Quantitative risk analysis of the novel H7N9 virus in environments associated with H9 avian influenza virus, Zhejiang province, China

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SUMMARY

H9 avian influenza virus played a key role during generation of the novel H7N9 virus. A surveillance programme was conducted to assess the H9 virus in relation to the risk of H7N9 virus contamination in the environment. Risk of H7N9 virus contamination in the presence of H9 virus was higher than without (adjusted odds ratio 4·49, 95% confidence interval 3·79–5·31). Adjusted odds ratios of the H7N9 virus associated with co-presence of H9 virus and interacting factors were 4·93 (rural *vs.* urban area), 46·80 (live poultry markets *vs.* other premises), 6·86 (Huzhou *vs.* Hangzhou prefecture), 40·67 (year 2015 *vs.* 2013), and 9·63 (sewage from cleaning poultry *vs.* poultry faeces). Regular surveillance on gene variability of H7N9 and H9 viruses should be conducted and extra measures are needed to reduce co-circulation of H7N9 and H9 viruses in the environment.

Key words: H7N9 virus, H9 virus, reassortment, risk factor, surveillance.

INTRODUCTION

A novel reassortant avian influenza A(H7N9) virus was detected in China in February 2013 [1]. It led to 552 human infections and about 200 deaths by 23 February 2015 in mainland China [2]. The reported case numbers are still increasing [3]. Evidence supports the origination hypothesis of triple reassortment between H9N2 virus and H7 and N9 viruses [4–7].

The widespread incidence of H9N2 virus in Chinese poultry stock provides an ideal reassortment environment for H7N9 virus [8, 9]. Previous studies verified the contribution of H9 virus in the process of H7N9 virus evolution [10, 11]. However, to our knowledge, no study has assessed the role of H9 virus in relation to the risk of H7N9 virus contamination in the environment. In this study, we assessed the risk of H7N9 virus contamination in environments associated with H9 virus based on a surveillance programme from March 2013 to April 2015 in Zhejiang province, China.

METHODS

Specimen and data collection

A surveillance programme on H7N9 virus in poultry related to the environment has been in effect since the first H7N9 case emerged in March 2013 in Zhejiang province. The province consists of 11 prefectures, which are further divided into 90 counties and finally into 1329 towns. In each prefecture, one-third

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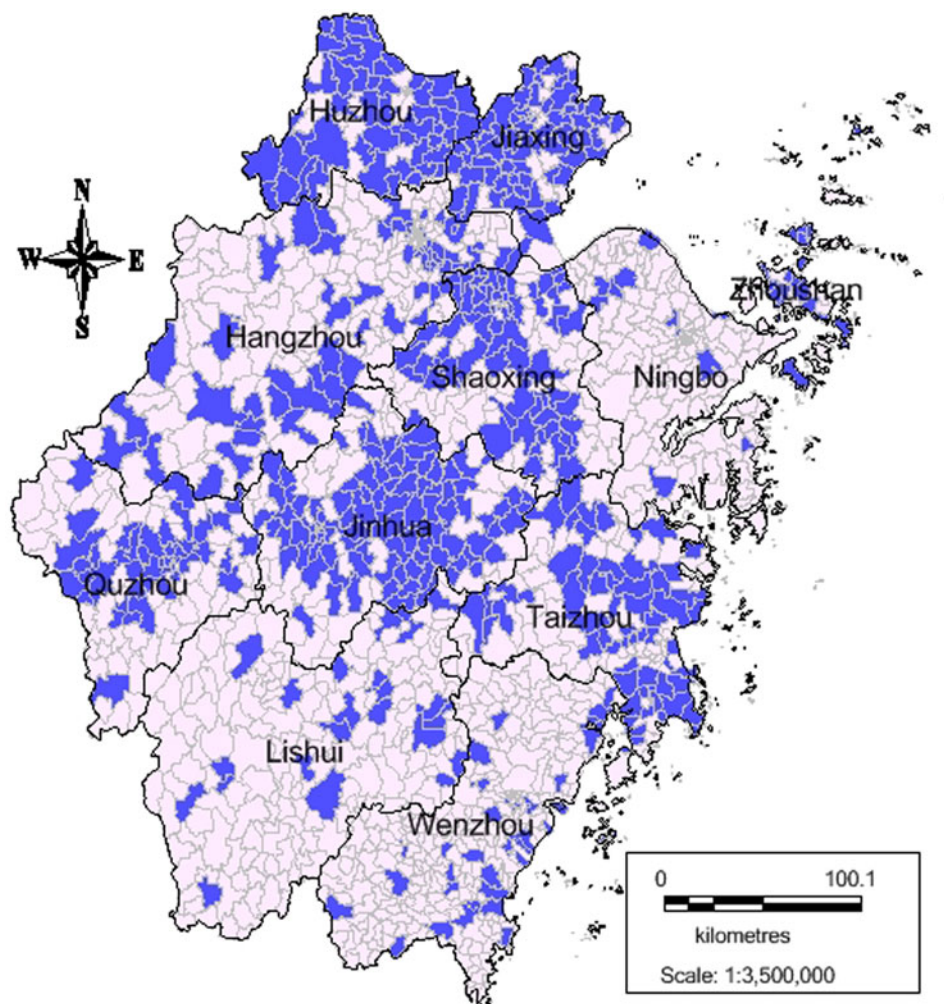† These authors contributed equally to this work.

Fig. 1. Distribution of towns surveyed for H7N9 virus in Zhejiang province, China, March 2013–April 2015.

of the counties were surveyed each month, thus all counties could be covered quarterly. As shown in Figure 1, 568 out of the 1329 towns in Zhejiang province were surveyed from March 2013 to April 2015.

The surveillance premises included live poultry markets, poultry-rearing farms, concentrated areas of backyard poultry farms, slaughtering and processing plants, habitats for migratory birds, and other poultry-related sites (such as living quarters of human H7N9 cases, restaurants, supermarkets, and markets for cold fresh poultry). In each prefecture, at least 30 specimens were collected monthly for each type of sample, which included poultry faeces, poultry cage swabs, drinking water for poultry, sewage from cleaning poultry, and swabs of tables used for slaughtering or holding poultry.

The number of H7N9 cases was collected from the China Information System for Disease Control and Prevention [12].

Laboratory testing

Viral RNA was extracted from specimens with the RNeasy mini kit (Qiagen, USA), and real-time RT–PCR was then performed with H7N9-specific primers and probes according to the manufacturer's protocol. The cycle threshold was defined as ⩽37·0. Real-time RT–PCR was performed at 11 local influenza network laboratories of Zhejiang province to detect H7, N9 and H9 viruses, and validated by the Zhejiang provincial influenza network laboratory [13, 14].

Statistical analyses

Student's t test was used to compare the mean positive rate of H7N9 virus between towns or premises with positive H9 virus detected and those with negative results. Fisher's exact test was used to compare the positive rates of H7N9 virus in different environmental specimens. Spearman's correlations were calculated for
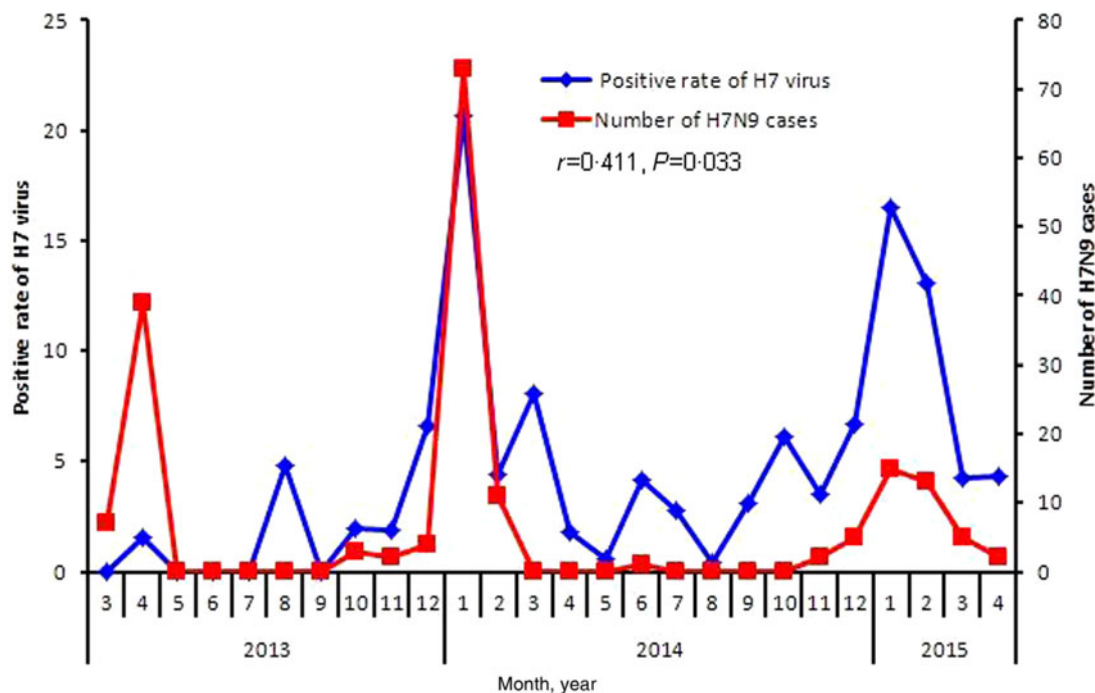
Fig. 2. The correlation of the positive rate of H7 virus and the number of H7N9 cases in Zhejiang province, China, March 2013–April 2015.

the positive rate of H7 virus and the number of H7N9 cases aggregated monthly. Multivariate logistic regression was used to calculate the adjusted odds ratios (aOR), with 95% confidence intervals (CI) as a measure of association between the H9 and H7N9 viruses. Stratified analyses and interaction terms were used to examine the effect of modification of H9 virus in areas, premises, prefectures, surveillance years and sample types. All analyses were performed with PASW software v. 18.0.0 (http://pasw.en.malavida.com/).

RESULTS

Of 568 towns under surveillance in Zhejiang province from March 2013 to April 2015, 207 tested positive for H9 virus and 175 for H7N9 virus. The mean positive rate of H7N9 virus in towns positive for H9 virus (10·87%) was higher than for those without H9 virus (3·80%) ($t = -5·418$, $P < 0·001$). Of 1723 premises distributed in the 568 towns surveyed, 369 tested positive for H9 virus and 248 for H7N9 virus. The mean positive rate of H7N9 virus was 12·12% in premises positive for H9 virus and 2·84% for those that were negative ($t = -7·852$, $P < 0·001$).

Of 16 275 environmental specimens collected, 1400 (8·6%) and 959 (5·9%) tested positive for H7 and H7N9 viruses, respectively. The number of H7N9 cases counted

monthly was significantly correlated with the positive rate of H7 virus ($r = 0·411$, $P = 0·033$) (Fig. 2). The positive rates of H7N9 virus were much higher in specimens acquired in 2015, from live poultry markets, in Hangzhou and Huzhou prefectures, in sewage used for cleaning poultry and on tables used for slaughtering or holding poultry (detailed information of H7N9 virus for all specimens summarized by year, prefecture and sample types provided in Supplementary Table S1). Moreover, the positive rate of H7N9 virus in environmental specimens with positive H9 virus was over four times higher than for those that were negative (Table 1).

Associations between characteristics of environmental specimens and H7N9 virus contamination are shown in Table 2. When we analysed premises and prefectures, we combined some categories with similar H7N9-positive rates because of their small number. Multiple logistic analyses showed that type of premises, prefecture, surveillance year and sample type were confounding factors in the association between H9 and H7N9 viruses in environmental specimens. There was a significant association between H9 and H7N9 viruses (aOR 4·49, 95% CI 3·79–5·31) after adjustment for these confounding factors.

As shown in Table 3, a significant multiplicative interaction ($P < 0·001$) was found between area and H9 virus, with nearly a fivefold increased risk of

Table 1. *Positive rates of the H7N9 virus in different environmental specimens, Zhejiang Province, China, March 2013–April 2015*

Characteristic	No. of total specimens	No. of positive specimens	Positive rate (%)	*P* value
Area type				
Urban	5832	329	5·6	0·314
Rural	10 443	630	6·0	
Premises type				
Live poultry markets	10 034	886	8·8	<0·001
Poultry-rearing farms	2703	6	0·2	
Concentrated areas of backyard poultry farms	1964	2	0·1	
Slaughtering and processing plants	398	12	3·0	
Habitats for migratory birds	346	0	0·0	
Others	830	53	6·4	
Prefecture				
Hangzhou	1150	153	13·3	<0·001
Huzhou	1518	284	18·7	
Jiaxing	2085	108	5·2	
Jinhua	2721	238	8·7	
Lishui	2072	72	3·5	
Ningbo	717	0	0·0	
Quzhou	1017	40	3·9	
Shaoxing	1633	0	0·0	
Taizhou	958	35	3·7	
Wenzhou	1192	9	0·8	
Zhoushan	1212	20	1·7	
Year				
2013	2490	32	1·3	<0·001
2014	9036	391	4·3	
2015	4749	536	11·3	
Sample types				
Poultry faeces	6875	317	4·6	<0·001
Poultry cage swab	3358	206	6·1	
Drinking water for poultry	1646	70	4·3	
Sewage from cleaning poultry	1354	114	8·4	
Swabs of tables for slaughtering or holding poultry	1961	186	9·5	
Others	1081	66	6·1	
H9 subtype				
Positive	1657	379	22·9	<0·001
Negative	14 618	580	4·0	

H7N9 contamination in environments of rural areas positive for H9 virus compared to urban areas with no H9 virus detected (aOR 4·93, 95% CI 3·90–6·23). The expected aOR for the combination of rural area and H9 virus was 3·40 in the additive model and 3·25 in the multiplicative model.

There was also significant multiplicative interaction (*P* < 0·001) between the premises and H9 virus. The risk of H7N9 virus contamination associated with the combination of H9 virus and live poultry markets (aOR 46·80, 95% CI 31·83–68·81), was higher than that expected in the additive model (aOR 34·10) and

was one-sixth of that expected in the multiplicative model (aOR 280·26).

In prefectures, we also identified a significant multiplicative interaction (*P* < 0·001). The OR of H7N9 virus contamination in environments with H9 virus in Huzhou prefecture was nearly seven times that in environments without H9 virus in Hangzhou prefecture (aOR 6·86, 95% CI 4·61–10·21). The estimate for the combination of Huzhou prefecture and H9 virus was more than twice that expected from adding (aOR 2·94) and multiplying (aOR 2·94) the independent associations.

Table 2. *Odds ratios for associations between environmental specimens and H7N9 virus contamination, Zhejiang province, China, March 2013–April 2015*

Characteristic	Univariate analysis			Multiple analysis		
	cOR	95% CI	*P* value	aOR	95% CI	*P* value
Area type (ref. urban)						
Rural	0·93	0·81–1·07	0·31	0·94	0·79–1·11	0·45
Premises type (ref. other premises)						
Live poultry markets	8·18	6·43–10·41	<0·001	6·92	5·33–8·99	<0·001
Prefecture (ref. Hangzhou)			<0·001			<0·001
Huzhou	1·50	1·21–1·86	<0·001	1·21	0·93–1·59	0·16
Jiaxing	0·36	0·28–0·46	<0·001	0·40	0·30–0·53	<0·001
Jinhua	0·62	0·50–0·78	<0·001	0·51	0·39–0·66	<0·001
Lishui, Quzhou, Taizhou	0·25	0·19–0·31	<0·001	0·14	0·11–0·18	<0·001
Ningbo, Shaoxing, Wenzhou, Zhoushan	0·04	0·03–0·06	<0·001	0·04	0·03–0·07	<0·001
Year (ref. 2013)			<0·001			<0·001
2014	3·47	2·42–5	<0·001	3·62	2·49–5·26	<0·001
2015	9·77	6·82–14·01	<0·001	10·38	7·11–15·15	<0·001
Sample type (ref. poultry faeces)			<0·001			<0·001
Poultry cage swab	1·35	1·13–1·62	<0·001	1·47	1·19–1·82	<0·001
Drinking water for poultry	0·92	0·71–1·2	0·53	1·51	1·11–2·05	0·01
Sewage from cleaning poultry	1·90	1·52–2·38	<0·001	2·12	1·64–2·74	<0·001
Swabs of tables for slaughtering or holding poultry	2·17	1·80–2·62	<0·001	1·69	1·36–2·10	<0·001
Others	1·35	1·02–1·77	0·03	2·68	1·96–3·68	<0·001
H9 subtype (ref. negative)						
Positive	7·18	6·23–8·27	<0·001	4·49	3·79–5·31	<0·001

cOR, Crude odds ratio; aOR, adjusted odds ratio; CI, confidence interval.

Risk of H7N9 virus contamination in environments with H9 virus in 2014 and 2015 was significantly higher than in those without H9 virus in 2013; the aORs were 7·73 (95% CI 5·03–11·87) and 40·67 (95% CI 27·11–61·02), respectively. The expected aORs for environments positive for H9 virus in 2014 and 2015 were 1·58 and 4·77 in the additive models, respectively, and were both zero in the multiplicative models.

Regardless of sample type, the aORs of H7N9 virus for positive H9 specimens were much higher than for negative H9 specimens. When compared to poultry faeces without H9 virus, the aOR of H7N9 virus contamination increased from 5·58 to 26·15 across all specimen types with H9 virus.

DISCUSSION

To our knowledge, this study was the first to assess the association of H9 virus with the risk of H7N9 virus contamination in the environment. We found that environments positive for H9 virus had significantly higher risk of H7N9 virus contamination than those that were not. Risk of H7N9 virus contamination associated with positive H9 virus was much greater in 2015 and in rural areas, live poultry markets, Huzhou prefecture and sewage used for cleaning poultry.

H9 virus plays an important role in the reassortment of H7N9 virus in China [7, 15]. The co-existence of H9 and H7N9 viruses in an environment will generate appropriate conditions for reassortment, leading to rapid evolution of H7N9 virus, which in turn enables it to change its genetic architecture rapidly, increasing viability in the environment and transmissibility in poultry or humans. The six internal genes of H7N9 virus, which originate from the H9N2 virus that circulates in chickens in eastern China [7, 16, 17], might affect the infectivity of H7N9 virus [18]. A recent study showed that a genotype (G57) of H9N2 virus had changed antigenicity and improved adaptability to chickens, which facilitated the genesis of the H7N9 virus [5]. Our study found that the presence of H9 virus was associated with an almost fivefold increased risk of H7N9 virus contamination in the environment. This implies that we might be able to predict epidemics caused by H7N9 virus by monitoring the prevalence and variation of H9 virus

Table 3. *Odds ratios for H7N9 virus according to H9 virus and characteristics of the environment, Zhejiang province, China, March 2013–April 2015*

Characteristics	H9(−)					H9(+)				
	H7N9(−)	H7N9(+)	aOR	95% CI	*P* value	H7N9(−)	H7N9(+)	aOR	95% CI	*P* value
Area type										
Urban	4871	227	1·00	Ref.		632	102	3·46	2·64–4·53	<0·001
Rural	9167	353	0·94	0·77–1·14	0·529	646	277	4·93	3·90–6·23	<0·001
Premise type										
Live poultry markets	8131	548	12·39	8·57–17·9	<0·001	1017	338	46·80	31·83–68·81	<0·001
Other premises	5907	32	1·00	Ref.		261	41	22·62	13·31–38·44	<0·001
Prefecture										
Hangzhou	919	107	1·00	Ref.		78	46	2·94	1·87–4·61	<0·001
Huzhou	1151	166	1·00	0·74–1·35	0·988	83	118	6·86	4·61–10·21	<0·001
Jiaxing	1938	93	0·34	0·25–0·47	<0·001	39	15	2·66	1·3–5·44	0·007
Jinhua	2135	95	0·40	0·3–0·55	<0·001	348	143	2·36	1·72–3·22	<0·001
Lishui, Quzhou, Taizhou	3385	97	0·17	0·12–0·23	<0·001	515	50	0·35	0·24–0·52	<0·001
Ningbo, Shaoxing, Wenzhou, Zhoushan	4510	22	0·04	0·02–0·06	<0·001	215	7	0·28	0·12–0·63	0·002
Year										
2013	2259	32	1·00	Ref.		199	0	0·00		0·995
2014	7987	292	2·58	1·77–3·76	<0·001	658	99	7·73	5·03–11·87	<0·001
2015	3792	256	5·77	3·93–8·48	<0·001	421	280	40·67	27·11–61·02	<0·001
Sample type										
Poultry faeces	6168	187	1·00	Ref.		390	130	6·51	4·92–8·63	<0·001
Poultry cage swab	2927	142	1·80	1·41–2·3	<0·001	225	64	5·58	3·89–7·99	<0·001
Drinking water for poultry	1418	38	2·00	1·37–2·91	<0·001	158	32	6·00	3·8–9·47	<0·001
Sewage from cleaning poultry	1080	63	2·52	1·83–3·46	<0·001	160	51	9·63	6·49–14·28	<0·001
Swabs of tables for slaughtering or holding poultry	1499	105	2·00	1·54–2·62	<0·001	276	81	7·58	5·42–10·59	<0·001
Others	946	45	2·58	1·79–3·72	<0·001	69	21	26·15	13·6–50·26	<0·001

aOR, Adjusted odds ratio; CI, confidence interval.

[5]. Moreover, the risk of H7N9 virus prevalence could also be decreased by reducing the amount of H9 virus in the environment.

Our study showed that the combination of a rural area and H9 virus was associated with an almost fivefold increased risk of H7N9 virus contamination in the environment. We also found that the H7N9 virus risk associated with rural areas was null in environments with no H9 virus detected. This indicates that the risk of H7N9 virus contamination in rural areas is the same as in urban areas if the H9 virus is absent. This interaction effect implies that H9 virus control is helpful for H7N9 virus control in rural areas.

Evidence based on the exposure histories of cases [19, 20] and similarity of gene sequences [21] supports the conclusion that live poultry markets are the main sources of H7N9 virus infections in humans [22]. In China, the special environment of live poultry markets, including the polyculture of a number of hosts in a high-density setting, provides ideal conditions for reassortment and transmission of the H7N9 virus [23]. Interestingly, our study shows that the independent risk of H7N9 virus contamination associated with H9 virus is much higher than that associated with live poultry markets, and that the combination of H9 virus and live poultry market led to a 47-fold increased risk of H7N9 virus contamination in the environment. If this finding can be replicated, it would mean more effort is needed to monitor the prevalence of H9 virus and to explore the effects of measures to reduce the levels of H9 virus in the environment.

Zhejiang province was one of the worst-hit areas of the H7N9 epidemic in China. Huzhou prefecture in Zhejiang province, located in the geographical centre of the Yangtze River Delta region, is considered one of the regions of H7N9 virus origin [24]. Our study indicated that H9 virus in the environment in Huzhou prefecture led to a high risk of H7N9 virus. The co-circulation of avian influenza virus subtypes H7, H9, N2 and N9 in poultry in Huzhou prefecture provides an ideal dynamic environment for the ongoing generation of H7N9 virus [25, 26]. The risk of H7N9 virus contamination in the environment in Huzhou prefecture could be similar to that in Hangzhou prefecture, neglecting the influence of H9 virus.

Evolutionary temporal dynamics suggested that there were at least two rounds of reassortment events during the generation of H7N9 virus. The N9 gene was first reinserted into the H9N2 genome. The H7 gene was then reassorted into the genome of the reassortant intermediate H9N9 virus [25]. Benefiting from the short cycle of reassortment and the wide range of co-existence for H9 and H7 viruses, the amount of H7N9 virus in the environment increased rapidly. This study found a rapid increase in risk for H7N9 virus from 2013 to 2015 in environments with H9 virus. Moreover, this grave situation could be exacerbated if this co-circulation of H9 and H7 viruses continues without control. Unfortunately, due to absence of detection of the N2 gene in the surveillance programme, we were unable to learn the distribution of the parental H9N2 virus and the evolutionary intermediate of the H9N9 virus, which restricted a detailed assessment of the evolutionary consequence of co-circulation of H7N9 and H9N2 viruses.

In summary, this study provided a quantitative association between H9 virus and increased risk of H7N9 virus contamination in the environment. The prevalence of H9 virus in the environment will significantly increase the risk of H7N9 virus. Moreover, it might continue to be a source of further genetic diversity for H7N9 viruses [27]. In light of these findings, we recommend that the gene variability of the H7N9 and H9 viruses should be constantly monitored. Moreover, effective measures are required to control environments favouring reassortment of H7N9 virus, especially those with co-circulation of H7N9 and H9 viruses.

SUPPLEMENTARY MATERIAL

For supplementary material accompanying this paper visit http://dx.doi.org/10.1017/S0950268816002168.

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DECLARATION OF INTEREST

None.

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