7 (5.3–12.1)
Lymphocytes (10^9 cells/L)	0.4 (0.2–0.99)
CRP (mg/L)	46.6 (19.5–89)
PCT (ng/ml)	1.03 (0.22–4.75)
$CD3^+$T cell count (cells/ul)	215 (141.5–564)
$CD3^+$T %	63.6 (51.75–81.65)
$CD3^+4^+$ T cell count (cells/ul)	97 (59.5–181.5)
$CD3^+4^+$ T %	31.4 (17.1–39.45)
$CD3^+8^+$ T cell count (cells/ul)	89 (63.5–201.5)
$CD3^+8^+$ T %	24.9 (21–42.9)
Radiologic findings	
Consolidation	7
Interstitial/patchy involvement	8
Immunodeficiency	
Nephrotic syndrome	3
Renal transplantation	2
Sjogren Syndrome	2
Adult onset Still's disease	1
Dermatomyositis	1
Rheumatic arthritis	1
Systemic vasculitis	1
Psoriasis	1

Table 2
Next-generation sequencing of BALF for the patients with pneumonia.

Case number	Pathogens identified by SP	Pathogens identified by NGS	Raw Reads	SMRN	Genomic coverage (%)
1	None	*Pneumocystis Jiroveci*	1883	1810	2.63
		Cytomegalovirus	4	4	6.09
2	None	*Pneumocystis Jiroveci*	2183	2060	2.85
		Cytomegalovirus	16	16	0.84
3	Human Bocavirus	Human Bocavirus	25	24	43
4	None	*Aspergillus Fumigatus*	848	608	0.3
		Cytomegalovirus	25834	24833	95
5	*Stenotrophomonas Maltophilia*	*Stenotrophomonas Maltophilia*	8641	6153	18
6	*Acinetobacter Baumannii*	*Acinetobacter Baumannii*	3322	2415	8.43
		Cytomegalovirus	4	4	0.20
7	*Candida Albicans*	*Candida Albicans*	30	28	0.03
8	None	*Pneumocystis Jiroveci*	96	95	0.15
9	None	Human Simple Virus I	26192	25472	95
10	*Acinetobacter Baumannii*	*Acinetobacter Baumannii*	1181	747	1.43
		Pneumocystis Jiroveci	3975	3791	2.32
11	None	*Pneumocystis Jiroveci*	105	103	0.06
12	*Acinetobacter Baumannii*	*Acinetobacter Baumannii*	186860	139486	85.44
13	None	None			

SMRN: strictly map reads number.

with PJP or CMV respectively by NGS. NGS detected pathogens in 12 patients (12/13) and one sample with non-infectious etiologies was confirmed by NGS (Table 2). Importantly, five PJP infections and one *Aspergillus fumigatus* infection were recognized by NGS. Moreover, among the six fungi-positive samples, CMV was also identified in three of them (two PJP and one *Aspergillus fumigatus*). Being relatively uncommon pathogens among the immunocompromised, PJP were identified in 5 cases and CMV in 4 cases by NGS. However, CMV serology for IgM were negative in these patients. No methenamine silver staining was performed in these cases, for the detecting item has not been carried out in our hospital. Overall, four bacteria, one fungus and one virus were detected by SP. By comparison, four bacteria, seven fungi and five viruses were identified by NGS (Table 2).

In terms of bacteria detection, NGS showed no significant advantages in this study. It is noteworthy that our study demonstrated NGS has a distinct advantage in the field of fungi and viral testing, especially PJP and CMV detection.

The definitive PJP diagnosis usually requires demonstration of fungus in tissue, BALF, or induced sputum samples. Methenamine silver staining and PCR in BALF samples have good sensitivity and specificity and is an emerging modality for diagnosis in the correct clinical setting.[7,8] There are a few laboratory tests to detect CMV infection.[7] Histopathologic analysis or viral culture from tissue, blood, urine, or respiratory specimens is less utilized because of poor sensitivity and longer time-consuming. CMV serology for IgG and IgM antibodies is another diagnostic tool but may be negative and inconclusive in immunocompromised hosts. The other methods such as PCR and pp65 antigenemia assays have not been carried out widely.[7,9] NGS technology showed its remarkable advantages in terms of opportunistic pathogens in patients with immunocompromised states.

In recent years, clinicians have been increasingly aware of the problem of mixed infection in immunocompromised hosts.[10] However, to diagnosis the co-occurrence of two commonly seen opportunistic infections is difficult in immunocompromised patients with lung infiltrates. It is worth noting the distinct advantage of NGS in concurrent detection of bacteria, fungi and viruses. In our study, standard procedures performed poorly in detecting mixed infections, whereas five cases with coinfection of bacteria, fungi or viruses were identified by NGS. This advantage may help clinicians more comprehensive evaluation of patients and make effective treatment.

In conclusion, our study explored the application of NGS in the microbiologic diagnosis in CAP patients with immunosuppression. NGS technology showed its remarkable advantages in detecting opportunistic pathogens and mixed infection in those patients.

Ethical approval

The study protocol was approved by the Ruijin Hospital Ethics Committee, Shanghai Jiaotong University School of Medicine, China. Formal consent was obtained from the patient or the next of kin.

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Conflicts of interest

The authors declare no conflicts of interest.

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