Two major mumps genotype G variants dominated recent mumps outbreaks in the Netherlands (2009–2012)

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During three seasons of mumps outbreaks in the Netherlands (September 2009–August 2012), 822 mumps cases were laboratory-confirmed at the National Institute for Public Health and the Environment (RIVM). Most patients were vaccinated young adults. Given the protracted endemic circulation, we studied the genetic diversity and changes of mumps virus over a period of 3 years. Phylogenetic analysis of the small hydrophobic (SH) gene (316 bp) was performed on a representative set of 808 specimens that tested positive for mumps via PCR. Additionally, the haemagglutinin/neuraminidase (HN) gene (1749 bp) and fusion (F) gene (1617 bp) were sequenced for a subset of samples (n = 17). Correlations between different sequence types and epidemiological and clinical data were investigated. The outbreaks in the Netherlands were dominated by two SH gene sequence types within genotype G, termed MuVs/Delft.NLD/03.10 (variant 1) and MuVs/Scheemda.NLD/12.10 (variant 2). Sequence analysis of the HN and F genes indicated that the outbreaks were initiated by separately introduced genetic lineages. The predominance of variant 2 by the end of the first outbreak season could not be explained by any of the epidemiological factors investigated. Orchitis was more frequently reported in males infected with variant 2, irrespective of age and vaccination status. These findings illustrate genetic heterogeneity of an emerging mumps genotype, and raise questions about the mechanisms driving mumps epidemiology and immunity in relation to vaccination.

INTRODUCTION

Mumps is a highly contagious self-limiting infection that is spread via airborne droplets from the upper respiratory tract and by direct contact with saliva from an infected person. Disease often starts with non-specific symptoms, followed in ~70% of cases by the characteristic unilateral or bilateral swelling of the parotid glands. Although mumps is mostly self-limiting, complications such as orchitis, pancreatitis, deafness, meningitis and encephalitis can occur (WHO, 2007).

Following the introduction of the measles–mumps–rubella (MMR) vaccination containing the Jeryl Lynn mumps virus strain in the Netherlands in 1987, the incidence of mumps decreased significantly. However, during the past decade, several mumps outbreaks were observed in the Netherlands as well as in other countries that adopted the MMR vaccine in their national childhood immunization programmes. As the recent mumps outbreaks were mainly caused by genotype G, whereas the Jeryl Lynn vaccine strain belongs to genotype A, one hypothesis could be that the recent mumps outbreaks resulted from the escape of vaccine-induced antibodies. However, WT mumps strains were effectively neutralized by vaccine-induced antibodies (Barskey et al., 2012; Carr et al., 2010; Rubin et al., 2008; Santak et al., 2012). There are also indications for a general lack of enduring humoral immunity, because mumps-specific antibodies induced by MMR vaccination were shown to wane both in concentration and in avidity (Kontio et al., 2012).

The first mumps outbreak in the Netherlands occurred in 2004 among a highly vaccinated student population at an international hotel school (Brockhoff et al., 2010). The second mumps outbreak occurred between 2007 and 2009 within an orthodox religious community with low...
vaccination coverage (Wielders et al., 2011). The most recent mumps outbreak started at the end of 2009 and was scaled-up by a large student party, after which many mumps cases were reported among students who had received two MMR doses during childhood. Thereafter, mumps virus persisted and spread throughout the country, and many cases were reported in student cities (Greenland et al., 2012; Whelan et al., 2010).

Given the protracted endemic circulation of mumps in the Netherlands, we studied the genetic diversity and changes of mumps virus over a period of 3 years (September 2009–August 2012). Mumps genotypes are identified based on the sequence of 316 nt encoding the small hydrophobic (SH) protein (WHO, 2012). The SH protein seems not to be essential for viral infection and replication, but may interfere with tumour necrosis factor-α signalling, thus potentially influencing the host response to infection (Takeuchi et al., 1996; Wilson et al., 2006; Xu et al., 2011). Additionally, we chose to sequence the genes encoding the haemagglutinin/neuraminidase (HN) protein and the fusion (F) protein for a representative subset of patients to monitor possible molecular changes of immunologically relevant mumps proteins and to increase the molecular resolution between separate genetic lineages. The HN protein – the major glycoprotein of mumps virus – is a viral attachment protein that is considered to be an important target for neutralizing antibodies and therefore potentially under selective pressure from vaccine-derived immunity (Orvell, 1984). The F protein is a surface protein that is involved in membrane fusion and viral entry (Tecle et al., 2000). Combining phylogenetic analyses of the SH, HN and F gene sequences, we aimed to determine whether the different sequence types were a result of separate introductions of mumps virus or ongoing virus transmission and evolution. Furthermore, we analysed the molecular genetic data combined with the epidemiological and clinical data in order to investigate if solely epidemiological factors could explain the predominance of sequence types circulating during this outbreak and to determine the differences in clinical impact between the mumps virus strains.

RESULTS

Mumps diagnostics

From 1 September 2009 to 31 August 2012, samples from 822 individuals were tested positive for mumps via either PCR (n=813) or serological analyses (n=7) or a combination of PCR and serology (n=2) at the National Institute for Public Health and the Environment (RIVM). All individuals positive for mumps virus had at least one mumps symptom, i.e. parotitis, orchitis or meningitis. Of the individuals with reported vaccination status (n=672), 86.3% were vaccinated at least once and 73.8% had received at least two MMR vaccine doses. Of the 822 mumps-positive patients, complete and adequate SH gene sequences were generated from the samples of 808 patients (98.3%). Samples included oropharyngeal swabs, saliva and urine. Of patients for whom gender was known (n=791), 58.0% were males. Orchitis was the most frequently reported complication and was reported in 7.3% of the males who tested positive for mumps virus.

Diversity of mumps virus SH gene sequences

Phylogenetic analysis based on the SH gene sequences of 808 mumps-positive patients revealed that the majority of the outbreak strains belonged to genotype G (98.51%). Other genotypes that were observed during the outbreak were J (0.99%), H (0.25%) and D (0.25%). Within genotype G, two large clusters were identified: MuVs/Delft.NLD/03.10 (22.11%) and MuVs/Scheemda.NLD/12.10 (65.32%), further referred to as variants 1 and 2, respectively (Fig. 1).

Sequence changes among main clusters

The SH gene sequence of variant 1 was identical to the SH gene sequences of genotype G mumps strains found in other countries, such as MuVs-GBR0300796-G5 (Cui et al., 2009), MuV/NewYork.USA/40.09/4 and MuV/NewYork.USA/01.10. Variant 2 differed in 2 nt from variant 1 (nt 167 and 205), resulting in one silent and one missense mutation, without any alterations in the amino acid character (aa 52, Ser → Asn).

None of the recent SH nucleotide sequences registered in GenBank from mumps genotype G subtypes circulating before March 2010 were similar to the SH sequence of variant 2. However, the amino acid sequence was identical to a mumps isolate from the UK in 2005 (MuVs-GBR05-1700315-G5; Cui et al., 2009). After the appearance of variant 2 in the Netherlands, mumps strains with identical SH sequences were found in Belgium and Germany.

Distribution of genotypes over time

Throughout the three mumps outbreak seasons (September 2009–August 2012), a shift in the distribution of the two major circulating clusters was observed. In the first mumps outbreak season, variant 1 was the most prevalent subtype detected (78.4%), whereas during the second and third seasons, variant 2 was most prevalent (74.2 and 87.8%, respectively). In addition to these two large genotype G clusters, smaller clusters were observed with a maximum of 21 sequenced cases per cluster (Fig. 1). These subvariants appeared and circulated for a few months, but could not be related to specific student events (Fig. 2). Identification of those clusters was based on changes in 1 or 2 nt in the SH gene background from either mumps variant 1 or 2 and they probably originated from one of those two dominant subtypes. Owing to these uncertainties, the subvariants were excluded from further analyses.
Fig. 1. UPGMA (unweighted pair group method with arithmetic mean) tree based on the nucleotide sequence of the SH gene of 808 samples obtained between September 2009 and September 2012. The three different outbreak seasons are indicated by light grey, dark grey and black shading. Genotypes and variants are indicated on the right. For each cluster, the number of
Genotyping based on the HN gene

Phylogenetic analysis based on the HN gene sequences showed different clustering compared with genotyping based on the SH gene. Genotype G variant 2 had 6 nt substitutions compared with variant 1, resulting in one amino acid substitution (aa 203, Lys → Asn) (Fig. 3a). The amino acid sequence of variant 2 was identical to the amino acid sequences of the New York strains. In contrast to the SH gene of variant 1, the HN gene sequence was not identical to genotype G outbreak strains from New York. Combined, these data suggest that the two dominant clusters are distinct genetic lineages.

Genotyping based on the F gene

In line with the phylogenetic analyses based on the SH and HN genes, F gene sequences of variants 1 and 2 were distinguishable. The New York strains formed a cluster with variant 2 sequences, whereas variant 1 contained 10 mutations at the nucleotide level, resulting in two amino acid substitutions (aa 14, Phe → Val and aa 97, Ser → Leu). However, aa 492 differed between New York strains (Val) and variant 2 (Gly), but this substitution was not found in variant 1 (Val) (Fig. 3b). Combined with the SH and HN gene sequence analyses, these data support the notion that the two dominant genotype G clusters in this outbreak were indeed distinct genetic lineages.

Comparison of patient characteristics between the two major mumps genotype G clusters

Patients infected with genotype G variants 1 and 2 were compared with respect to age, gender, region, vaccination status and clinical parameters to investigate whether the dominance of variant 2 during the second and third mumps outbreak seasons in the Netherlands could be explained by any of the epidemiological factors assessed in this study (Table 1). Most patients with either variant 1 or 2 were males, but males were more predominant among variant 2-infected patients ($P=0.047$). Age and vaccination status did not differ significantly between patients infected with one of the two genotype G variants ($P>0.5$).

Proportionally more variant 1 cases compared with variant 2 cases were reported among students or student contacts and in cities with universities ($P<0.001$). During the first outbreak season, large clusters of variant 1 were reported in the Dutch student cities of Delft, Leiden, Groningen, and Utrecht, whereas variant 2 circulated mainly in the student city of Groningen. During the second and third mumps outbreak seasons, large variant 2 clusters were reported in various regions of the country, including the major student cities, whereas only a few patients with variant 1 were reported during this period (Figs 1 and 2).

Among fully MMR-vaccinated males infected with either mumps variant 1 or 2 ($n=249$), there were 21 cases (8.4 %) who developed orchitis. Eight other orchitis patients were unvaccinated (17.4 % out of 46 unvaccinated males), one male with orchitis had received only one MMR dose and for three males the vaccination status was unknown. Orchitis was more frequently reported for cases infected with variant 2 compared with variant 1 (Table 1). When adjusting for age and vaccination status, the association between the mumps variants and occurrence of orchitis remained independently significant ($P=0.045$; odds ratio 3.5; 95% confidence interval: 1.03–12.1). Of the patients with mumps variant 1, three out of the 92 male patients (3.3 %) were reported with orchitis. One of these males was unvaccinated and the other two males were fully vaccinated. Regarding the variant 2 mumps strain, orchitis was reported in 30 out of the 306 male patients (9.8 %) during the whole outbreak, including 19 fully vaccinated cases and six unvaccinated cases. When comparing only fully vaccinated cases, two out of 55 fully vaccinated male patients with variant 1 had orchitis (3.6 %), compared with 19 out of 194 vaccinated male patients infected with variant 2 (9.8 %) ($P=0.147$).

**DISCUSSION**

To find possible epidemiological explanations for the protracted endemic circulation of mumps genotype G in the Netherlands (September 2009–August 2012), we studied the genetic diversity and changes of mumps virus over a period of 3 years. Phylogenetic analysis based on SH gene sequences defined two major clusters within genotype G, termed variants 1 and 2, and background epidemiological and clinical data for patients infected with these two lineages differed.

The proportion of students and the percentage of patients living in cities with a university was significantly larger in variant 1 patients compared with variant 2 patients. This is probably explained by the fact that variant 2 was the dominant strain during the second and third outbreak seasons when mumps was also reported more often in non-student cities, whereas during the first outbreak season variant 1 was the dominant mumps strain and mumps was primarily circulating in the student cities (Sane et al., 2014). Students have more social contacts than non-students and they may therefore be more likely to transmit mumps. However, this difference does not explain the dominance of mumps variant 2 during the second and
third outbreak seasons, because during these seasons proportionally fewer students were infected with mumps. Orchitis was reported in 8.3% of the males infected with either mumps variant 1 or 2, which is comparable with the rates of orchitis in males reported during other recent mumps outbreaks among vaccinated adults (Barskey et al., 2012; Marin et al., 2008). We found that orchitis was more often reported among males who were infected with variant 2. No epidemiological explanation or bias could be found that might explain these differences, which suggests that the risk for developing mumps-associated orchitis is higher for vaccinated individuals infected with mumps virus variant 2 compared with variant 1. Whether this is also accompanied by genotype-specific differences in viral pathogenesis and viral transmission needs to be determined. Single amino acid changes in the HN, F and polymerase proteins have been associated with neuroattenuation, but no association between specific mutations and orchitis has been described so far (Malik et al., 2007, 2009; Rubin et al., 2003).

Although mumps is a notifiable disease in the Netherlands, we expect that the majority of cases have not been reported. This probably results in an under-representation of certain clusters within genotype G. Furthermore, it might be that patients suffering from orchitis are over-represented in our databases, since those patients are more likely to be reported than patients without any further complications. The high orchitis incidence during this outbreak is not related to a larger proportion of male patients, because after excluding orchitis cases, >50% of the patients still were males.

During the mumps outbreaks in the Netherlands between 2009 and 2012, primarily vaccinated young adults were affected, which points towards waning immunity (Dayan & Rubin, 2008). Furthermore, the mumps strains during the recent outbreak were from a different genotype than the vaccine strain. Poorer cross-protection in individuals with waning antibody concentrations or specific immunopathogenic factors might make mumps genotype G more infectious than other mumps genotypes. However, this has yet to be determined.

Fig. 2. Mumps genotype G variants by week of onset of disease from 1 September 2009 to 31 August 2012. (a) Reported patients with MuVs/Delft.NLD/03.10 (variant 1) and subvariants. (b) Reported cases of MuVs/Scheemda.NLD/12.10 (variant 2) and subvariants. The sequences of the subvariants differed in 1 or 2 nt from the major genotype G variants 1 and 2 (see also Fig. 1). The three epidemic seasons were defined as: 1 September 2009–31 August 2010 (season 1), 1 September 2010–31 August 2011 (season 2) and 1 September 2011–31 August 2012 (season 3).
Sequencing of the HN and F genes indicates that mumps genotype G variant 2 may not have directly emerged from variant 1 in the recent mumps outbreak. The amino acid substitution in variant 1 compared with variant 2 and genotype G strains circulating in other countries was reported previously (Kulkarni-Kale et al., 2007; Lim et al., 2003), but the function of this region in the HN gene has not been described. Although we found some mutations in the SH, HN and F gene sequences between two mumps genotype G subtypes that have different orchitis incidence rates, we have no indication that those particular mutations are associated with pathogenicity. More research is needed to assess whether the substitutions described above have an effect on neutralization of the virus resulting in a selective advantage. Furthermore, the discrepancy between phylogenetic analyses of the SH, HN and F gene sequences between two mumps genotype G subtypes that have different orchitis incidence rates, we have no indication that those particular mutations are associated with pathogenicity. More research is needed to assess whether the substitutions described above have an effect on neutralization of the virus resulting in a selective advantage. Phylogeny of mumps virus genotype G in the Netherlands

**METHODS**

**Clinical samples and patient data.** Clinical and epidemiological data of the mumps cases were obtained from an enhanced mumps surveillance by the RIVM in collaboration with municipal health services, as well as from confirmed cases reported by peripheral laboratories. The enhanced surveillance was based on the sampling of oral fluid and urine specimens from suspected cases, and these specimens were tested for the presence of mumps virus RNA by quantitative PCR. Serum, throat swabs and/or urine samples were collected for serology and virological testing. Laboratory-confirmed cases were reported by the municipal health services to the national registration system for notifiable infectious diseases in the Netherlands (Osiris) between 1 September 2009 and 31 August 2012. Notification criteria for mumps virus infection include at least one related symptom (acute onset of painful swelling of the parotid or other salivary glands, orchitis, or meningitis) and laboratory confirmation or an epidemiological link to a laboratory-confirmed case (Whelan et al., 2010).

From the laboratory database, cases were selected for whom oropharyngeal swabs, oral fluid or urine specimens were available. In total, during the study period, samples from 822 individuals were tested positive for mumps virus by the national laboratory at the RIVM. From these mumps-positive individuals, SH genes of 808 samples could be sequenced completely and adequately, and were therefore used for phylogenetic analysis. The HN and F gene sequences were analyzed using the neighbor-joining algorithm in MEGA6 software.

**Fig. 3.** UPGMA trees based on the nucleotide sequences of the HN and F genes of a subset of 17 outbreak samples and isolates from the 2009–2012 outbreak. (a) UPGMA tree based on the nucleotide sequence of the HN gene. (b) UPGMA tree based on the nucleotide sequence of the F gene. Reference strains included in the analyses were MuV/New York.USA/40.09/4 and MuV/New York.USA/01.10. Percentages at the branches indicate bootstrap values (1000 replicates). Black and grey shading represent identical genotype G variants based on SH gene sequences, with all SH sequences identical to MuVs/Delft.NLD/03.10 (variant 1) in black and all SH sequences identical to MuVs/Scheemda.NLD/12.10 (variant 2) in grey.
sequences were also obtained from a selection of outbreak samples (n=17). Distribution of the epidemiological variables from the laboratory database was largely similar to the national Osiris database, which includes all laboratory-confirmed cases and cases that could be linked directly to a laboratory-confirmed case (n=1557 for the study period). Data from the laboratory database were linked to the notification database by matching patients by dates of onset of disease, postal codes, gender and year of birth. In accordance with Dutch law, no informed consent was required for this study.

RNA extraction, cDNA synthesis and quantitative PCR. RNA was extracted from specimens with either the High Pure Viral Nucleic Acid kit or the MagNA Pure 96 (Roche Diagnostics) following the manufacturer’s protocol. An aliquot of 20 μl RNA was transcribed into cDNA with 200 U murine leukemia virus reverse transcriptase (Applied Biosystems), 0.2 mM dNTP mix (Roche Diagnostics), PCR buffer (Applied Biosystems), 1.5 mM magnesium chloride (Applied Biosystems), 20 U RNase inhibitor (Applied Biosystems) and 2.5 μM random hexamers (Applied Biosystems). cDNA mix was incubated at room temperature for 10 min, 37 °C for 50 min, 95 °C for 5 min and then cooled down to 4 °C. The SH gene was used as the target for the quantitative PCR up to March 2010. For all samples tested from then onwards, the mumps F gene was used as the target. Quantitative PCR was performed with either the LightCycler 2.0 (until April 2011) or the LightCycler 480 (Roche Diagnostics).

Sequencing. For sequencing of the 316 nt encoding the SH gene, 20 μl cDNA from samples with positive PCR results was used as a template for the first PCR, which was followed by a nested PCR that amplified 5 μl first PCR product. For the first PCR, primers BV6132 SH1 (nt 6133–6152) and BV2013 SH5 (nt 6539–6557) were used. Primers BV6138 SH3 (nt 6139–6159) and BV2011 SH6 (nt 6535–6555) were used for the nested PCR. After purification of the product with ExoSAP-IT (GE Healthcare), 4 μl amplified fragments was sequenced with primers BV6138 SH3 (nt 6139–6159) and BV2011 SH6 (nt 6535–6555).

For sequencing of the 1749 nt encoding the HN gene, a first PCR was performed with 20 μl cDNA and primers FW-HN1 (nt 6535–6555) and RV-HN1 (nt 8442–8460), followed by a nested PCR with 5 μl

Table 1. Characteristics [n (%)] of mumps cases by mumps virus genotype G variant

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Variant 1 (N=179)</th>
<th>Variant 2 (N=514)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>92 (51.4)</td>
<td>306 (59.5)</td>
<td>0.047</td>
</tr>
<tr>
<td>Female</td>
<td>86 (48.0)</td>
<td>202 (39.3)</td>
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<tr>
<td>Unknown</td>
<td>1 (0.6)</td>
<td>6 (1.2)</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
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<td></td>
<td></td>
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<td>0–3</td>
<td>1 (0.6)</td>
<td>1 (0.2)</td>
<td>0.769</td>
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<tr>
<td>4–12</td>
<td>2 (1.1)</td>
<td>9 (1.8)</td>
<td></td>
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<tr>
<td>13–17</td>
<td>13 (7.3)</td>
<td>40 (7.8)</td>
<td></td>
</tr>
<tr>
<td>18–25</td>
<td>120 (67.0)</td>
<td>320 (62.3)</td>
<td></td>
</tr>
<tr>
<td>&gt;25</td>
<td>23 (12.8)</td>
<td>77 (15.0)</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>20 (11.2)</td>
<td>67 (13.0)</td>
<td></td>
</tr>
<tr>
<td>Vaccination status (no. doses)</td>
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<tr>
<td>0</td>
<td>20 (11.2)</td>
<td>60 (11.7)</td>
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<tr>
<td>1</td>
<td>15 (8.4)</td>
<td>41 (8.0)</td>
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<tr>
<td>2</td>
<td>114 (63.7)</td>
<td>316 (61.5)</td>
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<td>≥3</td>
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<td>6 (1.2)</td>
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<tr>
<td>Vaccinated but unknown dose</td>
<td>2 (1.1)</td>
<td>15 (2.9)</td>
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<tr>
<td>Unknown</td>
<td>27 (15.1)</td>
<td>76 (14.8)</td>
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<td>&lt;0.0001</td>
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<td>Not a student and no contact with student</td>
<td>9 (5.0)</td>
<td>80 (15.6)</td>
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<td>University student or contact with university student</td>
<td>96 (53.6)</td>
<td>212 (41.2)</td>
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<tr>
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<td>10 (5.6)</td>
<td>71 (13.8)</td>
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<tr>
<td>Unknown</td>
<td>64 (35.8)</td>
<td>151 (29.4)</td>
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<tr>
<td>Residence in a student city*</td>
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<td>Student city</td>
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<td>58 (32.4)</td>
<td>245 (47.7)</td>
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<td>3 (0.6)</td>
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<td>Orchitis cases†</td>
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</tr>
<tr>
<td>Orchitis</td>
<td>23 (3.3)</td>
<td>30 (9.8)</td>
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<tr>
<td>No orchitis</td>
<td>89 (96.7)</td>
<td>276 (90.2)</td>
<td></td>
</tr>
</tbody>
</table>

P values were calculated using the χ² test excluding the unknowns.

*Student cities include Amsterdam, Delft, Eindhoven, Enschede, Groningen, Leiden, Maastricht, Nijmegen, Rotterdam, Stichtse Vecht, Tilburg, Utrecht and Wageningen.

†Only males were included.

‡Adjusted for vaccination status and age using logistic regression.
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first PCR product and primers FW-HN2 (nt 6539–6557) and RV-HN2 (nt 8435–8454). Sequencing PCR was performed with 4 μl nested PCR product, which was first purified. The six primers used for the sequencing PCR were FW-HN2, FW-HN712 (nt 7172–7191), RV-HN7233 (nt 7212–7233), FW-HN7795 (nt 7795–7814), RV-HN7842 (nt 7823–7842) and RV-HN2.

For sequencing of the 1617 nt encoding the F gene, the sequence was divided into three overlapping parts. For the first PCR, 10 μl cDNA was added to a mix containing primers F-FW1A (nt 4292–4313) and F-RV1A (nt 4967–4989) for fragment 1, F-FW2A (nt 4836–4886) and F-RV2A (nt 5520–5541) for fragment 2, and F-FW3A (nt 5477–5500) and F-RV3A (nt 6174–6194) for fragment 3. For all fragments, a nested PCR with 5 μl first PCR product, and primers F-FW1B (nt 4298–4319) and F-RV1B (nt 4963–4985) for fragment 1, F-FW2B (nt 4930–4949) and F-RV2B (nt 5579–5600) for fragment 2 and F-FW3B (nt 5480–5502) and F-RV3B (nt 6172–6191) for fragment 3 was performed. Sequencing PCR was performed with 4 μl nested PCR product, which was first purified. The six primers used for the sequencing PCR were F-FW1B, F-RV1B, F-FW2B, F-RV2B, F-FW3B and F-RV3B. For all sequencing PCRs, Big Dye Terminator version 3.1 was used (Applied Biosystems). PCR fragments were analysed on the 3730 DNA Analysyzer (Applied Biosystems).

Phylogenetic analysis. BioNumerics 7.1 software (Applied Maths) was used to compare both nucleotide and amino acid sequences and to recreate phylogenetic trees. Nucleotide sequences were translated with an online translation tool (http://web.expasy.org/translate/).

Statistical analysis. The χ² test was used for comparison of genotype G subtypes. To compare orchitis cases, logistic regression analysis was performed, adjusting for age and vaccination status of the patients. P<0.05 was considered statistically significant and all reported P values are two-tailed, spps (version 19) and GraphPad Prism (version 6) were used for all analyses.

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