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International linkage of two food-borne hepatitis A clusters through traceback of mussels, the Netherlands, 2012

IL Boxman [1], L Verhoef [2], H Vennema [2], S Ngui [3], IH Friesema [4], C Whiteside [5], D Lees [6], M Koopmans [2,7]

1. Laboratory for Feed and Food Safety, Food and Consumer Product Safety Authority (NVWA), Wageningen, the Netherlands
2. Laboratory for Infectious Diseases and Screening, National Institute of Public Health and the Environment (RIVM), Bilthoven, the Netherlands
3. Virus Reference Department, Microbiology Services Division - Colindale, Public Health England, London, United Kingdom
4. Epidemiology and Surveillance Unit, Centre for Infectious Disease Control, National Institute for Public Health and the Environment, Bilthoven, the Netherlands
5. Public Health Wales, United Kingdom
6. Centre for Environment, Fisheries and Aquaculture Science, Weymouth, United Kingdom
7. Department of Viroscience, Erasmus MC, Rotterdam, the Netherlands

Correspondence: Ingeborg L.A. Boxman (i.l.a.boxman@nvwa.nl)

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This report describes an outbreak investigation starting with two closely related suspected food-borne clusters of Dutch hepatitis A cases, nine primary cases in total, with an unknown source in the Netherlands. The hepatitis A virus (HAV) genotype IA sequences of both clusters were highly similar (459/460 nt) and were not reported earlier. Food questionnaires and a case–control study revealed an association with consumption of mussels. Analysis of mussel supply chains identified the most likely production area. International enquiries led to identification of a cluster of patients near this production area with identical HAV sequences with onsets predating the first Dutch cluster of cases. The most likely source for this cluster was a case who returned from an endemic area in Central America, and a subsequent household cluster from which treated domestic sewage was discharged into the suspected mussel production area. Notably, mussels from this area were also consumed by a separate case in the United Kingdom sharing an identical strain with the second Dutch cluster. In conclusion, a small number of patients in a non-endemic area led to geographically dispersed hepatitis A outbreaks with food as vehicle. This link would have gone unnoticed without sequence analyses and international collaboration.

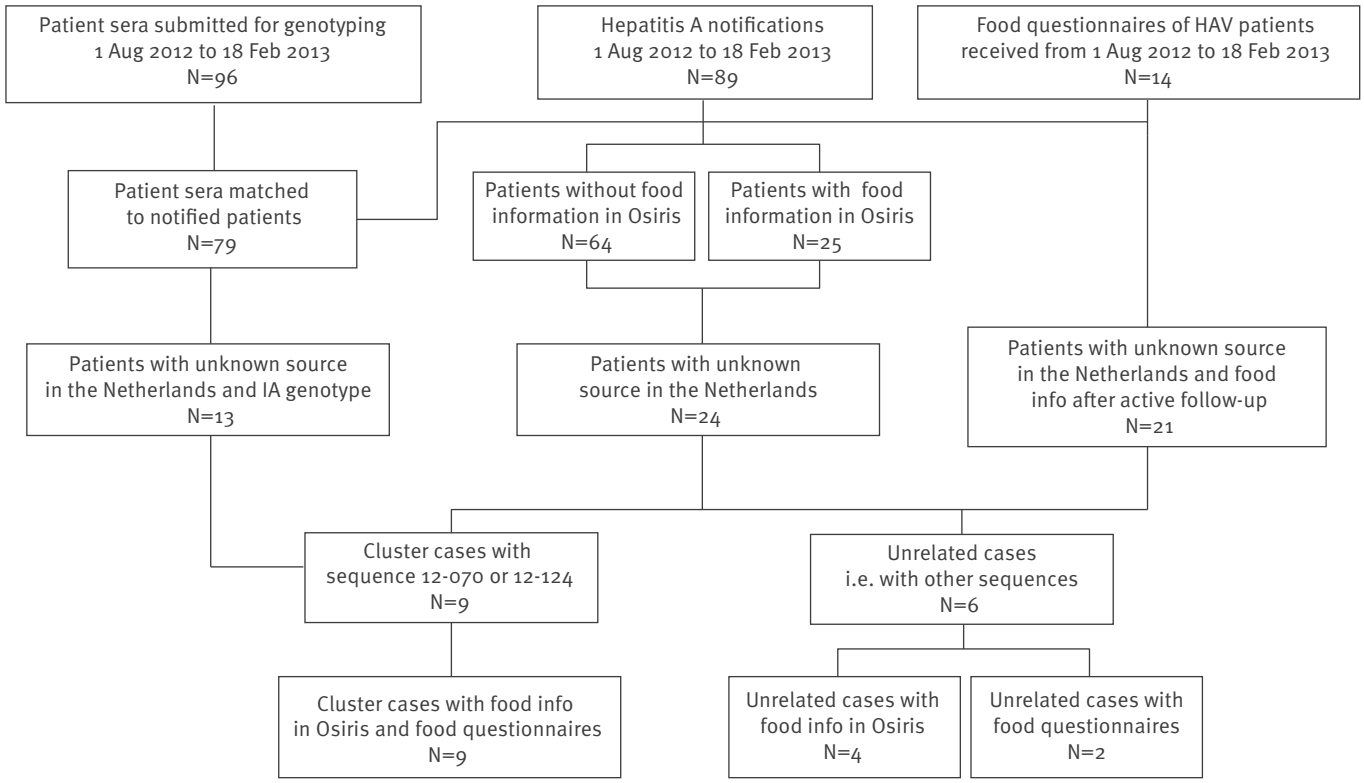
Introduction

Hepatitis A virus (HAV) is a faecal-orally transmitted pathogen causing acute self-limiting hepatitis. Risk factors for infection include exposure to infected persons, contact with faecally contaminated surfaces, food or water. The incidence of hepatitis A in industrialised countries has decreased due to improved sanitary and living conditions. The decreasing immunity in younger age groups has led to increasing age and severity of first infection [1]. Hepatitis A could therefore re-emerge in regions such as North America or western Europe, where it is not endemic, affecting mostly adults, with more severe course of infection [1]. In the Netherlands the incidence rate for hepatitis A in 2010 was 1.3 cases per 100,000 population [2]. Hepatitis A is a notifiable disease in the Netherlands. Cases, including routine demographic and epidemiological data, are reported according to standardised national criteria [3] by regional public health services using Osiris, a national electronic registration system for infectious diseases hosted by the National Institute for Public Health and the Environment (RIVM). Surveillance is intensified for cases with no travel history to endemic countries and an unknown source of infection. For these cases, sequences are actively collected and additional hypothesis-generating (trawler) questionnaires are routinely administered, aiming to identify a potential common (food-borne) source of infection [2-4].

Recently, food-borne transmission of HAV has been implicated in several multinational outbreaks or involving multiple states in the United States (US), occurring in rapid succession in 2013 and 2014 [5-8]. Such diffuse outbreaks that are characterised by cases that are geographically or temporally dispersed benefit from combining epidemiological data with viral sequence data. Finding the source is challenging due to the long incubation period of two to six weeks [9]. Recall can be assisted by the use of structured questionnaires including known risk factors or risk products [3,4]. Absolute confirmation of a food source is rare, in part due to difficulties with detection of the viral RNA in

Figure 1

Flowchart of available case data from different sources of information, food-borne hepatitis A clusters, the Netherlands, 2012



HAV: hepatitis A virus.

Osiris is the electronic registration system for infectious diseases hosted by the Dutch National Institute for Public Health and the Environment.

food or the absence of leftovers, while international traceback of food can be complex [8]. The high numbers of notifications in the Rapid Alert System for Food and Feed (RASFF) Portal website related to the presence of norovirus or hepatitis A virus in food in 2013 and 2014 compared with previous years indicate an increased awareness of the importance of food-borne viruses.

In this study we describe a thorough outbreak investigation by multiple institutes in two European Union (EU) Member States that yielded evidence for a common source of infection for nine Dutch hepatitis A patients and one patient in the United Kingdom (UK). The investigation was initiated after identification of a cluster consisting of four seemingly unrelated Dutch hepatitis A cases with an identical but unique HAV sequence (genotype IA) in August 2012. In November 2012, another cluster of seemingly unrelated hepatitis A cases, again with an identical HAV IA sequence, was identified. The HAV IA sequence of the second cluster was closely related (459/460 nt similarity) to the strain that caused the first cluster. The source was assumed to be food-borne, as hepatitis A is not endemic in the Netherlands, there was no known direct contact between the cases, and detailed case histories were negative for other common risk factors of hepatitis A. The onset of illness in each cluster occurred within a short period of two weeks with no recognised cases in the two to three month period in between these clusters. The final linkage between the Dutch and UK cases could only be conclusively made after combining traceback results of the suspected food items for each of the clusters and information provided by international partners.

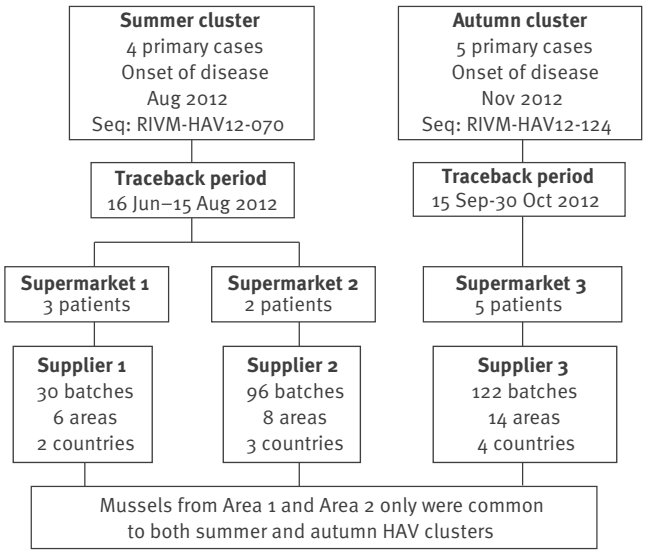
Methods

Active case finding by laboratory surveillance network

For cases with no travel history to endemic countries and an unknown source, additional routine hypothesis-generating questionnaires were administered. This questionnaire addresses a broad list of over 70 food products, 15 occupations and six health conditions, and is available upon request [3,4]. IgM positive sera or faecal samples from ca 70% of confirmed hepatitis A cases are sent by diagnostic laboratories to RIVM

Figure 2

Outcomes of traceback activities for potentially implicated mussels, food-borne hepatitis A clusters, the Netherlands, 2012



for genotyping [3]. Subsequently, a 460 nt fragment of the VP1/2A region [10,11] is compared with sequences recorded in an international HAV sequence database of HAVNET and GenBank. HAVNET is a global network of scientists working in hepatitis A reference laboratories. The network shares molecular and epidemiological data on hepatitis A.

Case definition hepatitis A cases in the Netherlands registered between 1 August 2012 and 18 February 2013 with an unknown source in the Netherlands were classified as follows: (i) summer cluster cases were cases infected by HAV strain RIVM-HAV12–070; (ii) autumn cluster cases were cases infected by HAV strain RIVM-HAV12–124; (iii) unrelated cases were unvaccinated persons infected by a HAV strain dissimilar from RIVM-HAV12–070 and RIVM -HAV12–124 with difference of at least 7/460 nt; (iv) Other cases: cases for which no HAV sequence is available (not included in the analysis).

Statistical analyses of risk factors from questionnaire data

The food items addressed in the hypothesis-generating food questionnaire or in the Osiris system were transformed to binary variables, i.e. consumed two to six weeks before disease onset vs not consumed or consumed longer ago than six weeks before disease onset. Using a univariate logistic regression model (SAS version 9.3, SAS Institute Inc, USA) outcomes for HAV-cluster-cases were compared with unrelated cases. The significance of the identified food items was further investigated using data on consumption patterns in healthy people unrelated to any outbreak [12]. For this, data were collected in the flexible Dutch population control group over a four-year period from 2008 to 2011 (n = 1,402).

Traceback investigation

Fresh mussel suppliers to specific supermarket chains, as derived from questionnaire data, were identified as well as the harvesting areas of mussels potentially involved, and the shipping dates to the Netherlands, when applicable. Information on shipments was requested from the Dutch Fish Product Board for the period six weeks before disease onset in the first patient until two weeks before disease onset in the last patient for both clusters. The registration documents of the mussel consignments revealed the number of batches, harvesting areas and countries for each supplier during these two periods.

International enquiry

Enquiries for any information on possible matches with the unique patient sequences were sent out to international contact points within the HAV reference laboratory network (HAVNET sent out enquiries on 15 January 2013 and 7 June 2013) and the international network Epidemic Intelligence Information System of Food and Waterborne Diseases (EPIS-FWD, 15 January 2013 and 13 December 2013) of the European Centre for Disease Prevention and Control (ECDC). Additional enquiries for any information on possible matches with the unique patient sequences or on any information on HAV monitoring in shellfish were sent out to national contact points (3 June 2013 and 31 October 2013) and shellfish reference laboratories (26 February 2013) in countries identified to be possibly involved. Furthermore, the RASFF portal database was checked for HAV notifications in 2012.

Results

Descriptive epidemiology

From 1 August 2012 to 18 February 2013, 89 hepatitis A cases were reported to the RIVM (Figure 1). Of these, 24 cases acquired their infection from an unknown source in the Netherlands as no travel history had been reported. The remaining 65 cases were reported with travel history to endemic regions, or with an epidemiological link to a confirmed case with recent travel history to an endemic region. In the same period, the RIVM received 79 samples from notified cases for typing. In total, 62 strains could be successfully typed, and 15 of these were from hepatitis A cases with an unknown infection source within the Netherlands. These 15 cases diverged into the summer and autumn clusters, each with an identical sequence which was further investigated in this study, and six unrelated cases with dissimilar sequences (i.e. unrelated cases).

The summer cluster of cases infected with RIVM-HAV12–070 consisted of three male and one female primary cases, with ages ranging from under 10 years to under 70 years of age, and onset of illness within 10 days in August 2012. One primary case needed hospitalisation. In addition, two secondary cases were reported, both relatives of primary cases.

Figure 3

Timeline of disease onset with cases and relevant harvesting periods, food-borne hepatitis A clusters, the Netherlands, 2012

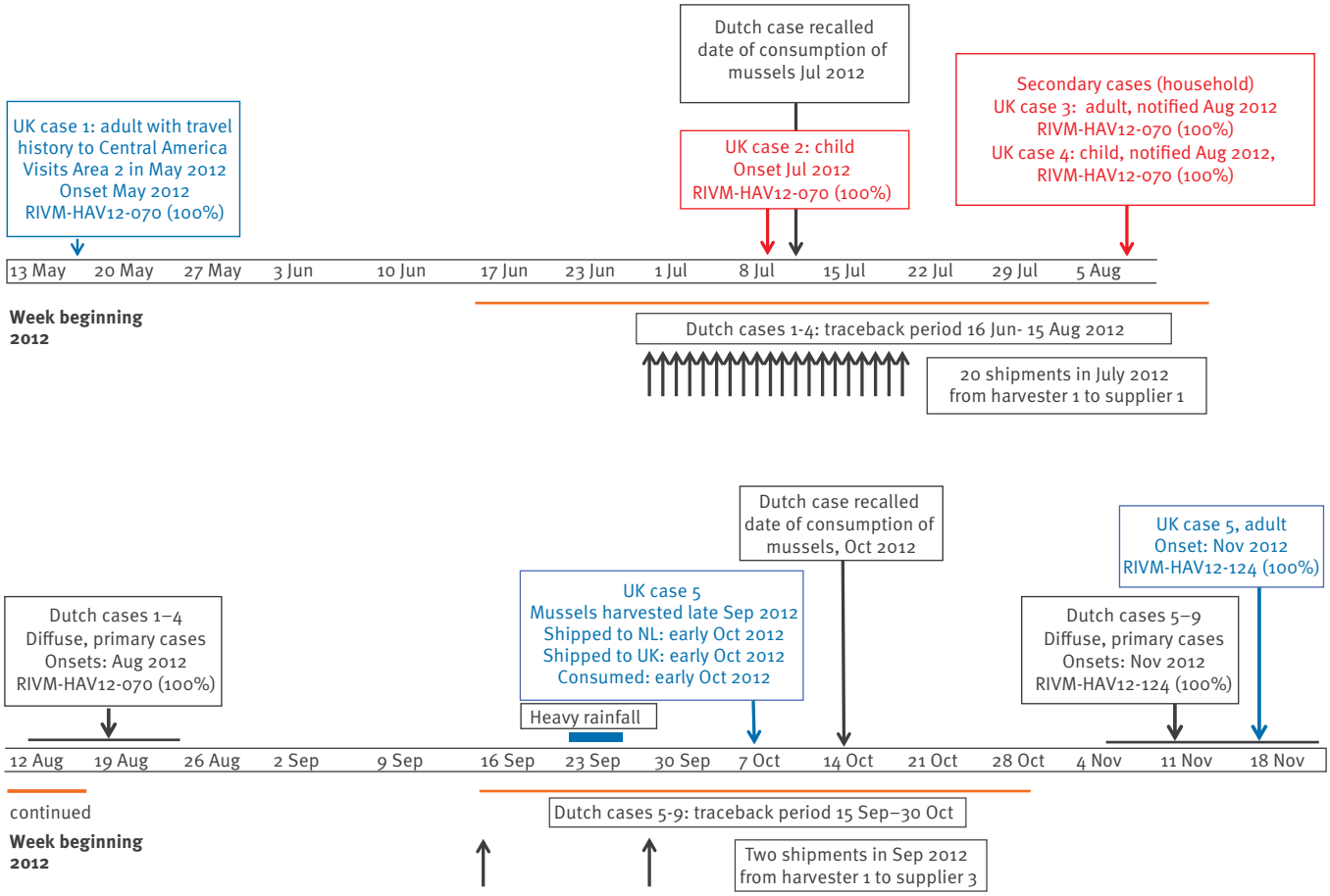


Table 1

Food items consumed six weeks before the onset of disease by confirmed hepatitis A cluster (n=9) and unrelated (n=6) cases, the Netherlands, 2012

Food item	OR (95% CI)	P value	Unrelated cases		Cluster cases	
			Yes	No	Yes	No
Food questionnaire			(4 missing)		(0 missing)	
Mussels	>100 (0.0–999)	p=0.99	0	2	9	0
Spring onions, raw	>100 (0.0–999)	p=0.99	0	1	9	0
Onions, raw	>100 (0.0–999)	p=0.99	0	1	9	0
Prawns	8.0 (0.3–256)	p=0.24	1	1	8	1
Iceberg lettuce	8.0 (0.3–256)	p=0.24	1	1	8	1
Leafy green lettuce	7.0 (0.2–226)	p=0.27	1	1	7	1[a]
Osiris notification			2 missing		0 missing	
Mussels	>100 (0.0–999)	p=0.99	0	4	9	0
Combined food questionnaire and Osiris			2 missing		0 missing	
Mussels	>100 (0.0–999)	p=0.99	0	4	9	0
Iceberg lettuce	24.0 (1.1–519)	p=0.04	1	3	8	1
Leafy green lettuce	8.0 (0.5–139)	p=0.15	2	2	8	1

CI: confidence interval; OR: odds ratio.

Osiris is the electronic registration system for infectious diseases hosted by the Dutch National Institute for Public Health and the Environment.

[a] Leafy lettuce data missing for one of the cluster cases.

Table 2

Food items consumed during past 4 weeks per calendar month by a flexible Dutch population control group, the Netherlands, June 2008–December 2011 (n=1,402)

Month of report	Mussels n/N %[a]	Prawns n/N %[a]	Lettuce n/N %[a]
January	2/9 (22)	4/9 (44)	8/8 (100)
February	12/82 (15)	22/81 (28)	71/86 (83)
March	14/129 (11)	36/132 (27)	116/139 (83)
April	17/108 (16)	36/113 (32)	100/116 (86)
May	0/11 (0)	6/11 (55)	10/11 (91)
June	12/201 (6)	74/202 (37)	178/208 (86)
July	13/156 (8)	37/162 (23)	154/167 (92)
August[b]	32/125 (26)	51/126 (40)	111/121 (92)
September	5/23 (22)	14/23 (61)	19/23 (83)
October	29/184 (16)	59/183 (32)	165/189 (88)
November[b]	13/143 (9)	44/145 (30)	121/151 (80)
December	14/138 (10)	43/139 (30)	108/131 (82)

[a] Data missing for mussels (n=93), prawns (n=76) and lettuce (n=52).

[b] Data reported for August and November (i.e. consumption during past four weeks) to be compared with data reported for cases during their incubation period.

The autumn cluster cases were infected with RIVM-HAV12–124 and consisted of two male and three female primary cases, with ages ranging from over 40 years to under 70 years of age, and onset of illness within 12 days in November 2012.

Six unrelated cases (four males and two females) with ages ranging between 20 and 60 years-old were infected by HAV strains dissimilar from RIVM-HAV12–070 and RIVM-HAV12–124. It is assumed that they are primary cases, since their sequences were not seen in previous cases. Dates of onset of disease were between early August and late December 2012.

Phylogenetics and international enquiry

The RIVM-HAV12–070 and -124 were both unique in the international HAV sequence database of HAVNET and the origin of the virus could not be mapped to a specific geographic region [3, and data not shown]. Only four EU Member States out of 38 EPIS-FWD contact points responded to international enquiries reporting non-related cases or not to have matching sequences. Furthermore, three RASFF notifications on HAV were identified in 2012. One notification was related to mussels from New Zealand that had only been distributed to Italy.

Two others were related to the same batch of frozen strawberry cubes from China. Traceback activities to find a possible link to the cluster cases indicated that strawberries from this batch had been on the Dutch market partly in the same period of the incubation period of the patients. The strawberries had been used as decoration for consumer-ready packed ice cream sold by one of the specific supermarket chains identified by two of the six cases. Unfortunately, no sequence information for the HAV strain detected on the frozen sliced strawberries was available.

Risk factors from questionnaire data

From the questionnaire data available, mussels, raw spring onions, raw onions, prawns, iceberg lettuce and leafy green lettuce were recalled by at least eight of the nine cases as having being consumed within the incubation period. Univariate logistic regression analyses showed clear association for mussels, prawns and iceberg lettuce (Table 1). All of the cluster cases had consumed pre-packed fresh mussels (all of which were heat-treated in the home before consumption), raw spring onions and raw onions, compared with none of the unrelated cases, not allowing statistical calculations.

In the period before the onset of illness, hepatitis A cluster cases were found to have consumed mussels significantly more often than individuals in the flexible control group (Table 2). In the two to six weeks before the onset of illness in August and November 2012, all nine hepatitis A cluster cases consumed mussels, compared with 32 of 125 (26%) and 13 of 143 (9%) for control groups during the 4 weeks before August and November 2012, respectively. Prawns were also consumed more often by cluster cases i.e. 8/9 (89%) compared with 51/126 (40%) and 44/145 (30%) for control groups during the four weeks before August and November 2012, respectively. Lettuce, however, was consumed by over 80% of the control group throughout all seasons (Table 2). Lettuce seemed therefore less likely to be the source of infection for these cases. Although other food items could not be excluded here, the known association of shellfish with HAV outbreaks informed the initiation of mussel traceback investigations.

Traceback investigation

All cluster cases had bought mussels at local supermarkets, but no batch numbers or original package labels were available for traceback activities. Therefore the traceback was targeted at suppliers within the incubation periods of the patients. Within this period the suppliers had sourced from a Dutch growing area and from other countries within the EU. No plausible sewage pollution-related link between any of the cluster cases and the Dutch growing area was found, nor could an epidemiological linkage be identified within the Netherlands due to uniqueness of the strains despite intensive surveillance. The registration documents from the mussel consignments to the Netherlands revealed the number of batches, harvesting areas and countries for each supplier in these two periods (Figure 2). All the identified areas were in countries non-endemic for HAV. Shellfish reference laboratories in these countries enquired for information reported that no routine surveillance for HAV in shellfish was performed. Archival mussel samples from July to November 2012 (n=28), randomly collected by the Dutch Food and Consumer

Table 3

Linking mussels consumed by patients to a specific harvester, food-borne hepatitis A clusters, the Netherlands, 2012

Dutch supplier	Harvesting area	Harvesting company	Date of shipments in 2012	Number of batches	Number of associated cases	Location of associated cases	Month of onset of disease
1	Area 1	N.a.	17 Jul–9 Aug	2	3	Netherlands	August 2012
2	Area 1	N.a.	18 Jul–16 Aug	13	2	Netherlands	August 2012
3	Area 1	N.a.	25 Sep–29 Oct	17	3	Netherlands	November 2012
1	Area 2	Harvester 1	2 Jul–20 Jul	20	3[a]	Netherlands	August 2012
2	Area 2	Harvester 2	21 Jun–8 Aug	32	2[a]	Netherlands	August 2012
3	Area 2	Harvester 1	17 Sep–30 Sep	2	5	Netherlands	November 2012
4	Area 2	Harvester 1	29 Sep	1	1	United Kingdom	November 2012

N.a.: not applicable.

[a] One of these patients consumed mussels from either Supplier 1 or 2.

Product Safety Authority for microbiological assessment of bivalves on the Dutch market, tested negative for HAV RNA using the method described in ISO/TS 15216–2 [13].

Combining the traceback results for both clusters revealed that two harvesting areas, Area 1 and Area 2, were common to both summer and autumn clusters (Figure 2). The dates of consignment for both areas were compared with a known date of mussel consumption by one of the cases. As shipments of mussels from Area 1 only began five days after this consumption date, whereas mussel shipments from Area 2 predated this consumption date, the mussels from Area 2 were identified as the most likely source.

International case identification

It was reported that mussels in harvest Area 2 in the UK were all locally produced. Therefore, the Virus Reference Department of Public Health England was asked to check for similarities in their HAV database with the RIVM-HAV12–070 or RIVM-HAV12–124 sequences that could belong to local cases. For this, epidemiological and sequence data from different departments were combined, revealing two patients with identical sequences. The first patient identified, (Figure 3, UK case number 4) was a child of school age with onset of symptoms in early August 2012, infected with RIVM-HAV12–070 (100%), who lived at a distance less than 10 km from Area 2. The child was a household member of two other confirmed HAV cases infected with RIVM-HAV12–070 strain (100%): another child and a middle-aged adult with onsets of illness on in early July 2012 and early August 2012, respectively (Figure 3, UK cases number 2 and 3). The household did not report recent history of mussel consumption. Searching the database for earlier local cases revealed a new archival sample that matched 100% with RIVM-HAV12–070 strain. This middle-aged adult (Figure 3, UK case number 1) had a travel history to a hepatitis A-endemic country in Central America while unvaccinated in April 2012. In May, the patient made a short visit to an area near the coast of Area 2, where the patient fell ill and was admitted to the local hospital in early June. Although direct contact between UK cases number 1 and 2 could not be demonstrated, the places of residence of these cases were within a few km of each other during the infectious period. The environmental and water authorities showed that biologically treated sewage from the residences and the hospital was discharged into rivers at a fluvial distances of less than 10 km from the nearest boundary of the designated mussel-harvesting Area 2. All sewage treatment plants were reported to be functioning normally during the relevant periods when contamination might have occurred.

The other reported case (Figure 3, UK case number 5) matching the RIVM-HAV12–124 strain had no links to the identified Area 2, but reported having consumed mussels in a pub in London within the incubation period for the illness. Local food safety officers reported that the consumed mussels were bought by the pub from a UK wholesaler that had received mussels from a Dutch supplier. This Dutch supplier, Supplier 4, had packed mussels in early October 2012 that had been harvested from Area 2 by Harvester 1 (Table 3). Registration documents demonstrated that Dutch Suppliers 1 and 3 had also bought mussels from this harvester. The mussel beds of Harvester 1 were those located closest to the implicated sewage treatment plant inputs of harvest Area 2.

Sequence analyses for UK case 4 showed a strong signal for the RIVM-HAV12–070, but also a weak signal for the subspecies RIVM-HAV12–124 suggestive of a subspecies within this patient which may subsequently have become the dominant strain in the autumn cluster of cases. Further Dutch cases and a UK case in autumn 2012 came after a period of heavy rainfall (23–25 September 2012) in Area 2. Heavy rain would have led to the activation of combined sewage overflow systems and thus the bypass of the sewage treatment works and potential release of untreated sewage into Area 2. This rainfall occurred one week before delivery of mussels to Supplier 4 that were consumed by UK case number 5 and before delivery of mussels to

Supplier 3 consumed by at least four of the five Dutch patients (Figure 3).

A second request to EPIS-FWD in December 2013 was posed specifically to five countries that might have imported mussels, according the Dutch company's website, in order to identify additional international related cases. None were reported by Austria, Belgium, Germany, or Switzerland. Previously (January and October 2013) none had been reported by France via HAVNET.

Discussion

This report describes a unique investigation of two closely related food-borne clusters of hepatitis A cases. Investigations following the traceback of the implicated mussels led to international matching of the nine primary and two secondary Dutch cases to three primary and two secondary cases in the UK with identical HAV sequences within the same time period. Consumption of a common source of mussels by Dutch cases and a UK case confirmed our findings. The investigation convincingly points towards the case with a travel history to Central America being the source of the HAV cluster, who then infected a subsequent household cluster, all of whom lived close to the mussel production area. This unique multinational outbreak investigation demonstrates that a small number of patients in a non-endemic area led to geographically dispersed HAV outbreaks via food as a vehicle. Such a link would have gone unnoticed without sequence analyses and the combined forces of diverse institutes.

The number of patients in this investigation was small compared with other HAV outbreaks recently reported [5-8], which limited the available epidemiological data. Notably, the identification of the clusters was not based on an increase in the total number of patients, but was the result of the existing intensive surveillance with molecular typing of HAV in patients in the Netherlands [3]. The rising numbers of reported food-borne HAV outbreaks and RASFFs are likely to promote interest in diagnostic sample typing. This would assist source tracing in outbreak investigation and may result in an improved estimate of the impact of contaminated food on public health.

Bivalve molluscs (e.g. oysters and mussels) have long been associated with viral food-borne disease. Several large outbreaks associated with consumption of HAV contaminated bivalve molluscs have been described [14-16], often traced back to harvesting areas within endemic areas. HAV infections through consumption of bivalve molluscs may be anticipated with global trade from endemic to less endemic HAV areas [17]. The implicated bivalve molluscs in this report were however harvested from a non-endemic area with no prior indication of recent local HAV outbreaks, as occurred in 1997 although the 1997 outbreak had many more cases [18]. Generally, mussels from non-endemic areas are considered to be at low risk for HAV infection, especially as they are usually consumed after domestic heat treatment, like the Dutch cases described here. Domestic cooking procedures usually involve heating by steaming until the shells open, rather than immersion for a defined period in boiling water. Internal temperatures reached during steaming have been shown to be ineffective for complete inactivation of infectious HAV in mussels [19,20], but the outcome depends on initial contamination levels. The degree of cooking to reach an internal temperature of 90 °C for 90 seconds as required by EU legislation for commercial heat treatment [21], which has been shown to reliably inactivate HAV [22], may however render bivalve molluscs unpalatable to consumers when applied in the domestic setting.

Area 2 is a source of mussels imported into the Netherlands, and mussels from this area have been previously tested for norovirus for microbiological assessment of bivalves on the Dutch market. The frequent occurrence of samples testing positive for norovirus indicates potential human faecal contamination, which is consistent with its class B classification (‹ 4,600 *Escherichia coli* per 100 g shellfish flesh in 90% of samples) under EU food safety regulations. (EU Regulation number 854/2004). It is therefore feasible that sewage containing faeces from the sporadic cases of HAV patients may have entered the mussel growing water and led to contamination of parts of the mussel bed. It is interesting to reflect that over 1,000 tonnes of mussels were harvested from this area during the relevant time period, the majority was exported to other European countries following processing and packaging in the Netherlands, and yet only 10 associated HAV cases were identified. This could suggest a low, heterogeneous or temporary contamination, the risk of which may have been reduced through heat treatment. Otherwise, the low number of associated HAV cases could point towards low recovery from laboratory surveillance, as sequence analyses of patient material is not common practice in most countries or is performed for some cases only [5,8] or is not systematically reported. In this study, it was only after more direct questioning regarding the residence of HAV cases and their proximity to mussel beds that samples taken from these particular cases were forwarded for sequencing. The occurrence of the two clusters, with the second cluster potentially associated with heavy rainfall, suggests environmental factors played a part in increasing the risk. The guidelines for the control of viruses in food [23] recommend that after heavy rainfall, and/or after overflow from sewage treatment plants, harvesting of bivalve molluscs should cease for a period, until the water and/or bivalve mollusc quality in the harvesting area has been assessed and has returned to normal background levels for the area. Similarly, official EU guidelines to Regulation 854/2004 require investigation following a pollution or extreme weather event and, if necessary, additional controls to protect public health [24]. Area 2, like other similar areas affected by human faecal pollution, remains vulnerable to

sporadic HAV cases shedding virus within the drainage catchment area. Monitoring of shellfish or effluents of treated sewage for HAV contamination during periods of elevated risk, for example following identification of sporadic or outbreak cases within the catchment, could potentially assist public health protection. This would require real-time exchange of data on hepatitis A cases or virus detection between regional agencies dealing with public health, food safety and water quality, as even sporadic cases can shed a large amounts of virus over a period of time, and these viruses are only partially reduced by water treatment plants, and viruses in bivalve molluscs are bioaccumulated [1].

International sharing of HAV sequences proved helpful in this as well as in other recent outbreak investigations [4-8]. A database such as HAVNET (www.havnet.nl) when used in real time may result in a rapid traceback of the product involved, followed by a recall, if still possible, or other preventive measures to increase food safety. Moreover, finding related cases in other countries could give an insight into the true size of food-borne outbreaks. Access to HAVNET can be requested by e-mailing havnet@rivm.nl.

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Conflict of interest

None declared.

Authors' contributions

IB and LV coordinated the investigation, and collected, analysed and interpreted the data. IB coordinated the traceback investigation and international contacts concerning food. IB drafted the manuscript. LV coordinated the international contacts with public health institutes. HV and SLN coordinated genotyping part and interpretation of the results. IF and LV worked on the analyses of the epidemiological data. DL and CW collected and interpreted the environmental data. MK participated as advisor in investigation and drafting of the manuscript. All authors participated in editing the manuscript, and read and approved the final manuscript.

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Research article

Mutations acquired during cell culture isolation may affect antigenic characterisation of influenza A(H3N2) clade 3C.2a viruses

DM Skowronski [1,2], S Sabaiduc [1], C Chambers [1], A Eshaghi [3], JB Gubbay [3,4], M Krajden [1,2], SJ Drews [5,6], C Martineau [7], G De Serres [7,8,9], JA Dickinson [10], A Winter [3], N Bastien [11], Y Li [11,12]

1. British Columbia Centre for Disease Control, Vancouver, Canada
2. University of British Columbia, Vancouver, Canada
3. Public Health Ontario, Toronto, Canada
4. University of Toronto, Toronto, Canada
5. University of Alberta, Edmonton, Canada
6. Alberta Provincial Laboratory, Edmonton, Canada
7. Institut National de Santé Publique du Québec (National Institute of Health of Quebec), Quebec, Canada
8. Laval University, Quebec, Canada
9. Centre Hospitalier Universitaire de Québec (University Hospital Centre of Quebec), Quebec, Canada
10. University of Calgary, Calgary, Canada
11. National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, Canada
12. University of Manitoba, Winnipeg, Canada

Correspondence: Danuta M. Skowronski (danuta.skowronski@bccdc.ca)

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As elsewhere, few (<15%) sentinel influenza A(H3N2) clade 3C.2a viruses that dominated in Canada during the 2014/15 season could be antigenically characterised by haemagglutination inhibition (HI) assay. Clade 3C.2a viruses that could be HI-characterised had acquired genetic mutations during in vitro cell culture isolation that modified the potential glycosylation motif found in original patient specimens and the consensus sequence of circulating viruses at amino acid positions 158–160 of the haemagglutinin protein. Caution is warranted in extrapolating antigenic relatedness based on limited HI findings for clade 3C.2a viruses that continue to circulate globally.

Introduction

During the 2014/15 influenza A(H3N2) epidemic, viruses belonging to phylogenetic clade 3C.2a predominated [1,2]. Viruses within this clade bore multiple (10–12) amino acid differences from the A/Texas/50/2012 (clade 3C.1) vaccine strain at antigenic sites of the surface haemagglutinin (HA) protein [2]. These differences included two clade-defining substitutions, a phenylalanine (F) to tyrosine (Y) substitution at residue 159 (F159Y) and a lysine (K) to threonine (T) substitution at the adjacent residue 160 (K160T), both in antigenic site B [1,2], a highly exposed region at the top of the HA protein where mutations create the potential for viral evasion of the antibody response [3,4]. Together with an asparagine (N) residue at position 158, conserved in all clade 3C viruses, the N158-Y159-T160 sequon in clade 3C.2a viruses represents a potential gain of glycosylation that can mask viral epitopes and reduce antibody access to the immuno-dominant antigenic site B [5,6]. This potential glycosylation motif at amino acid positions 158–160 of the HA protein is unique to clade 3C.2a viruses, and is not found in other recently circulating A(H3N2) genetic clades.

Koel et al. have previously highlighted positions 158 and 159 as among seven residues in the HA protein associated with all major antigenic cluster-transition events in A(H3N2) viruses since 1968 [7] and in a recent serological analysis, Chambers et al. highlighted the substitution at position 159 as likely to have been responsible for the 2014/15 antigenic drift [8]. Consistent with these molecular findings, mid-season vaccine effectiveness (VE) analyses from multiple countries, including the Canadian Sentinel Practitioner Surveillance Network (SPSN), the United States (US) and the United Kingdom (UK), reported negligible protection against the 2014/15 A(H3N2) clade 3C.2a epidemic strain [2,9,10]. In February 2015, the World Health Organization (WHO) recommended that the A(H3N2) component for the 2015/16 season be updated to an A/Switzerland/9715293/2013-like (clade 3C.3a) strain [11].

Influenza surveillance reports from reference laboratories globally have indicated that circulating A(H3N2) viruses belonging to clade 3C.2a are antigenically

Table 1

Haemagglutination inhibition assay titres and fold reductions relative to cell- and egg-passaged A/Switzerland/9715293/2013 reference virus for sentinel A(H3N2) virus isolates with known genetic clade, Canadian Sentinel Practitioner Surveillance Network, November 2014–April 2015 (n = 49)

Sentinel isolate (clade)	Cell-passaged A/Switzerland/9715293/2013 (n = 49)				Egg-passaged A/Switzerland/9715293/2013 (n = 35[a])			
	n	Sentinel isolate HI titre range	Homologous reference virus HI titre range	Fold reduction	n	Sentinel isolate HI titre range	Homologous reference virus HI titre range	Fold reduction
Clade 3C.2a	31	80–160	320–640	≤ 4	25	160–320	320–640	≤ 4
Clade 3C.3	1	160	320	2	1	320	640	2
Clade 3C.3a	2	160–640	320–640	≤ 4	2	320	320–640	≤ 2
Clade 3C.3b	15	80–1,280	320	≤ 4	7	80–640	320–640	≤ 8[b]

HI: haemagglutination inhibition.

[a] These 35/49 viruses were characterised in relation to both the cell-passaged and egg-passaged A/Switzerland/9715293/2013 reference virus. For the other 14/49 viruses initially characterised in relation to the cell-passaged A/Switzerland/9715293/2013 reference virus, there remained insufficient viral titre to support further characterisation in relation to the egg-passaged reference virus.

[b] One clade 3C.3b virus had an eightfold reduction to egg-passaged A/Switzerland/9715293/2013 reference virus; all other tested viruses had ≤ 4-fold reductions.

Table 2

Potential glycosylation motif at haemagglutinin positions 158-160 in influenza A(H3N2) clade 3C.2a viruses from original patient specimens and culture isolates prior to haemagglutination inhibition assay by the NML, Canadian Sentinel Practitioner Surveillance Network, November 2014–April 2015 (n = 234)

HI assay characterisation	HA amino acid sequence at positions 158-159-160					
	Original patient specimens n = 234			Culture isolates n = 234		
	N-Y-T[a] n = 219	Not N-Y-T n = 2	Sequence not available n = 13	N-Y-T[a] n = 156	Not or poly N-Y-T n = 71	Sequence not available n = 7
Sufficient HA titre for HI assay[b] (n = 31)	26 (12)	1	4	0 (0)	28 (39)	3
Insufficient HA titre for HI assay[b] (n = 203)	193 (88)	1	9	156 (100)	43 (61)	4

HA: haemagglutinin; HI: haemagglutination inhibition; NML: National Microbiology Laboratory; poly: polymorphic for the N-Y-T amino acid sequence (i.e. partial loss of the potential glycosylation motif).

Values displayed are: number (% column).

[a] A potential glycosylation motif is defined by the amino acid sequon: N-X-T/S; where N is asparagine, X is any amino acid other than proline and T/S is either threonine or serine [5,6]. The consensus sequence for clade 3C.2a viruses is N158-Y159-T160, conferring a potential gain of glycosylation.

[b] In the presence of 20 nM oseltamivir.

related to A/Switzerland/9715293/2013, despite the fact that only a small proportion could be characterised by conventional haemagglutination inhibition (HI) assay [1,12,13]. Influenza A(H3N2) viruses have been difficult to characterise antigenically by HI assay due to variable agglutination of erythrocytes or loss of ability to agglutinate erythrocytes, a particular problem for clade 3C.2a viruses [1]. For the majority of A(H3N2) viruses that could not be HI-characterised, reference laboratories have imputed antigenic relatedness based on sequencing findings, assuming that viruses that could be characterised within a given genetic group or clade are broadly representative of circulating strains [12,13].

The European Centre for Disease Prevention and Control (ECDC) has earlier highlighted that clade 3C.2a viruses that had sufficient HA titre to agglutinate erythrocytes and that could be characterised by HI assay had either lost or were polymorphic for the clade-defining glycosylation motif at positions 158–160 [14]. To assess the representativeness of clade 3C.2a viruses that could be characterised by HI assay, we examined amino acid identity at positions 158–160 for the presence of this potential glycosylation motif in original patient specimens collected by the Canadian SPSN compared with the corresponding sequence after cell culture isolation of virus during the 2014/15 season.

Methods

Nasal/nasopharyngeal specimens collected from patients within seven days of influenza-like illness onset through the Canadian SPSN between 1 November 2014 and 30 April 2015 were tested for influenza by RT-PCR. Influenza-positive specimens were inoculated into Madin-Darby Canine Kidney (MDCK), MDCK-SIAT1 or Rhesus Monkey Kidney (RMK) cells to attempt culture isolation as per provincial reference laboratory protocols. Cell culture isolates were submitted to Canada's National Microbiology Laboratory (NML) for antigenic characterisation by standard HI assay protocols using guinea pig erythrocytes and post-infection ferret antisera supplied by the US Centers for Disease Control and Prevention (US CDC) raised against cell- and egg-passaged A/Switzerland/9715293/2013 reference viruses [2]. To circumvent any neuraminidase (NA)-mediated binding of A(H3N2) viruses to erythrocytes, HI assays were conducted in the presence of 20 nM oseltamivir carboxylate following, where indicated, a further single passage in MDCK-SIAT1 cells at the NML to improve viral titres [15,16]. Antigenic relatedness of a sentinel isolate to A/Switzerland/9715293/2013 reference virus was defined as a ≤4-fold reduction in HI antibody titre compared to the titre of the homologous reference virus [17].

Sanger sequencing of the viral HA gene was conducted on the original patient specimens to establish clade designation and to detect amino acid substitutions in HA antigenic sites. For the current study, sequencing was also conducted on cultured isolates of clade 3C.2a viruses before and after further passage in MDCK-SIAT1 cells (if indicated) prior to HI characterisation at the NML to assess amino acid identity relative to the clade 3C.2a N158-Y159-T160 consensus sequence and to sequences based on the corresponding original patient specimen. Ethics boards in each participating province approved the SPSN VE study of which this virological sub-analysis is a component; virus characterisation was also conducted as part of national surveillance activities.

Results

Clade distribution and HI characterisation

Of the 460 influenza A(H3N2) detections by the SPSN during the 2014/15 season with known clade information, 265 (58%) virus isolates were cultivated by provincial reference laboratories and submitted to the NML for antigenic characterisation by HI assay. Of the 265 virus isolates, 197 (74%) were grown by provincial laboratories in MDCK, 44 (17%) in MDCK-SIAT1 and 24 (9%) in RMK cells. Submitted A(H3N2) virus isolates included 234 (88%) viruses belonging to clade 3C.2a, 25 (9%) belonging to clade 3C.3b, four (2%) belonging to clade 3C.3 and two (1%) belonging to clade 3C.3a, reflecting the overall clade distribution and clade 3C.2a predominance among sentinel A(H3N2) detections previously reported [2].

Of these 265 virus isolates with known clade information, 49 (18%) had sufficient HA titre to agglutinate erythrocytes and could be characterised by HI assay. These included only 31 (13%) of the 234 virus isolates belonging to clade 3C.2a. By comparison, of the 31 non-clade 3C.2a virus isolates, 18 could be characterised by HI, including 15 of 25 belonging to clade 3C.3b, one of four belonging to 3C.3, and both of the virus isolates belonging to clade 3C.3a.

All 49 virus isolates that could be HI-characterised were considered antigenically related to the cell-passaged A/Switzerland/9715293/2013 vaccine prototype recommended for the 2015/16 vaccine. A subset of 35 of the 49 viruses was additionally characterised against the egg-passaged A/Switzerland/9715293/2013 vaccine reference and 34 of them were considered antigenically related; one clade 3C.3b virus showing eightfold titre reduction was considered antigenically distinct (**Table 1**).

Clade 3C.2a viruses and the 158–160 sequon

Of the 234 clade 3C.2a virus isolates submitted to NML, sequencing of the viral HA at positions 158–160 based on original patient specimens was successful for 221 (94%) viruses (**Table 2**). Of these 221 viruses from original patient specimens, 219 (99%) bore the clade 3C.2a consensus sequence N158-Y159-T160 consistent with the potential glycosylation motif and two (1%) instead bore K160 found otherwise in clade 3C.2 viruses.

Following cell culture isolation at provincial reference laboratories, 229 of 234 (98%) clade 3C.2a virus isolates had sequencing information available before MDCK-SIAT1 passage (if indicated) at the NML. Of these, 63 (28%) viruses had lost or partially lost (i.e. become polymorphic for) the N158-Y159-T160 consensus sequence, including 45 (25%) of 178 grown in MDCK, 10 of 30 grown in MDCK-SIAT1 and eight of 21 grown in RMK cells.

Of the 31 clade 3C.2a viruses that could be HI-characterised, 17 had undergone a single further passage in MDCK-SIAT1 cells at the NML prior to HI assay. Sequencing information was available for 15 of these 17 viruses that required additional MDCK-SIAT1 passage and 13 of the 14 viruses that did not require further MDCK-SIAT1 passage. Based on available sequencing information, all 28 viruses had lost or were polymorphic for the potential glycosylation motif (**Table 2**). For all but two of the virus isolates that were further passaged in MDCK-SIAT1 cells at the NML, sequences were identical before and after that passage. Of the two viruses modified with MDCK-SIAT1 passage at the NML, one isolate that was polymorphic after initial cell culture lost the potential glycosylation motif and one isolate that maintained the original consensus sequence after initial cell culture became polymorphic after MDCK-SIAT1 passage.

Table 3

Amino acid sequence at haemagglutinin positions 158–160 of influenza A(H3N2) clade 3C.2a viruses with respect to the potential glycosylation motif in final culture isolates prior to haemagglutination inhibition assay by the NML, Canadian Sentinel Practitioner Surveillance Network, November 2014–April 2015 (n = 234)

HA amino acid sequence			Glycosylation motif	Frequency n (%)	Interpretation
158	159	160			
Consensus sequence in circulating clade 3C.2a viruses					
N	Y	T	+CHO		
Sequence after final cell culture[a]					
A(H3N2) clade 3C.2a viruses with sufficient HA titre for HI assay[b] (n = 31)					
N	Y	K	– CHO	8	Reversion to clade 3C.2 K160
N	Y	A	– CHO	7	New T160A mutation
N	Y	T/K	Polymorphic[c]	4	Partial reversion to clade 3C.2 K160
K	Y	T	– CHO	3	New N158K mutation
N	Y	I	– CHO	3	New T160I mutation
D	Y	T	– CHO[d]	1	New N158D mutation
H	Y	T	– CHO	1	New N158H mutation
N/S	Y	T	Polymorphic	1	Polymorphic for new N158S mutation
Sequence not available			NA	3	NA
A(H3N2) clade 3C.2a viruses with insufficient HA titre for HI assay[b] (n = 203)					
N	Y	T	+CHO	156 (77)	Consensus clade 3C.2a sequence
N/K	Y	T	Polymorphic	6 (3)	Polymorphic for new N158K mutation
N/D	Y	T	Polymorphic	5 (2)	Polymorphic for new N158D mutation
N	Y	T/I	Polymorphic	5 (2)	Polymorphic for new T160I mutation
N	Y	T/A	Polymorphic	5 (2)	Polymorphic for new T160A mutation
N	Y	T/K	Polymorphic	5 (2)	Partial reversion to clade 3C.2 K160
N	Y	A	– CHO	3 (1)	New T160A mutation
N	Y	I	– CHO	3 (1)	New T160I mutation
N	Y	K	– CHO	3 (1)	Reversion to clade 3C.2 K160
S	Y	T	– CHO	2 (1)	New N158S mutation
D	Y	T	– CHO	1 (0)	New N158D mutation
K	Y	T	– CHO	1 (0)	New N158K mutation
N/K	Y	T/I	Polymorphic	1 (0)	Polymorphic for new N158K and T160I mutations
N/R	Y	T/I	Polymorphic	1 (0)	Polymorphic for new N158R and T160I mutations
N/S	Y	T/A	Polymorphic	1 (0)	Polymorphic for new N158S and T160A mutations
N/K/R/S	Y	T	Polymorphic	1 (0)	Polymorphic for new N158K/R/S mutation
Sequence not available			NA	4 (2)	NA

CHO: carbon-hydrogen-oxygen (i.e. glycosylation); HA: haemagglutinin; HI: haemagglutination inhibition; NA: not available; NML: National Microbiology Laboratory.

+CHO: potential glycosylation motif in clade 3C.2a viruses defined by the amino acid sequon: N158-Y159-T160 [5,6]; – CHO: loss of this potential glycosylation motif; Polymorphic: partial loss of the glycosylation motif.

Mutations at residues 158–160 compared with the consensus sequence for clade 3C.2a viruses are shaded in blue: dark blue shading indicates amino acid mutation relative to the consensus sequence; light blue shading indicates polymorphism relative to the consensus sequence. Two viruses were N158-Y159-K160 (– CHO) in the original patient specimen, including one that could and one that could not be HI-characterised.

[a] Final available sequence of virus isolates prior to HI characterisation is shown. Of the 234 viruses sent to the NML, 220 were re-passaged in MDCK-SIAT1 cells to attempt to improve virus titre, including 17 of 31 that could be HI-characterised and all 203 that could not be HI-characterised. Sequences for cell culture isolates as submitted from provincial reference laboratories are shown for the 13 of 14 viruses with available sequence information that could be HI-characterised without further passage in MDCK-SIAT1 cells at the NML.

[b] In the presence of 20 nM oseltamivir.

[c] One of these four viruses was T160 (i.e. + CHO) in the cell culture isolate before further MDCK-SIAT1 passage at the NML but became polymorphic after MDCK-SIAT1-passage with partial reversion to T/K160 (i.e. N158-Y159-T/K160).

[d] This virus was polymorphic for the glycosylation motif with N/D158 in the cell culture isolate before MDCK-SIAT1 passage at the NML but lost the potential glycosylation motif (i.e. became – CHO) after MDCK-SIAT1 passage with D158 (i.e. D158-Y159-T160).

Table 4

Reference haemagglutinin sequences from the GISAID EpiFlu database used to assess the 158–160 sequon in the southern hemisphere 2016 influenza A(H3N2) cell- and egg-passaged vaccine reference strain A/Hong Kong/4801/2014 (clade 3C.2a)

Segment ID	Collection date	Isolate name[a]	Originating laboratory	Submitting laboratory	Authors	Passage history	158–159–160 sequon[b]
EPI539576	26 Feb 2014	A/Hong Kong/4801/2014	Government Virus Unit	National Institute for Medical Research		MDCK-SIAT1	N-Y-T
EPI578430	1 Jan 2014	A/Hong Kong/4801/2014	Crick Worldwide Influenza Centre	Centers for Disease Control and Prevention		E5/E1	N-Y-K
EPI643118	26 Feb 2014	A/Hong Kong/4801/2014	Crick Worldwide Influenza Centre	National Institute of Infectious Diseases (NIID)	Takashita, Emi; Fujisaki, Seiichiro; Shirakura, Masayuki; Watanabe, Shinji; Odagiri, Takato	E6(Am1AI)/E1+1	N-Y-K
EPI614406	1 Jan 2014	A/Hong Kong/4801/2014 X-263	New York Medical College	Centers for Disease Control and Prevention		EX	N-Y-K
EPI614421	1 Jan 2014	A/Hong Kong/4801/2014 X-263A	New York Medical College	Centers for Disease Control and Prevention		EX	N-Y-K
EPI614414	1 Jan 2014	A/Hong Kong/4801/2014 X-263B	New York Medical College	Centers for Disease Control and Prevention		EX	N-Y-K

GISAID: Global Initiative on Sharing All Influenza Data.

[a] Originating country for all isolates displayed is Hong Kong Special Administrative Region (SAR).

[b] The consensus sequence for clade 3C.2a viruses is N158-Y159-T160, shown as N-Y-T for the cell (MDCK-SIAT1)-passaged A/Hong Kong/4801/2014 (clade 3C.2a) reference virus (first row in Table) and conferring a potential gain of glycosylation. In the egg-passaged reference A/Hong Kong/4801/2014 (clade 3C.2a) reference viruses (rows 2–6 in Table), the potential glycosylation motif is lost due to reversion to clade 3C.2 K160.

Of the 203 clade 3C.2a viruses that could not be HI-characterised, all had undergone a single further passage in MDCK-SIAT1 cells at the NML to improve viral titre. Before MDCK-SIAT1 passage, 165 of the 199 (83%) virus isolates with available sequencing information pre-MDCK-SIAT1 passage had the potential glycosylation motif associated with the clade 3C.2a consensus sequence N158-Y159-T160. After passage in MDCK-SIAT1 cells, 156 (78%) of the 199 viruses that had sequence information available post-MDCK-SIAT1 passage had the potential glycosylation motif (**Table 2**).

Among viruses with available sequencing information (n=227), the absence of the potential glycosylation motif at positions 158–160 in the final virus isolate was significantly associated with the ability to HI-characterise viruses (0 with the glycosylation motif among 28 that could be HI-characterised vs. 156 (78%) with the glycosylation motif among 199 that could not be HI-characterised; chi-square = 70.2, degrees of freedom = 1, p-value < 0.001).

Specific mutations at positions 158 and 160 of final virus isolates influencing the potential glycosylation motif prior to HI characterisation are shown in **Table 3**; the F159Y clade marker for 3C.2a viruses was conserved in all isolates.

Discussion

Similar to reports elsewhere, only a small proportion (<15%) of clade 3C.2a viruses collected through the Canadian SPSN during the 2014/15 season were able to agglutinate guinea pig erythrocytes for antigenic characterisation by HI assay [1,12,13]. All clade 3C.2a virus isolates that could be characterised were considered antigenically related to the 2015/16 vaccine strain, although more variability in HI results, particularly in relation to the egg-passaged reference virus, has been reported by other surveillance systems [1]. Our findings, however, suggest that the small proportion of clade 3C.2a viruses that could be characterised by HI assay were not representative of circulating viruses with respect to the clade-defining potential glycosylation motif at positions 158–160.

We show that cell culture passage, whether in MDCK, MDCK-SIAT1 or RMK cells, that is required for HI characterisation, can fully or partially alter the N158-Y159-T160 sequon. This sequon in circulating clade 3C.2a viruses is associated with a predicted gain of glycosylation that may be relevant for antibody binding [6]. While these findings corroborate an earlier report of this effect by the ECDC [14], here we provide direct quantification and comparison of viral genomic sequences in original patient specimens compared with culture isolates, highlighting loss of the potential glycosylation

motif as an artefact of in vitro cell culture isolation. This type of assessment has not been widely reported elsewhere, in part because most viral sequences, including those posted to public databases, are based on culture isolates and are not directly compared with primary specimens. However, understanding how viral culture impacts genetic identity before antigenic characterisation is critical to interpreting and extrapolating relatedness among vaccine and circulating strains, for the purpose of anticipating vaccine performance and for vaccine strain selection.

Limitations of our analysis include the well-recognised variability in the HI assay [17], and the small number of viruses that could be antigenically characterised for sequence comparison. Antigenic characterisation of a greater number of A(H3N2) viruses, particularly those belonging to clade 3C.2a, may be possible through use of assays that do not rely on agglutination of erythrocytes, such as neutralisation assays [14,18]. The ECDC and others have considered the N158-Y159-T160 sequon to be a potential gain of glycosylation in the majority of clade 3C.2a viruses [1,5,6]; the glycosylation potential of this motif based on the clade 3C.2a consensus sequence is 0.65 using NetNGlyc 1.0, where the threshold for glycosylation potential is 0.5 [19]. To further delineate the N-glycosylation effect of the K160T mutation, in vitro studies should be done to specifically assess the interplay between this mutation, its resulting glycosylation potential and antibody binding at antigenic sites. We show statistically significant effects of the N158-Y159-T160 sequon on the ability to characterise viruses by HI assay, but it is unclear how much this potential glycosylation motif contributes to challenges in antigenic characterisation using antibody titration assays. A proportion of clade 3C.2a viruses (43/199; 22% in this analysis, **Table 2**), as well as other A(H3N2) genetic subgroups, that lack or are polymorphic for this glycosylation motif have also been difficult to antigenically characterise by HI assay, suggesting that other factors, such as viral load and avidity to sialic acid receptors, are also likely to contribute [20]. Our goal, however, was not to investigate those factors but to assess the representativeness of clade 3C.2a viruses that could be characterised by HI assay in relation to the majority that could not be characterised, with respect to the potential glycosylation motif at pivotal antibody binding positions 158–160 of antigenic site B.

Our findings suggest that caution is warranted in extrapolating antigenic relatedness based on limited HI results for A(H3N2) clade 3C.2a viruses. Clade 3C.2a viruses have continued to predominate among A(H3N2) detections throughout the 2015 southern hemisphere influenza season and early into the 2015/16 northern hemisphere season [1,18]. Despite vaccine reformulation, clade 3C.2a viruses still differ from the northern hemisphere 2015/16 clade 3C.3a vaccine strain by 10–12 amino acids at antigenic sites, including the same N158-Y159-T160 glycosylation motif that distinguished them from the 2014/15 vaccine [2]. For the southern hemisphere's 2016 influenza season, the WHO has recommended change to an A/Hong Kong/4801/2014(H3N2)-like (clade 3C.2a) representative vaccine virus [18], for which the egg-passaged reference strain bears K160 rather than T160 and thus also seems to have lost the potential glycosylation motif (**Table 4**). Clarifying the significance of the N158-Y159-T160 potential glycosylation motif in circulating clade 3C.2a strains thus remains critical to the interpretation of antigenic relatedness and to expectations of vaccine-induced antibody protection, for which ongoing epidemiological monitoring of VE will be important.

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Conflict of interest

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Authors' contributions

Principal investigator (epidemiology): GDS (Québec); JAD (Alberta); DMS (National and British Columbia); ALW (Ontario). Principal investigator (laboratory): SD (Alberta); JBG (Ontario); MK (British Columbia); CM (Québec); NB and YL (national). Data analysis: CC, AE, SS, DMS. Preparation of first draft: DMS. Draft revision and approval: all.

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Research article

Genotyping and strain distribution of *Mycobacterium avium* subspecies *hominissuis* isolated from humans and pigs in Belgium, 2011–2013

C Vluggen [1], K Soetaert [1], L Duytschaever [2 3], J Denoël [2], M Fauville-Dufaux [1], F Smeets [2], N Bruffaerts [4], K Huygen [4], D Fretin [3], L Rigouts [5 6], C Saegerman [2], V Mathys [1]

1. Bacterial Diseases Service, Operational Direction Communicable and Infectious Diseases, Scientific Institute of Public Health (WIV-ISP), Brussels, Belgium
2. Research Unit for Epidemiology and Risk Analysis applied to Veterinary Sciences (UREAR-ULg), Fundamental and Applied Research for Animal and Health (FARAH), Liège, Belgium
3. Bacterial Zoonoses of Livestock Unit, Operational Direction Bacterial Diseases, Veterinary and Agrochemical Research Centre (CODA-CERVA), Brussels, Belgium
4. Immunology Service, Operational Direction Communicable and infectious Diseases, Scientific Institute of Public Health (WIV-ISP), Brussels, Belgium
5. Mycobacteriology Unit, Institute of Tropical Medicine, Antwerp, Belgium
6. Department Biomedical Sciences, University of Antwerp, Belgium

Correspondence: Vanessa Mathys (vmathys@wiv-isp.be)

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Mycobacterium avium represents a health concern for both humans and pigs. The characterisation of its subspecies is an important step improving the understanding of the epidemiology and the control of this pathogen. Ninety-two human *M. avium* strains were selected for a retrospective study. Subspecies determination by *rpoB* sequencing and IS*1245*/IS*901* analysis showed that 98.9% of Belgian human *M. avium* strains belong to the subspecies *hominissuis* (MAH). Some of these MAH strains present particular IS*1245*/IS*901* profiles (absence of IS*1245* and false IS901 detection provoked by the presence of IS*Mav6*). In addition, 54 MAH strains isolated from submandibular lymph nodes of Belgian pigs with lymphadenitis were included in this study. Genotyping of human and porcine isolates was performed using multispacer sequence typing (MST). In total, 49 different MST types were identified among pig (n = 11) and human (n = 43) MA isolates, with only five shared by both hosts. Among these MST types, 34 were newly identified. Our findings demonstrate the extensive genetic diversity among MAH isolates. Some genotypes were more prevalent in human or pigs but no correlation was observed between MST type and place of residence or the farm of origin for human and porcine isolates respectively, suggesting an environmental source of infection.

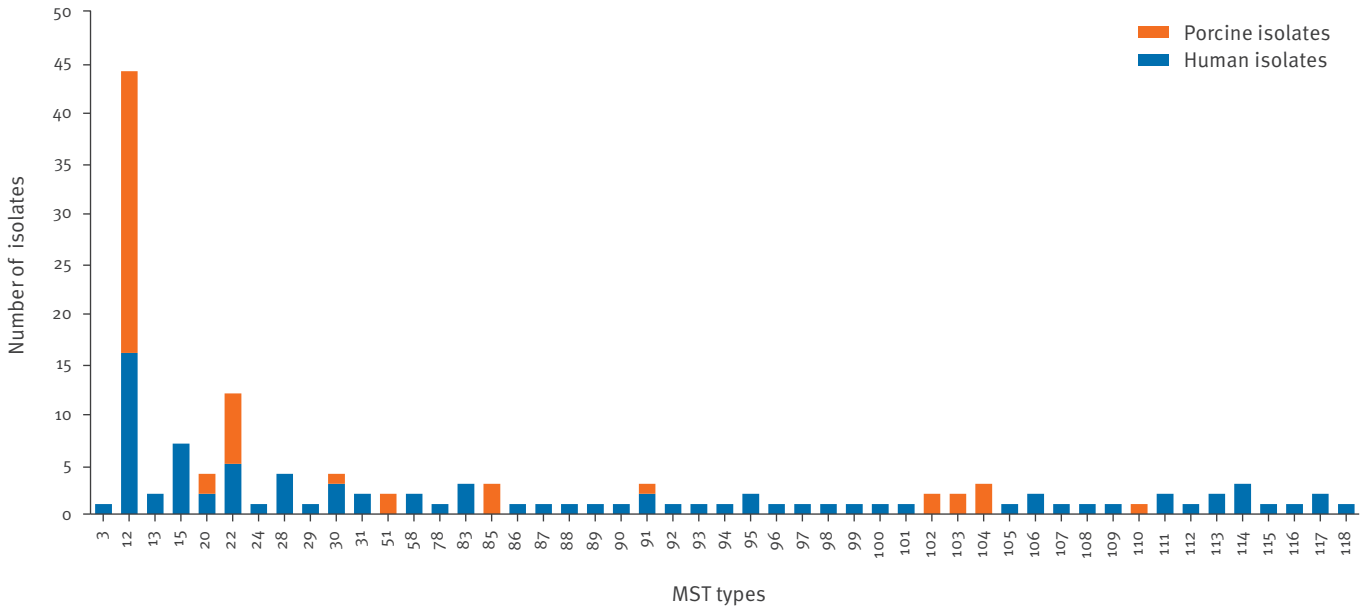
Introduction

Among non-tuberculous mycobacteria (NTM), *Mycobacterium avium* complex (MAC), composed of two major species *M. avium* and *M. intracellulare*, is the most frequently isolated from patients [1,2] and the most common NTM identified as responsible of pulmonary disease [3]. In humans, lymphadenitis due to NTM primarily affects children and is caused by a variety of species, with predominance of *M. avium* [4]. In industrialised countries, in the absence of antiretroviral therapy, MAC is also recognised as the most common opportunistic bacterial infection in patients with advanced AIDS [1,3], often with disseminated disease, rather than the typical lung involvement seen in immunocompetent patients [1].

The species *M. avium*, is divided into four subspecies; *M. avium* subsp. *avium* (MAA), *M. avium* subsp. *silvaticum* (MAS), *M. avium* subsp. *paratuberculosis* (MAP) and *M. avium* subsp. *hominissuis* (MAH) [5-7]. Recently, a phylogenetic study showed that MAH represents diverse groups of organisms from which two distinct groups, MAP and MAA/MAS, have evolved independently [8]. These subspecies of *M. avium* are genetically close, yet differ widely in their host range and pathogenicity. MAP is responsible for an intestinal illness in ruminants known as Johne's disease [9] and could be implicated in human Crohn's disease [10]. MAA and MAS mainly infect birds causing a tuberculosis-like disease, whereas MAH is a frequent agent of human and pig mycobacterioses [6,11]. Although MAH can infect a wide variety of animals, pigs are its primary animal host species, causing granulomatous lesions mainly in the lymph nodes of the digestive tract, which can reduce the value of carcasses [12]. Moreover, an association between MAH and human lymphadenitis has been described [13]. MAH represents an increasing

Figure

Distribution of multispacer sequence typing types among porcine (n = 52) and human (n = 87) isolates, Belgium, 2011–2013



public health concern given its pathogenicity for both humans and pigs, and so precise characterisation of MA subspecies and genotypes is essential for epidemiological studies, identification of the source of infection, and to establish or exclude its possible zoonotic role [14-16].

In the period from 2010 to 2013, 2,964 NTM cultures were sent for identification to the Belgian National Reference Centre for Tuberculosis and Mycobacteria (WIV-ISP), of which 1,104 belonged to the *M. avium* complex, with the following distribution between the two species: 574 (19.4% of the total) *M. avium* and 530 (17.9% of the total) *M. intracellulare*.

According to these data, *M. avium* is the predominant NTM isolated from Belgian patients. As the identification of *M. avium* isolates in Belgium is usually carried out by sequencing of the gene coding for 16SrRNA or by line probe assay (based on the analysis of the 23SrRNA gene), the distribution of the four *M. avium* subspecies remains largely unknown. In order to better characterise the *M. avium* strains circulating in Belgium, we selected clinical *M. avium* isolates from the above mentioned *M. avium* isolates and performed subspecies identification by sequencing of the *rpoB* gene and analysis of the IS*1245*/IS*901* elements. We also performed genotyping of the MAH isolates by using multispacer sequence typing (MST). As mycobacteriosis in pigs is mainly caused by MAH [16-18], we also genotyped previously identified MAH isolates from submandibular lymph nodes of Belgian pigs with lymphadenitis. The MST types of strains of human and porcine origin were compared.

Materials and methods

Bacterial isolates and study design

A total of 146 Belgian *M. avium* isolates from human patients (n = 92) and pigs (n = 54) were characterised in this study.

One hundred clinical *M. avium* isolates were selected from the collection maintained at WIV-ISP, Belgium. The isolates came from patients who had been diagnosed with *M.* avium in the last trimester of 2011 and in 2012, and for whom drug susceptibility testing (DST) had been requested after clinical, radiological and microbiological evaluation. Isolates were considered clinically relevant based on the criteria specified by the American Thoracic Society/Infectious Disease Society of America (ATS/IDSA) statement [19]. We aimed to exclude isolates representing environmental contamination of the samples. The selection represents 63% (100/159) of all *M. avium* isolates received at WIV-ISP for identification during the study period. WIV-ISP is the only Belgian laboratory performing DST for NTM. The *M. avium* species identification, carried out during routine activities at the reference laboratory, was performed by sequencing of the gene coding for the 16SrRNA, as previously described [20]. Eight isolates were eliminated from the study because of poor conservation quality, leaving a final sample of 92 human isolates.

The pig isolates were obtained from another ongoing study analysing submandibular lymph nodes from pigs with lymphadenitis sampled after veterinary inspection by the UREAR-ULg in all Belgian slaughterhouses. Isolates within the present study were sampled in 2012 and 2013. The first 54 *M. avium* isolates obtained were

Table 1

Mycobacterium avium subspecies identification of clinical isolates using IS1245/IS901 and rpoB sequencing, Belgium, 2011–2012, (n=92)

Interpretation based on IS*1245*/IS*901* detection			Interpretation based on *rpoB* sequencing	
No. of isolates	IS*1245*/IS*901* profile	Subspecies conclusion	No. of isolates	Perfect match with *rpoB* sequence of
87	IS*1245*+ / IS*901*-	*M. avium* subsp. *hominissuis*	72 1 1 4 9	*M. avium* subsp. *hominissuis* *Mycobcterium* sp. 09–7368 *Mycobacterium* sp. 11–0068 (KP098592) *Mycobacterium* sp. 11–006596, 10–5763 or 09–4604 *rpoB* PCR amplification impossible
3	IS*1245*+ / IS*901*+	*M. avium* subsp. *avium* / *silvaticum*	1 2	*Mycobacterium avium* subsp. *avium* *Mycobacterium avium* subsp. *hominissuis*
2	IS*1245* - / IS*901*+	Not *M. avium*	2	*M. avium* subsp. *hominissuis* strain TH135

IS: insertion sequence; *M. avium: Mycobacterium avium*; subsp.: subspecies; - : absence of the fragment; + : presence of the fragment.

included. They were isolated by the Belgian veterinary centre CODA-CERVA which supplied them for the study. *rpoB* gene sequencing was used to confirm the subspecies identification that had been made by the CODA-CERVA using the IS*1245*/IS*901* detection method. MST genotyping of these porcine isolates was performed as described above.

Clinical data and patient information

All clinical data recorded in the context of the present study had been collected not for research purposes but as part of the routine data collection for diagnosis. We collected the following data: origin of specimens (site of infection), date of collection, sex and age of patients and their city of residence (or postal code). Anonymity of data was ensured before analysis. The study was approved by the ethical committee of Hôpital Erasme (ULB) (reference P2014/028).

IS*901*/IS*1245* detection

M. avium species identification of the selected strains was confirmed by PCR detection of the insertion sequence (IS) 1245 (present in *M. avium* strains), and the *M. avium* subspecies determined by analysing the presence/absence of IS*901*. Avian MAA and MAS strains contain IS*901*, whereas mammalian MAH and MAP strains lack it. The following primers were used for the analysis of IS*1245* and IS*901* respectively: IS1245F (5' AGGTGGCGTCGAGGAAGAC 3') and IS1245R (5' GCCGCCGAAACGATCTAC 3'), IS901F (5' GCAACGGTTGTTGCTTGAAAGGAAT 3') and IS901R (5' GCGCACGCATGATGAGTGGACTTAC 3'). Both PCR assays were performed as previously described [21,22]. PCR products were analysed by electrophoresis on a 2% agarose gel and visualised by staining with ethidium bromide and UV illumination. Sequencing of some IS*901* amplicons was performed using the primers used for the PCR amplification.

Gene sequencing

Subspecies identification among *M. avium* isolates was also performed by sequencing of the *rpoB* gene as described by Ben Salah et al. [23]. Briefly, the primers Myco-F (5' GGCAAGGTCACCCCGAAGGG 3') and Myco-R (5' AGCGGCTGCTGGGTGATCATC 3') were used to amplify a variable region of the *rpoB* gene [23]. PCR products (10 µl) were visualised on a 2% agarose gel after staining with ethidium bromide. The remaining PCR products were purified using a QIAquick 96 PCR Purification Kit (Qiagen) and sequenced with primers Myco-F and Mycoseq-R (5' GGACATCATCCTGAACACCCACG 3'). Sequencing was performed by using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems) and analysed on an ABI 3130xl Sequencer (Applied Biosystems). The obtained forward and reverse sequences were re-assembled using the bioinformatics software Lasergene SeqMan Pro and used for sequence comparisons by BLAST with the NCBI database (National Centre for Biotechnology Information).

For a few strains, the subspecies identification was also confirmed by sequencing of the *hsp65* gene using the classification proposed by Turenne et al. [24] and the method described by Dirac et al. [25].

Multispacer sequence typing

Genotyping of the *M. avium* isolates was performed by MST. The PCR amplification of the spacers MST2, MST4, MST15 and MST16 was performed as described by Cayrou et al. [26]. Amplicon purification and sequencing were performed as described above, with the same forward and reverse primers as used for the different MST PCRs.

The MST type was determined by consulting the MST database [27].

Statistical analysis

A 95% confidence interval (CI) of prevalence of clinical *M. avium* isolates according to the affected organ was estimated using an exact binomial distribution. The comparison of age between lymphadenitis and non-lymphadenitis patients was assessed using a two-sample t-test with unequal variances, whereas comparison of the sex ratio was assessed using the chi-squared test (Chi 2), and comparison of province of origin by the Fisher exact test [28]. The number of porcine *M. avium* strains according to the MST type and farm was assessed using a Poisson regression. A p value of 0.05 was considered as significant and all analyses were performed in Stata (StataCorp. 2011. Stata Statistical Software: Release 12. College Station, TX: StataCorp LP).

Results

Clinical isolates

The subspecies of *M. avium* isolates is not determined routinely in Belgium. In order to better characterise these *M. avium* isolates, a retrospective study was carried out (n = 92).

Information on the biological origin of the isolate was available for 71 patients. The vast majority of these were isolated from the respiratory tract (n = 51; 95% CI: 59.9–81.9) followed by lymph nodes (n = 13; 95% CI: 10.1–29.3), abscesses (n = 4; 95% CI: 1.6–13.8), blood (n = 2; 95% CI: 0.3–9.8) and peritoneal fluid (n = 1; 95% CI: 0.04–7.6).

The mean age of the selected patients was 58 years (data available for 90 of 92 patients). However, a significant difference in age was observed between lymphadenitis (n = 13; mean = 15.3 years, standard deviation (SD) = 22.8 years) and non-lymphadenitis patients (n = 77; mean = 65.1 years, SD = 18.7 years) ($p < 0.0001$). The overall sex ratio was 1.04 (47 males and 45 females) but no significant difference was observed between patients with or without lymphadenitis (p value = 0.70). In addition, lymphadenitis and non-lymphadenitis patients did not differ in geographic origin (p value = 0.125).

Subspecies identification

Based on presence of IS*1245* and absence of IS*901* determined by PCR analysis, 87 isolates among the 92 analysed were identified as MAH, and three as MAA/MAS (Table 1). For the two remaining strains, conflicting results were observed between the IS*1245* PCR (absence of the fragment usually present in *M. avium* strains) and sequencing of the 16sRNA used for the selection of the *M. avium* strains included in the study.

Subspecies identification based on the *rpoB* sequence confirmed MAH subspecies for 72 of 87 isolates (Table 1). For the 15 remaining strains, nine were negative in *rpoB* PCR amplification (repeated three times), five strains were identified as *M. species* (M. sp. 11–006596 (JF804804), M. sp. 10–5763 (JF327744) or M. sp. 09–4604 (JF327745) with same blast score, and M. sp. 09–7368 (JF437546)), and one had no perfect match with the *rpoB* sequences currently recorded. The four *M. species* were isolated from a macaque, a pig, a cow and a steer, and characterised as close to *M. avium* subspecies in a study evaluating the usefulness of *rpoB* sequencing for identification of *Mycobacterium* isolates of veterinary origin [29]. The new *rpoB* sequence discovered in this study was deposited in the GenBank database under accession number KP098592. This strain was isolated from a lymph node of a 64-year-old patient with lymphadenitis.

Of the three strains identified as MAA/MAS based on the presence of both IS*1245* and IS*901*, one was confirmed by *rpoB* analysis as MAA, and the other two were identified as MAH. We therefore sequenced the IS*901* amplicons obtained for these three strains. Blast analysis of 173 nt sequenced confirmed the presence of the IS*901* in the isolate identified as MAA but revealed that one of the two MAH actually had the insertion sequence IS*Mav6*, presenting 95% similarity with IS*901* [30].

Finally, for the two strains presenting negative results for the IS*1245* amplification, the *rpoB* sequence was identical to the MAH strain TH135 of the NCBI bank. *hsp65* sequencing yielded codes 1 and 2 according to the classification of Turenne et al. [24]. These *hsp65* codes have been described in MAH strains. It should be noted that these strains were also positive for the IS*901* element, which is unusual for MAH strains. Sequencing of IS*901* revealed the presence of insertion sequence IS*Mav6* instead of the standard IS*901*.

Molecular typing

The 92 human *M. avium* isolates included in this study were genotyped by MST in order to establish their genetic diversity (unrelated clinical isolates) or relatedness (clustered clinical isolates). MST types were interpretable for 87 strains. The distribution of the obtained genotypes is presented in the Figure. Overall, 43 different MST types were identified. Some genotypes were more prevalent such as MST type 12 found in 16 isolates, MST15 in seven isolates, MST22 in five isolates and MST28 in four isolates. Among the human isolates, twenty-nine MST types (86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 105, 106, 107, 108, 109, 111, 112, 113, 114, 115, 116, 117, 118) were identified for the first time and recorded in the MST database developed by the Université de la Méditerranée. The MAA strain belonged to MST type 3, which was previously described as specific for MAA [26]. No association was observed between the MST types and sites of infection. Moreover, analysis of the geographical repartition of the patients revealed no correlation between the MST types and the patient's places/cities of residence.

Table 2

Repartition of the porcine *Mycobacterium avium* subspecies hominissuis isolates by multispacer sequencing type and farm, Belgium, 2012–2013

Farm	No. of porcine isolates							
	MST 12	MST 51	MST20	MST 103	MST 22	MST 104	MST 102	MST 51
1	6			1				
2	2	1	1					
3	1							
4	2							
5	1							
6	6							
7	10							
8		1						
9		1						
10			1					
11				1				
12					1			
13					6			
14						1		
15						1	1	
16						1	1	
17								1
18								1
Total 18	**28**	**3**	**2**	**2**	**7**	**3**	**2**	**2**

MST: multispacer sequencing type.
This table includes only MST types found in two or more isolates/pigs.

Porcine isolates

Within the ongoing Belgian project begun in 2012, the inspection of 125,000 randomly selected pig carcasses was performed by the UREAR-ULg. All submandibular lymph nodes from pigs with lymphadenitis were sampled and transmitted to CODA-CERVA. In the laboratory, a smear of all lymph nodes were stained by the Ziehl-Neelsen method and observed microscopically. The samples with acid-fast resistant stain, as well as a randomised selection of 266 samples selected to be able to detect an expected prevalence of gross lesions of 0.5% with an acceptable error of 0.06% and a confidence interval of 95%, were cultivated on Coletsos (Biorad) and Löwenstein-Jensen (Biomérieux) media. The first 54 porcine MAH isolates were transmitted to the WIV-ISP, where 52 could be MST-typed, yielding 11 different MST types, of which five were also detected in human isolates. The most frequent porcine genotypes, MST type 12 (28 isolates) and MST type 22 (seven isolates), were also among the most frequently detected in humans. On the other hand, the second most prevalent human type (MST 15) was not detected in pigs. As for the clinical isolates, new MST types (n = 6) were identified (85, 91, 102, 103, 104, 110) and recorded in the MST database. The Figure shows the distribution of the MST types among porcine and human strains.

Analysis at fattening farm level (18 farms) was performed for all pigs infected with a MAH strain belonging to a MST type for which at least two strains were detected in this study. This analysis showed in all cases (six different MST types), that strains with the same MST type could be isolated from pigs originating from at least two different Belgian farms (Table 2). For example, the 28 porcine MST12 strains were isolated from pigs fattened at seven different farms. Farms of origin (farms of birth) supplying piglets to the fattening farms were also different in all cases. Moreover, MAH strains with different MST types were found among pigs originating from the same fattening farm. In addition, the number of different MAH strains isolated in a farm was not related to the MST type or the farm origin (Poisson regression; p value › 0.12).

Discussion

MAC strains are the most frequently isolated NTM, and mycobacterial infections caused by these MAC strains are on the rise in both animals and humans. According to data from Belgium's National Reference Laboratory, *M. avium* currently represents about 20% (167/857) of all NTM isolated from patients. In 2000, this percentage was only 12% (63/506 NTM analysed) suggesting an increasing number of *M. avium* infections.

Although a true relative increase in *M. avium* infections is suspected, this observation could also be partially

attributed to the increased awareness of NTM disease in recent years and the increased availability and sensitivity of laboratory techniques [3]. Indeed, the development of rapid liquid culture methods and molecular tests, such as species-specific PCR or reverse hybridisation assay, has improved and facilitated the detection and identification of NTM infections [3].

The present study was undertaken to characterise *M. avium* isolated from patients living in Belgium. First, the subspecies of a collection of clinical *M. avium* isolates was determined by detection of IS*1245* and IS*901*, which is the most widely used technique for the (sub-)species identification of MAC. By using the strict IS*1245*/IS*901* interpretation model, the majority (87/92, 94.6%) of human isolates were identified as MAH. When applying multiple identification strategies, however, conflicting data were obtained. Three of the five remaining isolates were detected as positive for IS*901* and IS*1245*, which is the usual profile for MAA, but the MAA subspecies identification was only confirmed by *rpoB* sequencing for one of these three isolates, whereas the other two yielded a typical MAH *rpoB* profile. Moreover, the two other remaining isolates, showing positive results for IS*901* but absence of amplicon for the IS*1245* (usually used as a marker for *M. avium* strains) gave the identification MAH by *hsp65* sequencing and MAH strain TH135 by *rpoB* sequencing.

Strain TH135 was isolated in Japan from a HIV-negative patient with pulmonary disease. Genome analysis of this strain, carried out by Uchiya et al., revealed the presence of a new [31] insertion sequence, designated IS*Mav*6, which presents 95% sequence identity with the subspecies marker IS*901* [30]. PCR amplification of IS*Mav6* with primers designed for IS*901* was previously reported, explaining the positive IS*901* PCR results obtained for our two TH135 strains [32]. Moreover, *M. avium* isolates lacking IS*1245* were also reported [29,31,32]. The existence of *M. avium* strains lacking IS*1245* and MAH strains harbouring an IS homologous to IS*901* complicates the interpretation of IS*1245*/*901* results. In this study, we show that the presence of strains with this particular IS profile appears to be quite limited in Belgium with 2.2% (2/92) of *M. avium* strains lacking IS*1245* and 4.4% (4/91) of MAH strains giving false IS*901* PCR amplification. A more problematic situation was described in Japan with 16% and 67%, respectively [32].

Taking into account the MAH identification by *rpoB* sequencing for the four isolates presenting an unusual (but already described) IS*1245*/IS*901* profile, 91 (98.9%) of the 92 *M. avium* analysed isolates belong to the *hominissuis* subspecies, leaving only one isolate being identified as MAA. Contrary, subspecies identification based on *rpoB* sequencing alone, would result in a lower percentage of MAH (82.6%; 76/92). The differences between the two techniques are caused by *rpoB* amplification problems [9] and isolates that are identified as *M. species* by *rpoB* sequencing [6].

Regarding to the sampling design, different time periods in human (last trimester of 2011 and 2012) and pig (2012–2013) studies were applied for logistic reasons: availability of historical human samples and the opportunity of the ongoing pig study. These non-overlapping time frames may have caused a bias in inter-species strain diversity. To address this issue, human isolates from 2012 to 2013 should be analysed and compared with the current pig set.

Regardless of the technique used, we can conclude that the vast majority of *M. avium* strains isolated in Belgium belong to the subspecies *hominissuis* as described in other countries [13,32]. Our analysis also shows that application of either the IS*1245*/IS*901* or *rpoB* based strategy resulted in the determination of most *M. avium* subspecies, but it was the combination of the two techniques that allowed us to firmly conclude the species and/or subspecies identification for nineteen (20.6%) cases.

Concerning the MAH genotypes, 48 different MST types were identified among pig and human MAH isolates, comprising five types shared by both hosts. Moreover, among these MST types, 34 were newly identified within this study (28 in human, five in porcine and one in both hosts) showing that the genotypes of the Belgian MAH strains are quite different from those isolated in other countries and previously recorded in the MST database. This clearly demonstrates the large genetic diversity of MAH isolates.

There was no correlation between the place of residence of the patients and the MST types of the MAH strains. Indeed, strains with same MST types were isolated from patients living in dispersed places in the country, and for patients living in the same city often various MST types were identified. Also for the porcine strains there was no correlation of MST type with the geographical localisation of the farms. Indeed, analysis of the MST types compared with the pig farms showed that MAH strains with identical MST types could be isolated from pigs originating from different fattening farms, while pigs on the same farms can be infected by MAH strains of various MST types. This genetic diversity of the MAH strains has also been described in other epidemiological studies (using other genotyping tools like IS*1245*-RFLP or MIRU-VNTR), suggesting an environmental source of infection rather than direct transmission between the hosts [14,16,33]. Alternatively, transmission from pigs to humans by consumption of meat could be considered. Indeed, in this situation, no correlation between pig farms and the patients' place of residence is needed. Although this route of transmission cannot be excluded, there is as yet no clear evidence of meat-borne transmission of *M. avium* to humans [34].

While considerable attempts were made to include only *M. avium* human isolates responsible for disease (and

to avoid environmental contamination of the specimens) by selecting isolates of patients for whom DST had been requested by a physician, we consider it likely that the selection of pathogenic isolates studied may be underrepresented as some physicians probably treat their patients after species identification without asking the laboratory to perform DST. Another limitation of our study could be the small sample size, particularly due to the large genetic diversity detected among the MA strains. For instance, our data suggest that some MST types are only detected in *M. avium* isolated from humans or pigs. Analysis of a larger sample is required to formally test this hypothesis.

In conclusion, we report on the predominance of MAH among Belgian patients infected by a *M. avium* strain, and characterisation of Belgian MAH porcine isolates. Genotyping revealed a large genetic diversity of strains (whatever their origin; human or porcine) and the absence of a link between genotypes and the place of residence (human) or the farm of origin (pigs), suggesting an environmental source of infection. Some genotypes are however more frequent in human or in pigs, and some are only found in one of these two hosts. Moreover, the IS*Mav6* element was detected in some human isolates as described in another study [15]. As MAH represents an increasing public health concern, these host-strain particularities deserve more investigations.

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Conflict of interest

None declared.

Authors' contributions

VM, KH, DF and CS conceived and designed the experiments; CV and KS performed the experiments; CV, VM and CS analysed the data; KS, LD, JD, FS, DF, CS contributed reagents/materials/analysis tools; CV, LD, MFD, NB, KH, DF, LR, VM and CS wrote the paper.

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