# Unravelling Factors Contributing to Mumps Outbreaks

Sigrid Gouma

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# Unravelling Factors Contributing to Mumps Outbreaks

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# INTRODUCTION

# CHAPTER 1

## General introduction

## GENERAL CHARACTERISTICS OF MUMPS

Clinical mumps was first described by Hippocrates in the 5th century BC during a mumps outbreak on the island of Thasos (1). A causative agent for mumps was not demonstrated until 1934, when Johnson and Goodpasture showed that mumps is caused by a virus present in saliva of infected patients (2,3). The name mumps may be derived from the old English verb that means to grimace, grin, or mumble (4). In the pre-vaccination era, mumps was an endemic childhood disease with epidemic peaks every 2-5 years and the majority of cases among those aged 5-9 years (5). A Dutch seroepidemiological study from the pre-vaccine era shows that over 90% of children had acquired mumps before the age of 14 years and that only 5% of adults were seronegative (6). Classic mumps is characterized by parotitis and is usually a mild disease, although in the pre-vaccination era up to 15% of the mumps patients developed meningitis (7). Other complications included encephalitis, orchitis, oophoritis, mastitis, pancreatitis and deafness. Introduction of the measles, mumps, and rubella (MMR) vaccination in the Netherlands in 1987 has greatly reduced the morbidity rates of mumps as well as the number of hospitalizations due to mumps (8,9). However, during the last decade various mumps outbreaks occurred among MMR vaccinated populations in the Netherlands as well as in other countries worldwide, including the United States, Canada, Australia, Spain, Israel, Germany and Belgium (10–18). The majority of mumps patients in the recent mumps outbreaks among MMR vaccinated persons were adolescents and some outbreaks were specifically associated with educational settings or student events (15,19–21).

## GENOME ORGANIZATION AND PROTEIN FUNCTIONS

Mumps virus is a paramyxovirus that belongs to the *Rubulavirus* genus. The virus is enveloped and consists of a nonsegmented, negative-sense RNA genome of 15,384 nucleotides that includes 7 genes. Each gene encodes a single protein, with the exception of the V/P/I gene that encodes 3 different proteins (Figure 1). The viral genome is surrounded by the viral capsid, that consists of the nucleoprotein (N), the large protein (L) and the phosphoprotein (P) (Figure 1) (22,23). The L protein and the P protein are subunits of the viral RNA-dependent RNA polymerase and form replication complexes along with the N protein (24,25). The matrix protein (M), the haemagglutinin-neuraminidase protein (HN) and the fusion protein (F) form the viral envelope (Figure 1). The M protein links the replication complex to the viral membrane and is essential for virus assembly (26). The HN protein plays a critical role in viral entry via sialic acid binding. Furthermore, the protein acts as a neuraminidase by removal of sialic acid from

progeny virus particles to facilitate viral spread and promotes fusion activity of the F protein via cleavage (27–30). The HN protein is the major antigenic protein and various B cell and T cell epitope regions within the HN protein have been mapped (31,32). The F protein is important for both membrane fusion and virus-like particle production (26). Data on the antigenicity of the F protein is limited, but some B cell epitope regions have been mapped (33). The entropy for both the HN protein and the F protein is relatively low, which suggests that changes in these proteins might be a result of selection pressure (34). The SH protein is a membrane protein that is not essential for viral replication (35). This protein is important for viral pathogenesis, because it blocks apoptosis through inhibition of the tumor necrosis factor α (TNF-α) pathway, probably via its cytoplasmic tail (36–39). The mumps virus V protein plays also a role in mumps virus pathogenesis, because this protein blocks the interferon (IFN) and interleukin-6 (IL-6) signalling pathways (40–42). The role of the I protein in the virus life cycle is unknown. The V and I proteins are encoded by the same gene as the P protein. Via a process called “RNA editing” the generated mRNA is translated into 3 different open reading frames (ORFs), encoding the 3 proteins (43,44).

## MUMPS VIRUS GENOTYPES

Based on the nucleotide sequences of the SH gene and HN gene, 12 mumps virus genotypes have been defined so far, named A-N. Because the earlier proposed genotypes E and M are not validated by the phylogenetic analysis based on the most updated dataset, these genotypes do not longer exist (45). Mumps virus surveillance is usually performed based on solely SH gene sequences. However, the intra-genotypic variation in this gene is low, especially with respect to genotype G strains, as was shown during recent mumps outbreaks (34,46,47). Although the geographic and temporal distribution of mumps virus strains has not been well characterized, some global distribution through time has been described (34,45). Genotype G strains were first detected in the late 1990s and have been continually detected in many countries since then, contrary to most of the other genotypes that were only reported during a more defined period (34). Mumps virus genotype G strains caused many of the recent mumps outbreaks among persons vaccinated with the Jeryl Lynn mumps virus strain, that belongs to genotype A. Therefore, the recent mumps outbreaks among MMR vaccinated persons raise questions regarding potential differences between mumps virus genotype G strains and other wild type strains as well as differences between genotype G strains and the vaccine strain.

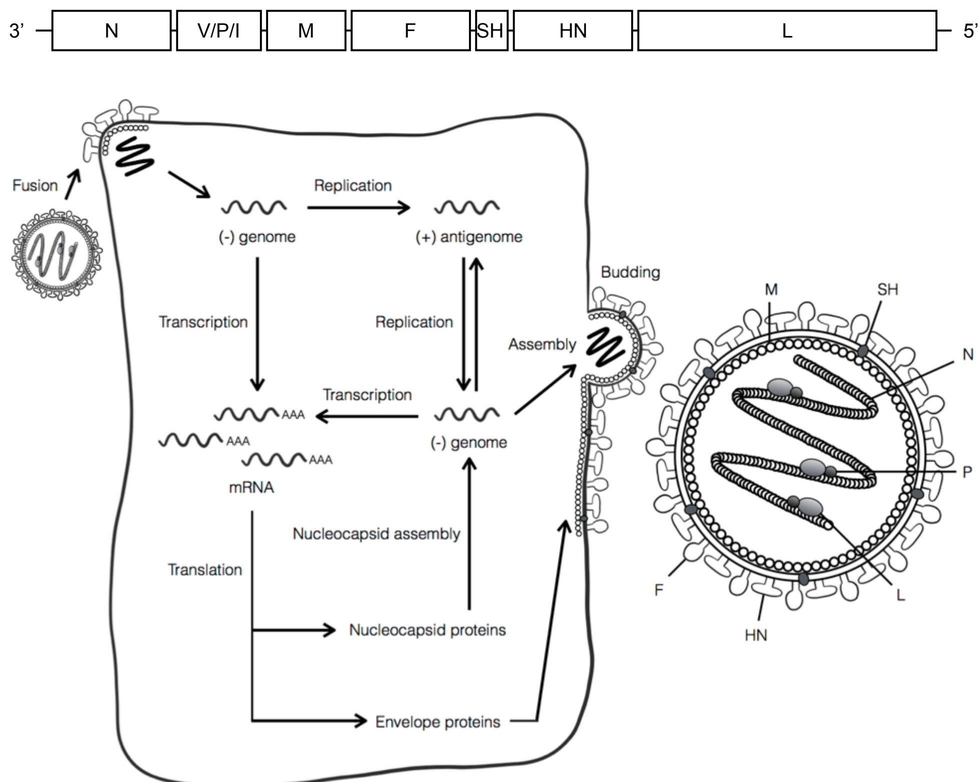


**Figure 1.** Schematic representation of the mumps virus genome structure, virion structure and replication cycle. The V and I proteins are non-structural proteins and are therefore not shown in the virion structure. N, nucleoprotein; V, V protein; P, phosphoprotein; I, I protein; M, matrix protein; F, fusion protein; SH, small hydrophobic protein; HN, haemagglutinin-neuraminidase protein; L, large protein.

## CLINICAL MANIFESTATIONS

The clinical hallmark of mumps is parotitis. Parotitis can be unilateral or bilateral and occurs in up to 98% of the clinical mumps cases (48–51). Other symptoms include fever, malaise, abdominal pain, nausea, headache and vomiting (48,50). However, many mumps virus infections run an asymptomatic course; about one third of the mumps virus infections are asymptomatic in unvaccinated persons (45). It has been suggested that the proportion of asymptomatic mumps virus infections is higher among MMR vaccinated persons, but the data about asymptomatic mumps virus infections are limited (52,53). The most common complication in adult males is orchitis. The clinical manifestations of orchitis are milder in vaccinated

patients than in unvaccinated patients and sterility after orchitis is rare (54,55). In adult females, oophoritis is reported at low frequencies (56). Other complications associated with mumps include meningitis, encephalitis, pancreatitis, mastitis and deafness (Table 1) (48,56). Myocarditis and nephritis have been rarely reported as complications associated with mumps (57). Long term complications and deaths associated with mumps are rare. Mumps virus reinfections have been reported, but are usually milder than primary mumps virus infections (58).

**Table 1.** Complications reported in mumps patients.

| | Mumps patients (%) | |
|---|---|---|
| Complications | Unvaccinated patients | Patients who received 2 doses of MMR vaccine |
| Meningitis | 0.4%-10% | 0.1%-1% |
| Encephalitits | 0%-0.7% | 0% |
| Pancreatitis | 0%-4% | 0%-0.6% |
| Orchitis* | 8%-38% | 2%-7% |
| Oophoritits† | 0%-4% | 0.8% |
| Mastitis† | 0%-31% | 0% |
| Deafness | 0%-4% | 0.1% |

* Percentage orchitis cases among males aged 12 years or older.
† Percentage oophoritis and mastitis cases among females aged 12 years or older.
References (5,9,48,50,56,59–61).

## MUMPS VIRUS PATHOGENESIS

Mumps virus is transmitted via direct contact or by airborne droplets and the incubation period varies between 2 and 4 weeks (62,63). The virus has been isolated from saliva from 7 days before until 8 days after onset of symptoms, which shows that the virus can be transmitted before disease onset (64). Mumps virus binds to sialic acid to enter the polarized epithelial cells in the upper respiratory tract from both sides (65). Apical entry facilitates transmission of virus to neighbouring cells, whereas infection from the basolateral side is probably important for secondary infection via the bloodstream (65). Mumps virus is predominantly released from the apical side of epithelial cells, which enables virus replication in the glandular epithelium and mumps virus shedding in saliva (65). So far, it is unclear how mumps virus breaks through the epithelial barrier. It has been suggested that mumps virus targets T cells, because the virus has a high affinity for T cells and efficiently replicates in these cells (66). Migrating mumps virus-infected T cells could facilitate spread from the respiratory tract to other sites of the body and might therefore play an important role in disease pathogenesis (66). Mumps virus infected cells might escape host immunosurveillance via degradation

of STAT1 and STAT3 by the mumps virus V protein. In this way, IFN and IL-6 signalling are blocked and the virus can evade both innate and adaptive antiviral responses (40–42,67,68). Furthermore, blockage of the IFN pathway enhances mumps virus replication, as IFN inhibitors promote mumps virus replication *in vitro* (69). However, the effect of the V protein on the magnitude of the IFN and IL-6 response is unclear, because IFN and IL-6 levels appear to be elevated in mumps patients, especially in patients with meningitis and/or encephalitis (70).

The mechanism behind the development of mumps parotitis and orchitis is unknown. It has been hypothesized that these complications result from lymphocytic infiltration and destruction of periductal cells that lead to blockage of the ducts in the salivary glands and the semeniferous tubules of the testes, respectively (71). The hypothesis that orchitis is caused by an immune mediated reaction is strengthened by the relatively rapid development of orchitis after MMR vaccination as was reported for 2 persons who were exposed to mumps in the past (72). The rapid development of post-vaccine orchitis in these cases may be a result of pre-existing immune responses in the testis, which react immediately upon exposure to mumps virus antigen. On the other hand, replicating mumps virus has been isolated from the testis and semen, which indicates that orchitis is the result of direct invasion of the testicular cells (73,74). Mumps virus shedding in urine is caused by dissemination of mumps virus to the kidneys and is associated with abnormal renal function (75,76). As these data show, further studies are required to obtain more knowledge about mumps virus pathogenesis and the potential role of T cells.

## IMMUNOLOGICAL RESPONSES

Mumps virus elicits the production of mumps virus-specific immunoglobulin (Ig)M and IgG by antibody-secreting B cells (ASCs). Primary immune responses are characterized by production of IgM, followed by low-avidity IgG. IgM peaks about one week after the onset of disease and declines during the first month, whereas IgG peaks about 3 weeks after the onset of disease and is detectable for many years (77–81). In secondary responses, IgM is often absent and high levels of high-avidity IgG are produced. Besides the production of antibodies by ASCs, memory B cells are generated. These memory B cells can rapidly produce antibodies during reinfection. Compared with the primary antibody response, the response during reinfection is typically faster and of greater magnitude (82). Mumps virus infection elicits relatively poor B cell memory, which may be a basis for susceptibility to reinfection (83). In the upper respiratory tract, mumps virus also elicits antibody responses at mucosal sites. Mumps-specific secretory antibodies, predominantly IgA, are produced in saliva after natural mumps virus infection (84).

IgA peaks about 1-2 weeks after onset of disease and is detectable for up to 10 weeks in saliva of mumps patients (84). Furthermore, mumps virus induces secretory IgA responses in the nasal cavity, which suggests that the nasal cavity is also an important protective barrier (71,85).

Besides humoral immune responses, cell mediated immunity is probably also important for elimination of mumps virus (71). Mumps-specific cytotoxic T cell responses peak about 2-4 weeks after onset of disease and the decline in cytotoxic T cell activity differs greatly between individuals (86). Cellular responses may be long-lasting, because significant lymphoproliferative responses, mumps-specific IFNγ and IL-10 production, and $CD4^+$ T cells were measured in adults who had a clinical mumps virus infection during childhood (81,87). The development of T cell responses upon mumps virus infection is probably independent of humoral responses, as there is no significant correlation between humoral responses and cellular responses both shortly after vaccination and on the long term (81,88). Furthermore, cellular immune responses may be sufficient to protect from mumps virus infection, because persons without detectable mumps-specific IgG concentrations do not always contract mumps infection upon heavy exposure to the virus (89).

## MEASLES, MUMPS, AND RUBELLA VACCINATION

Many different mumps virus strains have been used as component of the vaccine worldwide. The predominant mumps vaccine strains include Jeryl Lynn, RIT 4385, Urabe Am9, Leningrad-3, L-Zagreb, Rubini and S79 (90). In addition, a few other vaccine strains are used on a limited scale (63). The Rubini strain is the only strain that is recommended not to be used in national immunization programs (NIPs), because of its low effectiveness as compared with the other mumps vaccine strains, which could be attributed to the high number of passages used for attenuation (63,91). The vaccine strain used in the United States and in various European countries, including the Netherlands, is the Jeryl Lynn strain (90). In this thesis, we will focus on this widely used mumps vaccine strain when we discuss MMR vaccination.

The Jeryl Lynn strain was isolated in 1963 from a 5-year-old mumps patient, named Jeryl Lynn. Mumps virus was attenuated by passage in embryonated hens' eggs and in cell cultures of chick embryo (92). Nowadays, the nucleotide sequence of Jeryl Lynn is known and it appears that the vaccine is a mixture of 2 independently replicating mumps virus strains, designated JL2 and JL5 (93–95). The ratio of JL2 to JL5 is about 1:5 (94). Comparison of the nucleotide sequences of minor variant JL2 and major variant JL5 shows 414 nucleotide changes, resulting in 87 amino acid changes (96). This large number of differences suggests

that the variants derive from separate wild type mumps virus strains rather than from a common isolate (94).

MMR vaccination, with the Jeryl Lynn strain as mumps component, was introduced into the Dutch NIP in 1987 in a 2-dose vaccination schedule at 14 months and 9 years of age. Until 2008 all MMR vaccines administered via the NIP were produced by the Netherlands Vaccine Institute (NVI). From then onwards, Priorix (GSK) and MMR-Vaxpro (Sanofi Pasteur MSD) are used as MMR vaccine in the NIP. The high vaccination coverage of >95% for 1 dose and >92% for 2 doses of MMR resulted in a rapid decline in the number of reported mumps cases after introduction of the MMR vaccine into the NIP (8,97–99). However, mumps outbreaks among MMR vaccinated persons occurred in various countries worldwide during the last decade (12,15,17,18,100). Vaccine effectiveness (VE) for 2 MMR doses ranges from 66% to 95%, depending on the study population and exposure setting (18,19,21,101–106). Although the recent mumps outbreaks among MMR vaccinated persons show that the MMR vaccine does not fully protect against mumps virus infection, the vaccine provides protection against complications. VE for 2 MMR doses is estimated as 72%-81% against orchitis, which is the major complication reported during the recent mumps outbreaks (9,50,59,107).

MMR vaccination induces both humoral and cellular immune responses (87,88). Mumps-specific IgG concentrations in the blood are long-lasting, although antibody concentrations induced by MMR vaccination are lower than antibody concentrations induced upon mumps virus infection (81,108). A 20-year follow-up study shows that the decrease in both antibody concentration and avidity after MMR vaccination is bigger for mumps than for measles and rubella (109). Mumps vaccination induces strong memory T cell immunity, as lymphoproliferative responses, mumps-specific IFNγ and IL-10 production and the number of mumps-specific $CD4^+$ T cells are high in adults who received MMR vaccination during childhood (81,87). Since the cellular responses seem to last longer than the humoral responses, the waning in humoral responses as seen in adults who had received MMR vaccination during childhood does not necessarily mean that these adults become susceptible to mumps virus infection (81). Further studies are needed to investigate the importance of cellular immune responses induced by the mumps vaccine in protection against mumps virus infection.

## MUMPS OUTBREAKS AMONG MMR VACCINATED PERSONS

During the last decade, various mumps outbreaks among MMR vaccinated persons occurred worldwide. These outbreaks were mainly caused by mumps virus genotype G strains. The first mumps virus genotype G outbreak in the

Netherlands occurred in 2004 in a highly vaccinated student population at an international hotel school and was followed by another small outbreak in 2005 (16,110). After a mumps virus genotype D outbreak within an orthodox religious community with low vaccination coverage between 2007 and 2009, the next mumps virus genotype G outbreak in the Netherlands among MMR vaccinated persons started at the end of 2009 and was scaled-up by a large student party in February 2010, followed by a nationwide outbreak (17,19,111).

It is unlikely that the recent mumps outbreaks were caused by primary vaccine failure, because MMR vaccination results in high seroconversion rates (112,113). Furthermore, MMR vaccinated mumps patients have mumps-specific antibody responses prior to infection (53). Therefore, it is more probable that the recent mumps outbreaks were a result of secondary vaccine failure. MMR vaccination was introduced into the NIP in 1987, including a catch-up campaign for children born between 1983 and 1985 (98). The predominant age group affected during the mumps outbreaks were the adolescents between 18 and 25 years of age, so the majority of the mumps patients were in the first cohort of children who received an MMR vaccination in the 1980s. Since mumps incidence rapidly decreased after introduction of MMR vaccination into the NIP, these persons probably had never been exposed to mumps virus until the start of the mumps outbreaks in 2009. In the absence of natural mumps, the vaccine-induced immune responses were not boosted and therefore waned after the second MMR dose at 9 years of age (20,109,113,114). This hypothesis is supported by findings from a large Dutch cross-sectional population-based serosurveillance study performed in 2006-2007 (the so-called Pienter2 study), in which a small drop in seroprevalence to 87% was observed for the age cohort of 15-21 years (80). Furthermore, the risk for mumps disease during mumps outbreaks is positively associated with the time interval since last MMR vaccination and pre-outbreak antibody levels in serum are lower in mumps patients than in non-patients (53,115).

The recent mumps outbreaks among persons vaccinated with the Jeryl Lynn strain were mainly caused by mumps virus genotype G strains, whereas the Jeryl Lynn strain belongs to genotype A. A mismatch in both B cell epitopes and T cell epitopes between the vaccine strain and circulating wild type strains may contribute to the vaccine failure during the recent mumps outbreaks (32). Serological studies have shown that mumps virus genotype G strains are neutralized by vaccine-induced antibodies, although the level of neutralization is lower than for the vaccine strain (53,116). Investigation of differences in mumps virus surface proteins between the vaccine strain, genotype G and other wild type genotypes could contribute to the current understanding of mumps virus neutralization.

Since many of the mumps outbreaks were among students, the social behaviour of this group could play a role in the occurrence of the recent mumps outbreaks (19,117). It is believed that mumps virus transmission occurs through close contact, such as inhalation of infectious droplets or contact with contaminated surfaces (117). Many students live in a close contact environment, whereas prolonged exposure to someone with mumps is associated with an increased risk for mumps (118). Moreover, dormitory residents and attendees of large social events, including student parties, were at increased risk for mumps during the recent outbreaks (19,20,119). In these settings, sharing drinks and dancing in close proximity could enable mumps virus transmission via salivary droplets. Students in the Netherlands often travel between university cities, which may facilitate spread of the virus during a mumps outbreak. This was the case in 2010, when a student party in Leiden resulted in mumps virus spread to other university cities (17). However, further studies about mumps virus transmission in outbreak settings are needed to better understand factors that play a role in mumps virus transmission and to evaluate potential measures during future mumps outbreaks more effectively.

## THESIS OUTLINE

The aim of this thesis is to provide insights into possible causes for the recent mumps outbreaks among MMR vaccinated persons in the Netherlands. In this way, our research contributes to the evaluation of the MMR vaccine in the light of the recent mumps outbreaks worldwide and can be used to inform public health decisions. Specifically, the objectives of this thesis are to:

1) Investigate the role of epidemiological, virological and immunological factors in the occurrence of the recent mumps outbreaks;
2) Track mumps virus transmission using sequencing as a tool;
3) Study the effect of MMR vaccination on the development of mumps virus infection and severity of disease.

This thesis has been divided in 4 parts. In **Part II** the recent mumps outbreaks in the Netherlands and the molecular characterization of the circulating mumps virus strains are discussed, to investigate possible epidemiological and virological causes for the recent mumps outbreaks and to study mumps virus transmission. In **Chapter 2**, we analysed the epidemiology of a nationwide mumps epidemic in the Netherlands during 3 outbreak seasons, to study potential changes in transmission patterns over time and to investigate the effect of MMR vaccination on the risk for complications. In this way, we could provide information for future mumps prevention efforts. Additionally, the mumps virus strains that circulated during the

recent mumps outbreaks were phylogenetically analysed to study whether cases were caused by endemic circulation or not (**Chapter 3**). One of the sequence types became predominant during the outbreak and we investigated if solely epidemiological factors could explain this predominance or if its predominance was a result of virological differences between the mumps virus strains. Therefore, we increased the molecular resolution by adding F gene and HN gene sequencing to the recommended SH gene sequencing. Combining the nucleotide sequences from these genes enabled us to study mumps virus transmission, even in the absence of an epidemiological link (**Chapter 4**). Next, in **Chapter 5**, differences in the mumps virus surface proteins between the vaccine strain and wild type strains were studied to find a possible explanation for the occurrence of mumps genotype G outbreaks among MMR vaccinated persons.

In **Part III** the effect of MMR vaccination on mumps virus infection and severity of disease is evaluated. Paired pre- and post-outbreak mumps-specific IgG concentrations were measured in MMR vaccinated students to study attack rates and risk factors for mumps virus infection (**Chapter 6**). We also compared pre-outbreak mumps-specific IgG concentrations and strain-specific neutralization between mumps virus infected and non-infected students to identify a potential correlate of protection (**Chapter 7**). Furthermore, we compared clinical and laboratory data between mumps patients who had received 2 MMR doses and unvaccinated mumps patients to investigate if the MMR vaccine provides protection against mumps virus transmission and complications in mumps patients (**Chapter 8**).

In **Part IV** the implications of the data presented in this thesis are discussed (**Chapter 9**).

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# OUTBREAKS IN THE NETHERLANDS

# CHAPTER 2

## Epidemic of mumps among vaccinated persons, the Netherlands, 2009-2012

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## ABSTRACT

To analyse the epidemiology of a nationwide mumps epidemic in the Netherlands, we reviewed 1,557 notified mumps cases in persons who had disease onset during September 1, 2009-August 31, 2012. Seasonality peaked in spring and autumn. Most case-patients were males (59%), 18-25 years of age (67.9%), and vaccinated twice with measles-mumps-rubella vaccine (67.7%). Nearly half (46.6%) of cases occurred in university students or in persons with student contacts. Receipt of 2 doses of vaccine reduced the risk for orchitis, the most frequently reported complication (vaccine effectiveness [VE] 74%, 95% CI 57%-85%); complications overall (VE 76%, 95% CI 61%-81%); and hospitalization (VE 82%, 95% CI 53%-93%). Over time, the age distribution of case-patients changed, and proportionally more cases were reported from nonuniversity cities ($p<0.001$). Changes in age and geographic distribution over time may reflect increased immunity among students resulting from intense exposure to circulating mumps virus.

## INTRODUCTION

Mumps is an acute illness caused by mumps virus (family *Paramyxoviridae*) and characterized by fever, swelling, and tenderness of ≥1 salivary gland, usually the parotid gland. Complications associated with mumps include orchitis (inflammation of the testes), meningitis, pancreatitis, and deafness. Mumps virus is spread in respiratory droplets, and the incubation period is 15-24 days (median 19) (1).

Vaccination for mumps has been in use in industrialized countries for decades (2). The Netherlands began mumps vaccination in 1987, using the measles, mumps, and rubella combination vaccine (MMR). The vaccine, containing the Jeryl-Lynn mumps virus strain, is administered in a 2-dose schedule at 14 months and 9 years of age. Vaccination coverage of ≥1 dose of MMR has consistently been ≥93% since the introduction of the vaccination program (3). After the MMR program was launched, the incidence of mumps in the Netherlands decreased considerably; nevertheless, during the 2000s, several mumps outbreaks were detected. In 2004, an outbreak occurred among students at an international school (4), and in 2007–2008, an outbreak was detected mainly in a religious community that had low vaccination coverage (5). Since the end of 2009, a countrywide epidemic has been ongoing, affecting mainly student populations (6,7).

Mumps was notifiable in the Netherlands before 1999 and was made notifiable again in December 2008 (5). Mumps surveillance reports are released biweekly or monthly and include data on age and sex distribution, geographic distribution, vaccination, and contact status of case-patients. The report is distributed to public health professionals, including epidemiologists, virologists, and local-level health professionals, but comprehensive spatiotemporal characterization of the surveillance data has not been conducted. To provide information for future mumps prevention efforts, we used this surveillance data to assess the rates of illness and complications associated with the ongoing outbreak, to understand who is at risk for infection, and to assess whether transmission patterns have changed over time.

## METHODS

We reviewed data on mumps cases reported to the registration system for notifiable infectious diseases in the Netherlands (OSIRIS) during September 1, 2009–August 31, 2012. Notification criteria for mumps include >1 related symptom (i.e., acute onset of painful swelling of the parotid or other salivary glands, orchitis, or meningitis) and laboratory confirmation of infection or an epidemiologic link to a laboratory confirmed case (7). In addition to basic demographic information, notification data reported to OSIRIS included vaccination status and student or contact with student status. The questions on student/student contact status were

made more specific on April 19, 2010. For cases reported before that date, the information for the new variable was obtained from open-format questions. Laboratory confirmation criteria included >1 of the following: detection of mumps-specific IgM; detection of viral RNA; or isolation of the virus on cell culture. Genotyping targeting the gene encoding the small hydrophobic protein was performed on specimens submitted to the National Institute for Public Health and the Environment by using an in-house method.

We used the $\chi^2$ test for comparison of proportions and testing for trends over time and calculated a 3 week moving average to characterize trends and seasonality. Vaccine effectiveness (VE) was estimated as 1 – odds ratio. The odds ratio, which describes the association between complications/hospitalizations and vaccination status, was adjusted for age and sex (when outcome was orchitis, adjustment was done for age only) and estimated by using logistic regression. Associations with p values of $<0.05$ were considered statistically significant, and all reported p values are 2-tailed. Stata software version 12 (StataCorp, College Station, TX, USA) was used for the analyses.

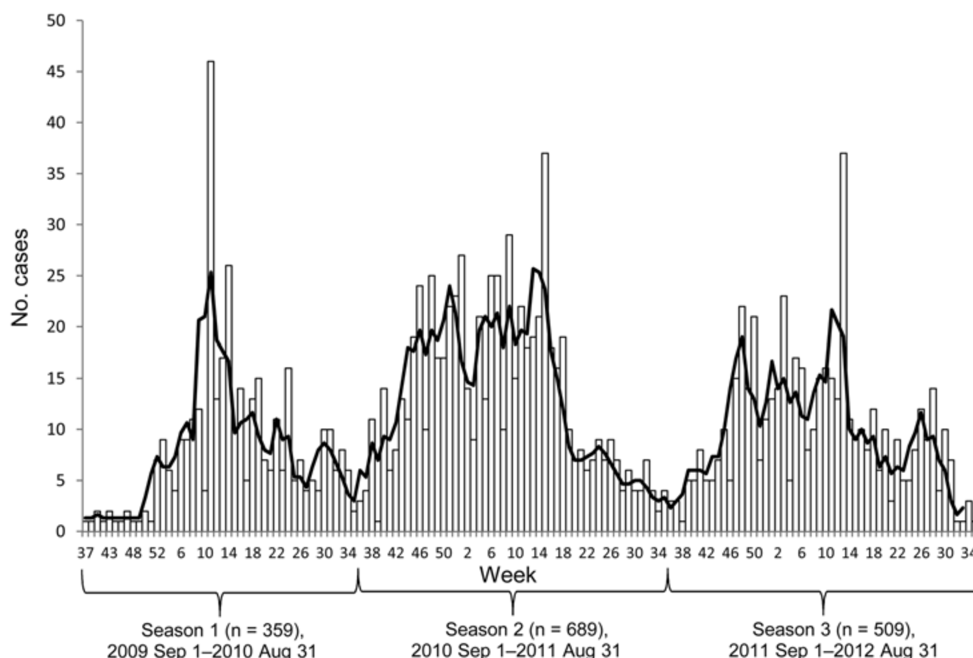


**Figure.** Numbers of notified mumps cases, by week of onset, The Netherlands, September 1, 2009-August 31, 2012 (N=1,557 cases). Seasons and number of cases (n) are indicated; black line indicates 3-week moving average.

## RESULTS

During September 1, 2009–August 31, 2012, a total of 1,557 cases of mumps were reported in the Netherlands (Figure); 1,254 (80.5%) of these were laboratory confirmed. Laboratory confirmation was most often by detection of viral RNA (68.8%), followed by antibody detection (21.9%) and virus isolation (7.3%). In 2% of cases, 2 methods were combined for diagnosis.

Most case-patients were male (59%) and 18–25 years of age (67.9%). The average annual incidence per 100,000 population was 0.5 for the 0–3-year age group, 0.8 for the 4–14-year age group, 4.5 for the 13–17-year age group, 21.4 for the 18–25-year age group, and 0.9 for the >25-year age group. Of the 1,474 cases for which patient vaccination status was reported, 998 (67.7%) case-patients had received 2 doses of MMR; 157 (10.6%) had received 1 dose, and 242 (16.4%) were unvaccinated. Genetic analysis of small hydrophobic gene sequences of 808 mumps-positive samples showed that most (98.5%) outbreak strains belonged to the G5 subtype.

Complications were reported in 126 cases (8.4% of 1,492 cases with known complication status) (Table 1). Most (78 [62%]) complications occurred in the 18–25-year age group. Orchitis was the most frequent complication (109 [12.7%] male case-patients >12 years of age) and occurred significantly more often among unvaccinated casepatients than among case-patients who had received 1 vaccine dose (p=0.04); vaccination with 2 doses of MMR reduced the risk for orchitis even further (p<0.01). Other reported complications were meningitis (n=6), pancreatitis (n=3), thyroiditis (n=1), and encephalitis (n=1). Three case-patients had permanent unilateral hearing loss that was probably caused by mumps virus infection. Deafness and meningitis occurred more frequently among unvaccinated than vaccinated persons, but those numbers were probably too low for statistical significance (Table 1).

A total of 31 patients (2.1% of 1,436 patients with known hospitalization status) were hospitalized. Risk for hospitalization was significantly lower among case-patients who had received 2 MMR doses than for unvaccinated case-patients (p<0.01); VE for preventing hospitalization was 82% (Table 1). Of the 31 hospitalized case-patients, 13 (42%) had orchitis. No deaths were reported.

Three distinct epidemic seasons occurred during the outbreak: seasonal peaks in spring and late autumn and a decline in number of cases during summer and, to some extent, during the Christmas holidays (Figure). Data on sex, age, vaccination status, residence in a city with a university, student status, and contact with student status by season are shown in Table 2. Overall, the age distribution of mumps case-patients differed significantly between the seasonal peaks (p=0.007). The number of cases increased proportionally over time for the 13–17-year age group

(p=0.003) and the >25-year age group (p=0.042) and decreased over time for the 18–25-year age group (p<0.001). The overall proportion of cases in vaccinated persons did not change (Table 2), and the proportion of complications or hospitalizations did not differ by season (data not shown).

We found significant seasonal differences in the proportion of cases occurring in students and in persons with student contacts (p<0.001). During early spring 2010, large clusters of cases were reported from university cities of Leiden and Delft, as described (6). However, during 2011 and 2012, proportionally more case-patients were not students and had no contact with students than during 2010 (p<0.001). The proportion of student case-patients enrolled in higher education other than university or case-patients who had contact with these nonuniversity students increased after 2010 (p<0.001). The absolute numbers of cases in these categories increased from 2010 to 2011 but stayed more or less constant, or decreased slightly, in 2012. The number of case patients who were university students or who had contact with university students decreased proportionally (p<0.001), and over time, proportionally more cases were reported from cities without universities (p<0.001). In addition, the total number of cases from nonuniversity cities was higher in 2012.

**Table 1.** Association between rates of mumps complications and hospitalization and MMR status, the Netherlands, September 1, 2009-August 31, 2012*

| Complication | No. MMR doses | No. (%) complications | Crude OR (95% CI) | p value | aOR† (95% CI) | p value | aVE† (95% CI) |
|---|---|---|---|---|---|---|---|
| Orchitis§ | 0 | 36 (15.5) | Ref | | Ref | Ref | Ref |
| | 1 | 10 (6.6) | 0.46 (0.22-0.97) | 0.04 | 0.46 (0.22-0.98) | 0.04 | 54 (2-78) |
| | 2 | 46 (4.7) | 0.26 (0.16-0.41) | <0.01 | 0.26 (0.15-0.43) | <0.01 | 74 (57-85) |
| Deafness | 0 | 2 (0.9) | Ref | | | | |
| | 1 | 0 | NA | NA | - | - | - |
| | 2 | 1 (0.1) | 0.12 (0.01-1.3) | 0.1 | - | - | - |
| Meningitis | 0 | 2 (0.8) | Ref | | | | |
| | 1 | 1 (0.6) | 0.76 (0.07-8.5) | 0.8 | - | - | - |
| | 2 | 2 (0.2) | 0.24 (0.03-1.7) | 0.2 | - | - | - |
| All complications | 0 | 44 (19.0) | Ref | | Ref | Ref | Ref |
| | 1 | 10 (6.6) | 0.30 (0.15-0.62) | <0.01 | 0.29 (0.14-0.62) | <0.01 | 71 (38-86) |
| | 2 | 55 (5.7) | 0.26 (0.17-0.39) | <0.01 | 0.24 (0.14-0.39) | <0.01 | 76 (61-86) |
| Hospitalization | 0 | 11 (4.8) | Ref | | Ref | Ref | Ref |
| | 1 | 3 (2.0) | 0.41 (0.11-1.5) | 0.18 | 0.43 (0.11-1.6) | 0.2 | 57 (-60 to 89) |
| | 2 | 10 (1.1) | 0.22 (0.09-0.52) | <0.01 | 0.18 (0.07-0.47) | <0.01 | 82 (53-93) |

* Only case-patients with known complications and vaccination status were included in the analyses. OR, odds ratio; VE, vaccine effectiveness; Ref, referent; NA, not applicable; -, not analysed (insufficient sample size).

† Adjusted for age (age groups <18 y, 18-25 y, >25 y) and sex, except orchitis, which was adjusted only for age.

§ Includes only male case-patients >12 y of age.

**Table 2.** Demographic characteristics and student status for 1,557 patients with mumps, by annual epidemic season, the Netherlands, September 1, 2009-August 31, 2012*

| | No. (%) case-patients | | |
|---|---|---|---|
| Characteristic | Season 1, 2009 Sep 1-2010 Aug 31, n=359 | Season 2, 2010 Sep 1-2011 Aug 31, n=689 | Season 3, 2011 Sep 1-2012 Aug 31, n=509 |
| Sex | | | |
| M | 205 (57.1) | 416 (60.4) | 296 (58.2) |
| F | 154 (42.9) | 271 (39.3) | 213 (41.8) |
| Unknown | 0 | 2 (0.3) | 0 |
| Age, y | | | |
| 0-3 | 3 (0.8) | 4 (0.6) | 3 (0.6) |
| 4-12 | 5 (1.4) | 22 (3.2) | 16 (3.1) |
| 13-17 | 17 (4.7) | 63 (9.1) | **54 (10.6)** |
| 18-25 | 270 (75.2) | 468 (67.9) | **318 (62.4)** |
| >25 | 64 (17.8) | 131 (19) | **118 (23.2)** |
| Unknown | 0 | 1 (0.2) | 0 |
| Vaccination status | | | |
| 0 doses | 57 (15.9) | 115 (16.7) | 70 (13.7) |
| 1 dose | 37 (10.3) | 69 (10.0) | 51 (10.0) |
| 2 doses | 225 (62.7) | 436 (63.3) | 337 (66.2) |
| ≥3 doses | 4 (1.1) | 4 (0.6) | 5 (1.0) |
| Vaccinated but unknown no. doses | 24 (6.7) | 25 (3.6) | 15 (3.0) |
| Unknown | 12 (3.3) | 40 (5.8) | 31 (6.1) |
| Residence in a city with university† | | | |
| Yes | 258 (71.9) | 351 (50.9) | **243 (47.7)** |
| No | 92 (25.6) | 322 (46.7) | **263 (51.7)** |
| Unknown | 9 (2.5) | 16 (2.3) | 3 (0.6) |
| Student/contact with students | | | |
| Not a student and no contact with students | 22 (6.1) | 171 (24.8) | **118 (23.2)** |
| University student or contact with university students | 229 (63.8) | 275 (39.9) | **221 (43.4)** |
| Other student‡ or contact with other students | 20 (5.6) | 144 (20.9) | **88 (17.3)** |
| Unknown | 88 (24.5) | 99 (14.4) | 82 (16.1) |
| Incidence estimates§ | | | |
| University students | 92.9 | 93.9 | 80.2 |
| Other students | 2.0 | 14.7 | 9.8 |
| Secondary school students | 0 | 0.7 | 5.4 |

* Boldface indicates significance trends by $\chi^2$ test, calculated by using proportions excluding unknowns.

† University cities: Amsterdam, Delft, Eindhoven, Enschede, Groningen, Leiden, Maastricht, Nijmegen, Rotterdam, Stichtse Vecht, Tilburg, Utrecht, and Wageningen.

‡ Students enrolled in higher education other than university.

§ Incidence per 100,000 students. Total student numbers by category obtained from www.cbs.nl.

## DISCUSSION

The epidemic of mumps in the Netherlands during late 2009 through 2012 affected mainly vaccinated students. However, vaccination evidently offered protection against mumps-associated complications. The epidemic showed a seasonal trend, although cases were identified throughout the years. Over time, age, student status, and geographic distribution changed, which suggests a slight shift in transmission trends from student populations to younger and older nonstudent populations and to cities without a university. This shift may relate to increased immunity in the primarily affected high-risk student population; exposure to wild-type mumps virus may have boosted individual immunity and thus contributed to increased herd immunity.

Mumps outbreaks among vaccinated populations have been reported in other countries during recent years: a 2006 outbreak in the United States (8), a 2009–2010 outbreak in Canada (9), and a 2012 outbreak in the United Kingdom (10). Description of an outbreak in 2009–2010 in the northeastern United States among a highly vaccinated population of Orthodox Jews indicated that intense exposure among boys in a religious school facilitated the transmission of mumps virus, which overpowered the vaccine-induced protection (11,12). Similar to our findings, transmission in that outbreak shifted from adolescents to younger and older populations over time. The intense social crowding among students (e.g., large indoor social gatherings) partly explains why secondary vaccine failure occurred in the outbreak described in this study. A subgroup of students, including those living with many other students and members of university fraternities, may be at increased risk for infection (6,7). Crowding in nonstudent populations may not be as intense as among students, and mixing is usually with more heterogeneous age groups. In these circumstances, herd immunity is sufficient to prevent more widespread transmission. A lower rate of crowding may be one explanation for the relatively low numbers of cases among 4–12-year-olds, despite the generally lower IgG titers in this group than in adolescent students (13). Still, even though lower antibody levels do not automatically mean higher risk for mumps virus infection (14), a higher rate of illness would have been expected in the 4–12-year age group. An additional explanation for the lower apparent illness rate among these younger children might be a higher frequency of unapparent and subclinical infections, which would lead to many undiagnosed cases in this age group.

Most of the persons affected in the epidemic were male, a finding also observed in other studies (15,16). The reasons for male predominance are unclear, but significantly higher mumps antibody titers in female than in male persons have been demonstrated (13,17); this finding, in turn, may be linked to gender-

associated genetic differences in immune response. Behavioural differences between sexes may also play a role.

Most cases occurred in persons who had received 2 doses of MMR, which suggests inadequate effectiveness of the vaccine. Recent studies indicate the effectiveness of MMR against mumps is moderate and lower than the clinical efficacy estimates (1,18). Postlicensure studies of 2 doses (Jeryl-Lynn strain) of MMR have provided a median VE estimate of 88% (range 79%–95%) (2). A recent study of an outbreak of mumps at a student party in the Netherlands estimated a VE of 68% for 2 doses of MMR (6). This estimate is, however, uncertain because of the low number of unvaccinated case-patients. We attempted to provide VE estimates against clinical mumps applying the screening method; however, because this method is most vulnerable to error when proportions of the population and case-patients vaccinated are high (19), as in this study, the estimates became inaccurate and thus are not included in our results. The possible causes for lower than expected VE include secondary vaccine failure (waning immunity), intense exposure to high virus inoculum, and a possible mismatch between the vaccine genotype and circulating strains (1,2,18,20). However, because the level of antibodies correlating with protection remains unknown (12,21), we are unable to further elucidate the role of these factors.

Orchitis was the most common complication, consistent with previous outbreaks in a population with a similar age structure (1). However, orchitis occurred significantly more often among unvaccinated than vaccinated case-patients, and the vaccine was effective in preventing orchitis, which has previously been shown in a study based in part on the same study population (22) and in other studies (11,23). Vaccination also significantly reduced the risk for complications overall and for hospitalizations. A previous report described 3 cases of deafness (0.19% of all notified infections), 2 in unvaccinated persons (24). The frequency of 0.005% for unilateral deafness commonly cited in the literature (25) is considerably lower than that found in our study, but this difference is likely attributable to a different denominator population. A higher incidence of deafness has been reported from Japan using more appropriate denominators (26).

One limitation of our study was the short time span for assessing changes over time. Mumps cases have continued to occur after our study period, but the number of cases reported after September 2012 (180 as of August 31, 2013) is much lower than that reported during the previous years. Recent numbers indicate that a similar trend in changing patterns of age and geographic distribution is ongoing; most of the more recent cases have occurred in nonstudents and in age groups other than 18–25 years (data not shown). However, because of lower case numbers, this comparison must be interpreted with caution.

A further limitation of our study is that it is likely that many mumps cases are not notified because they are subclinical infections or because of reluctance to seek medical care; thus, these cases are not included in our analyses. Furthermore, complications that occurred after the notification date are not included; however, because vaccination status is probably not associated with the reporting of complications, we regard our VE estimates against complications as unbiased.

Although VE for mumps vaccination is not optimal for preventing clinical disease, our results support previous findings that vaccination limits the severity of disease. Because complications are the primary mumps-associated public health problem, these findings support the current vaccination recommendations. Still, this epidemic demonstrates that mumps virus can cause large outbreaks even in highly vaccinated populations. The observation that the incidence after the third season studied has been considerably lower than during previous seasons is consistent with the development of herd immunity among high-risk students resulting from the high rate of natural symptomatic and asymptomatic infections. However, the annual inflow of new susceptible students – unvaccinated and vaccinated – who start their studies could again lower overall immunity. A recent study suggested that use of a third MMR dose might be an effective control measure in certain outbreak situations (27). Introduction of a third MMR dose to the vaccination schedule has been considered in the Netherlands (6) but was not recommended because of relatively low overall illness rates associated with mumps and other factors, including an expected low vaccine uptake. Although the vaccine remains effective in most settings and significantly reduces the risk for complications, further research is needed to understand the limitations of MMR, and modeling is warranted to understand the dynamics of mumps virus transmission in future.

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# OUTBREAKS IN THE NETHERLANDS

# CHAPTER 3

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

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## ABSTRACT

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 (1).

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 (2–5). 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 (6).

The first mumps outbreak in the Netherlands occurred in 2004 among a highly vaccinated student population at an international hotel school (7). The second mumps outbreak occurred between 2007 and 2009 within an orthodox religious community with low vaccination coverage (8). 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 (9,10).

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 (11). 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 (12–14). 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 (15). The F protein is a surface protein that is involved in membrane fusion and viral entry (16). 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.

## 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 (10).

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 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=1,557 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 ml 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 mM 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 ml cDNA from samples with positive PCR results was used as a template for the first PCR, which was followed by a nested PCR thaamplified 5 ml 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 ml 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 ml cDNA and primers FW-HN1 (nt 6535–6555) and RV-HN1 (nt 8442–8460), followed by a nested PCR with 5 ml first PCR product and primers FW-HN2 (nt 6539–6557) and RVHN2 (nt 8435–8454). Sequencing PCR was performed with 4 ml nested PCR product, which was first purified. The six primers used for the sequencing PCR were FW-HN2, FW-HN7172 (nt 7172–7191), RV-HN7233 (nt 7212–7233), FW-HN7795 (nt 7795–7814), RVHN7842 (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 ml 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 4863–4886) 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 ml 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 ml 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 Analyser (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 x2 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. SPSS (version 19) and GraphPad Prism (version 6) were used for all analyses.

## 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 (Figure 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 (17), MuV/New York.USA/40.09/4 and MuV/New York.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) (17). 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 (Figure 1). These subvariants appeared and circulated for a few months, but could not be related to specific student events (Figure 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.

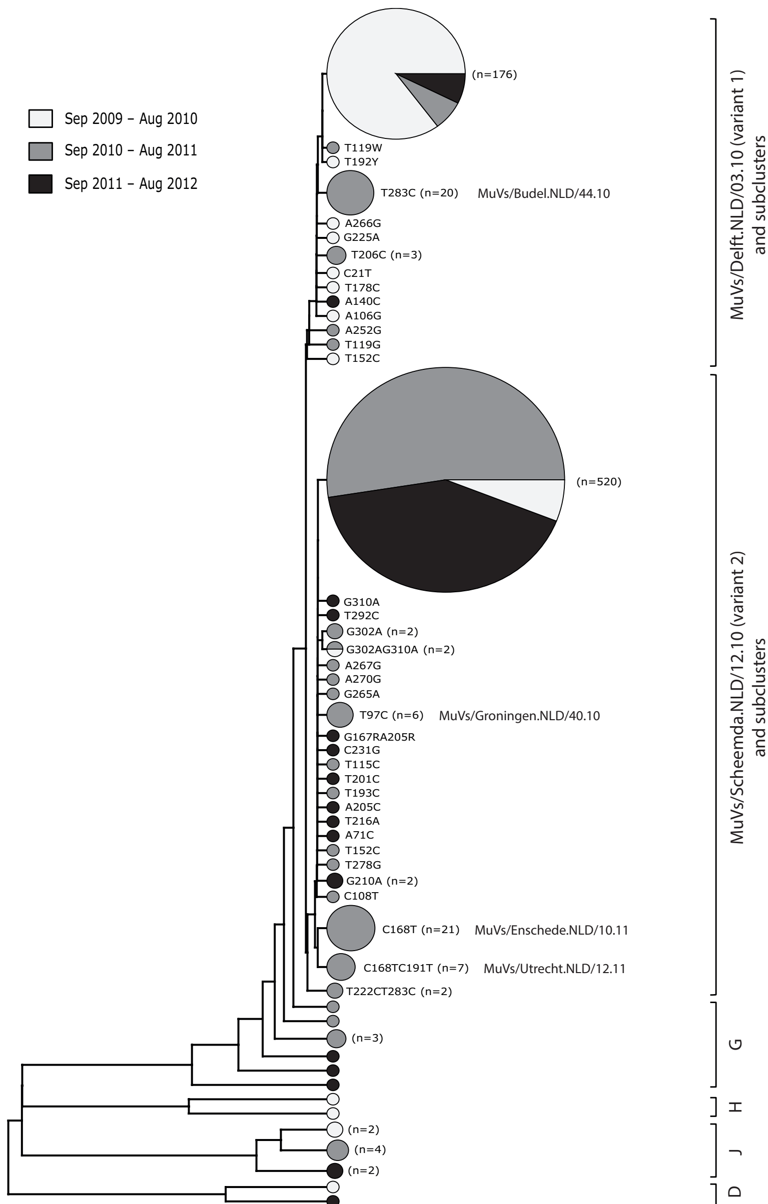



**Figure 1.** UPGMA (unweighted pair group method with arithmetic mean) tree based on the nucleotide sequence of the SH gene of 808 samples obtained between September 2009 and September 2012. The three different outbreak seasons are indicated by light grey, dark grey and black shading. Genotypes and variants are indicated on the right. For each cluster, the number of identical sequences is indicated in parentheses. Substitutions are shown for each subcluster, including nucleotide positions within the SH gene. The nucleotides that differ are indicated by single-letter codons.

## 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) (Figure 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.

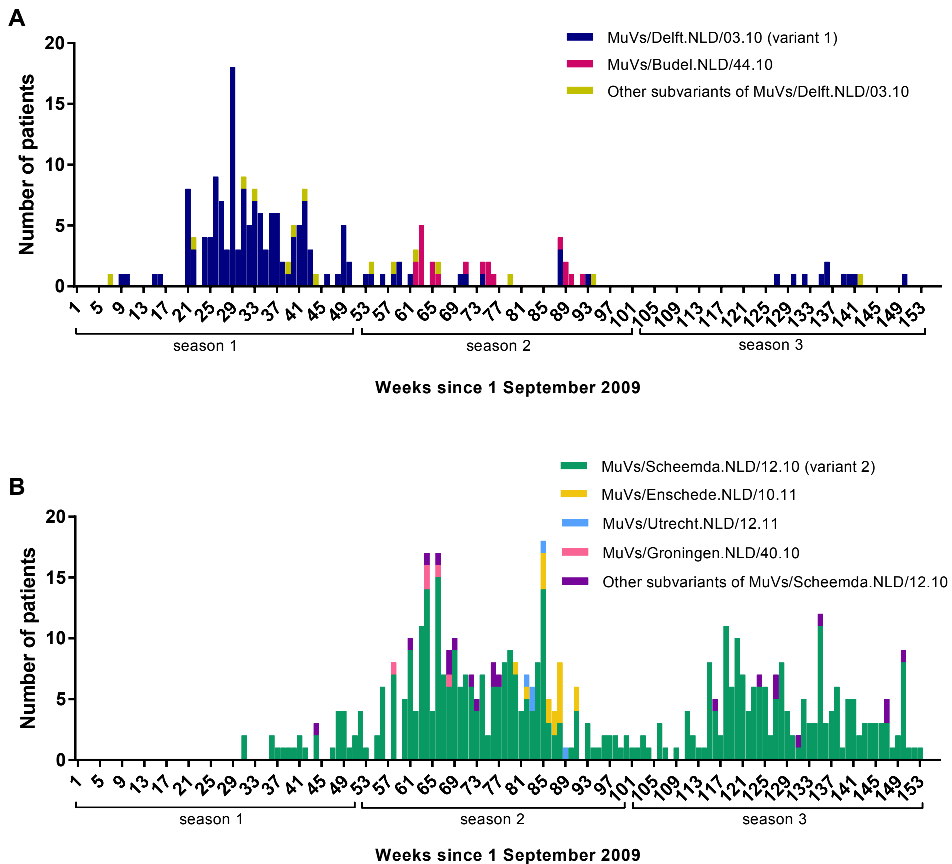



**Figure 2.** Mumps genotype G variants by week of onset of disease from 1 September 2009 to 31 August 2012. (A) Reported patients with MuVs/Delft.NLD/03.10 (variant 1) and subvariants. (B) Reported cases of MuVs/Scheemda.NLD/12.10 (variant 2) and subvariants. The sequences of the subvariants differed in 1 or 2 nt from the major genotype G variants 1 and 2 (see also Figure 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).

## 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) (Figure 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

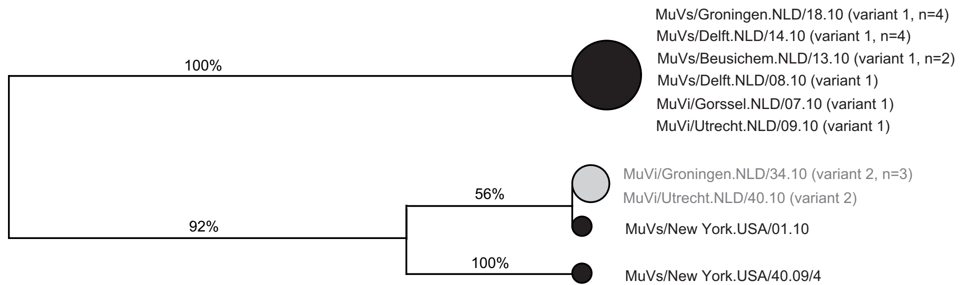
Patients infected with genotype G variants 1 and 2 were compared with respect to age, gender, region, vaccination status and clinical parameters to investigate whether the dominance of variant 2 during the second and third mumps outbreak seasons in the Netherlands could be explained by any of the epidemiological factors assessed in this study (Table). 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 (Figures 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). 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).

A




B

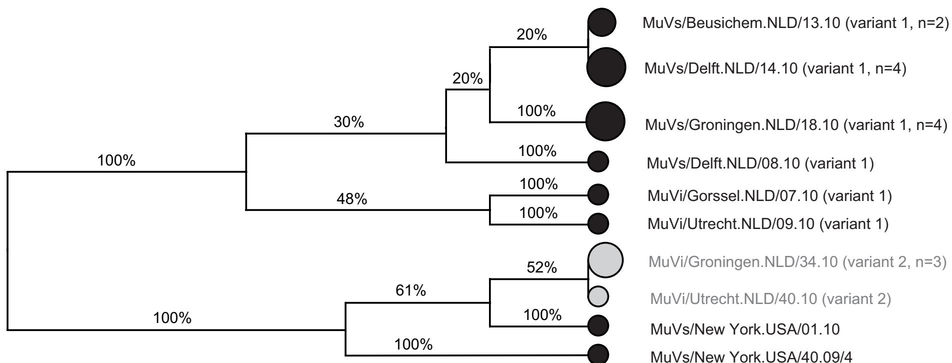



**Figure 3.** UPGMA trees based on the nucleotide sequences of the HN and F genes of a subset of 17 outbreak samples and isolates from the 2009-2012 outbreak. (A) UPGMA tree based on the nucleotide sequence of the HN gene. (B) UPGMA tree based on the nucleotide sequence of the F gene. Reference strains 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.

## 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 nonstudent cities, whereas during the first outbreak season variant 1 was the dominant mumps strain and mumps was primarily circulating in the student cities (18). Students have more social contacts than nonstudents 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 (2,19). 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 (20–22).

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.

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

| Characteristic | Variant 1 (n=179) | Variant 2 (n=514) | p value |
|---|---|---|---|
| Gender | | | 0.047 |
| Male | 92 (51.4) | 306 (59.5) | |
| Female | 86 (48.0) | 202 (39.3) | |
| Unknown | 1 (0.6) | 6 (1.2) | |
| Age (years) | | | 0.769 |
| 0-3 | 1 (0.6) | 1 (0.2) | |
| 4-12 | 2 (1.1) | 9 (1.8) | |
| 13-17 | 13 (7.3) | 40 (7.8) | |
| 18-25 | 120 (67.0) | 320 (62.3) | |
| >25 | 23 (12.8) | 77 (15.0) | |
| Unknown | 20 (11.2) | 67 (13.0) | |
| Vaccination status (no. doses) | | | 0.662 |
| 0 | 20 (11.2) | 60 (11.7) | |
| 1 | 15 (8.4) | 41 (8.0) | |
| 2 | 114 (63.7) | 316 (61.5) | |
| ≥3 | 1 (0.6) | 6 (1.2) | |
| Vaccinated but unknown dose | 2 (1.1) | 15 (2.9) | |
| Unknown | 27 (15.1) | 76 (14.8) | |
| Student and contact with students status | | | <0.0001 |
| Not a student and no contact with student | 9 (5.0) | 80 (15.6) | |
| University student or contact with university student | 96 (53.6) | 212 (41.2) | |
| Other student or contact with other student | 10 (5.6) | 71 (13.8) | |
| Unknown | 64 (35.8) | 151 (29.4) | |
| Residence in a student city* | | | 0.0004 |
| Student city | 120 (67.0) | 266 (51.8) | |
| No student city | 58 (32.4) | 245 (47.7) | |
| Unknown | 1 (0.6) | 3 (0.6) | |
| Orchitis cases† | | | 0.045‡ |
| Orchitis | 3 (3.3) | 30 (9.8) | |
| No orchitis | 89 (96.7) | 276 (90.2) | |

p values were calculated using the $\chi^2$ 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.

During the mumps outbreaks in the Netherlands between 2009 and 2012, primarily vaccinated young adults were affected, which points towards waning immunity (23). 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.

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 (24,25), 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 illustrates that sequencing of additional genes improves the resolution of the molecular data, especially for ongoing outbreaks in which one particular genotype such as genotype G is highly prevalent and frequent importations of the same genotype might occur. Further studies are needed to investigate if mumps genotype G variant 2 has a virological advantage over variant 1 with respect to viral transmission and pathogenesis.

## ACKNOWLEDGEMENTS

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# OUTBREAKS IN THE NETHERLANDS

# CHAPTER 4

## Mumps virus F gene and HN gene sequencing as a molecular tool to study mumps virus transmission

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## ABSTRACT

Various mumps outbreaks have occurred in the Netherlands since 2004, particularly among persons who had received 2 doses of measles, mumps, and rubella (MMR) vaccination. Genomic typing of pathogens can be used to track outbreaks, but the established genotyping of mumps virus based on the small hydrophobic (SH) gene sequences did not provide sufficient resolution. Therefore, we expanded the sequencing to include fusion (F) gene and haemagglutinin-neuraminidase (HN) gene sequences in addition to the SH gene sequences from 109 mumps virus genotype G strains obtained between 2004 and mid 2015 in the Netherlands. When the molecular information from these 3 genes was combined, we were able to identify separate mumps virus clusters and track mumps virus transmission. The analyses suggested that multiple mumps virus introductions occurred in the Netherlands between 2004 and 2015 resulting in several mumps outbreaks throughout this period, whereas during some local outbreaks the molecular data pointed towards endemic circulation. Combined analysis of epidemiological data and sequence data collected in 2015 showed good support for the phylogenetic clustering.

## INTRODUCTION

After introduction of the measles, mumps, and rubella (MMR) vaccination in the Netherlands in 1987, the incidence of mumps rapidly declined to less than 1/100,000 per year in the 1990s (1). However, during recent years various mumps outbreaks have occurred in the Netherlands, particularly among adolescents who had received 2 MMR doses (2). Mumps outbreaks among MMR vaccinated persons were also reported in various other countries (3–6). Genotyping of the virus plays an important role in mumps virus surveillance. The molecular data serve to distinguish wild type mumps virus strains from vaccine strains and to track the geographic and temporal distribution at a global level (7). Based on the nucleotide sequences of the small hydrophobic (SH) gene and the haemagglutinin-neuraminidase (HN) gene, 12 mumps virus genotypes have been defined so far (8). SH gene based typing is the reference method as the SH gene is considered to be the most variable gene in the mumps virus genome (9). However, genotype G strains show little diversity when using SH gene based typing and large clusters of mumps virus strains with identical SH gene sequences have been found in recent outbreaks in the Netherlands and elsewhere (10–12).

The first such outbreak in the Netherlands among a highly vaccinated population occurred in 2004 at an international hotel school (13). After this outbreak, mumps virus genotype G cases were repeatedly reported, but the incidence was relatively low. The second outbreak (2007-2009) occurred within an orthodox religious community with low vaccination coverage and was caused by mumps virus genotype D (14). During the largest mumps outbreak (2009-2012), which was caused by mumps virus genotype G, many twice MMR vaccinated students as well as non-students throughout the country were reported with mumps (2,10). Between 2013 and mid 2015, there were only small local mumps outbreaks as well as periods with sporadic mumps cases. The majority of these cases were due to mumps virus genotype G infections. These observations triggered the question whether mumps was endemic in the country, or if these outbreaks reflected repeated importations.

To address this question, we have sequenced the fusion (F) gene, SH gene and HN gene from 109 mumps virus genotype G strains obtained between 2004 and mid 2015 in the Netherlands to track mumps virus transmission and to identify possible mumps virus clusters, which was not possible based on the low resolution SH gene typing (10). The F gene and HN gene were chosen because these genes encode for surface proteins that play important roles in mumps virus pathogenesis and immunity. By combining sequences of the F gene, SH gene and HN gene, we have investigated whether the observed outbreaks resulted from locally circulating viruses or from new introductions. A well characterized mumps outbreak in 2015

was studied to investigate added value of molecular typing over epidemiological data.

## METHODS

### Clinical samples

Clinical samples that yielded a complete mumps virus F gene, SH gene and HN gene sequence from patients diagnosed between 2004 and 2015 were used in this study (Table 1). In all samples mumps virus had been detected via polymerase chain reaction (PCR). Only 1 sample per mumps patient was included. Analysed samples included saliva samples, throat swabs and urine specimens, depending on availability. Epidemiological data about geographical location, source of transmission and day of onset of disease was retrieved from the national registration system for notifiable diseases in the Netherlands (Osiris) to provide background for analysis of robustness of clustering based on molecular data. In addition, epidemiological data from 67 mumps patients who were reported in Osiris in the first half of 2015 was used to study transmission.

### RNA extraction and cDNA synthesis

RNA was extracted from specimens with either the High Pure Viral Nucleic Acid kit or the MagNA Pure 96 (Roche Diagnostics), according to manufacturer's protocol. cDNA was synthesized using random hexameres (Invitrogen) as described previously (10) or using specific primers FW-F4351 and FW-HN6497 G together with Superscript III reverse transcriptase (Invitrogen; Supplementary table 1).

### Sequencing

All primers used for sequencing are described in Supplementary table 1. The SH gene was amplified as described previously using primers BV6132 SH1 and BV2013 SH5 during the first PCR and primers BV6138 SH3 and BV2011 SH6 during the nested PCR (10). Thereafter, sequencing of the 316 nt encoding the SH gene was performed using primers BV6138 SH3 and BV2011 SH6. Sequencing of the 1749 nt encoding the HN gene was initially performed as described previously, using primers FW-HN1, RV-HN1, FW-HN2, RV-HN2, RV-HN7233, FW-HN7795, RV-HN7842 and RV-HN2 (10). Samples from mumps virus genotype G strains for which HN gene sequencing failed were repeated using mumps virus genotype G specific primers FW-HN6497 G and RV-HN8482 G during the first PCR and primers FW-HN6534 and RV-HN8433 during the nested PCR. The nested PCR primers were also used to generate sequences, together with primers FW-HN7172, RV-HN7233, FW-HN7795 and RV-HN7842. The 1617 nt encoding the F gene were sequenced using primers F-FW1A and F-RV3A during the first PCR and

primers F-FW1B and F-RV3B during the nested PCR. Sequences were generated using primers 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). All F gene, SH gene and HN gene sequences were submitted to the GenBank database and are available with the accession numbers KJ125045-KJ125051, KJ125053-KJ125059, KJ125061-KJ125067 and KU756625-KU756930.

### Phylogenetic analysis

BioNumerics software version 7.5 (Applied Maths) and MEGA software version 6.06 were used to analyse nucleotide sequences. Maximum likelihood trees with 1000 bootstrap replicates for branch support were created using MEGA software version 6.06. F, SH and HN gene sequences were merged for phylogenetic analyses of the combined sequences.

## RESULTS

### Phylogenetic analysis

Complete F gene, SH gene and HN gene sequences were obtained from a set of 109 mumps virus genotype G outbreak samples obtained between October 2004 and June 2015 (Table 1). The percentage of variable sites was largest in the SH gene (8.2%) as compared with the F gene (5.5%) and the HN gene (4.2%) (Table 2). Phylogenetic analysis based on solely the mumps virus SH gene sequences showed 14 clades and segregation in two groups consisting of mumps virus sequences from 67 and 29 mumps patients, respectively (Figure 1A).

Additional F gene and HN gene sequence information led to increased resolution, with 60 clades when the F gene, SH gene and HN gene sequences were combined (Figure 1B). The groups formed when F gene, SH gene and HN gene sequences were combined varied in size and included 2 clusters with samples from local mumps outbeaks. The first cluster consisted of 13 samples obtained between June 2013 and April 2014 (cluster 1). The majority of these samples with identical F, SH and HN gene sequences were from mumps patients from a local mumps outbreak that started in the summer of 2013 (n=12). The other sample in this cluster was from a different geographical area in the Netherlands without epidemiological link to any of the other cases in this cluster. The second cluster consisted of 10 samples that were from a local mumps outbreak between March and June 2015 (cluster 2). Nine of these samples had identical F, SH and HN gene sequences and 1 sample contained 2 mumps virus variants; one variant was identical to the other 9 mumps viruses and the other variant had a point mutation at nucleotide position 44 in the HN gene.

Sequencing both the F gene and the HN gene in addition to the SH gene resulted in the most detailed molecular information, but sequencing of either one of these genes also added substantial resolution to the data. Analysis of only the F gene sequences resulted in 40 clades, as compared with 36 clades when only the HN gene sequences were analysed (Figure 2). Beyond the increase in resolution, comparison of the phylogenetic trees based on the F gene sequences and HN gene sequences showed also discrepancies in tree topology. The variation in mumps virus F gene, SH gene and HN gene sequences during the various mumps outbreaks in the Netherlands suggested that these outbreaks were caused by multiple mumps virus introductions rather than by endemic circulation of mumps viruses.

**Table 1.** Clinical samples belonging to mumps virus genotype G sequenced in this study (n=109) in comparison with the total number of samples sequenced (n=1106) and the number of samples with mumps virus genotype G (n=929), stratified by year.

| Year | No. samples with SH gene sequenced (n=1106) | No. samples with mumps virus genotype G (n=929) | No. genotype G samples with F gene, SH gene and HN gene sequenced (n=109) |
|---|---|---|---|
| 2004 | 1 | 1 | 1 |
| 2005 | 7 | 6 | 2 |
| 2006 | 0 | 0 | 0 |
| 2007* | 10 | 0 | 0 |
| 2008* | 132 | 5 | 0 |
| 2009 | 25 | 14 | 3 |
| 2010 | 351 | 342 | 29 |
| 2011 | 282 | 281 | 14 |
| 2012 | 165 | 163 | 8 |
| 2013 | 85 | 79 | 32 |
| 2014 | 16 | 14 | 5 |
| 2015 | 32 | 24 | 15 |

* In 2007 and 2008 there was a mumps outbreak among unvaccinated children in the Netherlands, caused by mumps virus genotype D.

**Table 2.** Genetic information of the mumps virus F gene, SH gene and HN gene within genotype G mumps virus strains (n=109).

| | Length | | Variabe sites | |
|---|---|---|---|---|
| Gene | Nucleotide | AA | Nucleotide | AA |
| F | 1617 | 539 | 89 (5.5%) | 27 (5.0%) |
| SH | 316 | 76 | 26 (8.2%) | 6 (7.9%) |
| HN | 1749 | 583 | 74 (4.2%) | 13 (2.2%) |

**Figure 1.** Phylogenetic analysis of genotype G mumps virus samples (n=109) using the maximum likelihood method with 1000 bootstrap replicates based on (A) the SH gene and (B) the F gene, SH gene and HN gene combined. Bootstrap values are shown at corresponding branches. Colours represent the year of sampling. Two clusters with samples from local mumps outbreaks are indicated in the trees (clusters 1 and 2).

**Figure 2.** Phylogenetic analysis of genotype G mumps virus samples (n=109) using the maximum likelihood method with 1000 bootstrap replicates based on (A) the F gene and (B) the HN gene. Bootstrap values are shown at corresponding branches. Colours represent the year of sampling. Two clusters with samples from local mumps outbreaks are indicated in the trees (clusters 1 and 2).

### Epidemiological data supports phylogenetic clustering

From 1 January 2015 to 30 June 2015, 67 mumps patients were reported in the Netherlands, according to Osiris (Figure 3). Two clusters were epidemiologically identified. Cluster 1 (n=11) was related to a hockey club, including some team mates of the index case. Cluster 2 (n=6) was initially related to a pub in the same geographical area as the hockey club but then continued at a school, because a patient who visited the pub was a student at that particular school. Furthermore, there were 7 mumps cases reported who were thought to be primary or secondary import cases. The majority of mumps cases (n=43) could not be linked to one of the epidemiological clusters and had an unknown source of infection.

Out of the 15 samples from 2015 with complete F gene, SH gene and HN gene sequences, 9 samples had identical sequences and 1 sample had a nearly identical sequence with a double peak at 1 positions in the HN gene sequence. The cluster formed by these 10 samples was named molecular cluster A. Cluster A included 5 samples from cases who belonged to epidemiological cluster 1 and 1 sample from a case who belonged to epidemiological cluster 2, thereby indicating a link between these clusters. Mumps viruses belonging to cluster A were detected during a period of 3 months. Molecular cluster B included 3 samples from patients with day of onset of disease all within the same week, but from different geographical areas and without epidemiological link, which suggests that part of the chain of transmissions for this cluster was missed. Cluster A and cluster B were defined based on F gene and HN gene sequences, since the SH gene sequences were identical for samples in both clusters.

## DISCUSSION

Combining mumps virus F gene, SH gene and HN gene sequences enabled us to study mumps virus transmission within mumps virus genotype G outbreaks. Our data showed endemic circulaton of mumps virus during some local mumps outbreaks. However, the diversity in mumps virus strains throughout the recent outbreak seasons and the discrepancies in tree topology between the genes indicates that there were multiple mumps virus introductions, and therefore shows the potential for the identification of geographical clusters in regions where mumps is endemic. Currently, there is insufficient genomic data globally to verify this assertion, as most phylogenetic analyses are based on solely SH gene sequences and show limited variation (11,12).

The sequences in this study may not be fully representative for the mumps virus strains circulating during the study period. Although we have attempted to sequence a systematic selection of samples, we were limited by the availability of the samples due to asymptomatic mumps virus infections, underreporting of

clinical mumps cases and the lack of laboratory confirmation in mumps virus diagnosis. Mumps symptoms are often mild and treatment options are limited, which makes it likely that not all mumps patients are diagnosed and reported (15). Furthermore, mumps diagnosis in the Netherlands does not require laboratory testing per se, since mumps can also be diagnosed when there is an epidemiological link to a confirmed mumps case. Another limitation is that samples with low viral loads often failed for sequencing. The lack of mumps virus sequences from samples with low viral loads might lead to a bias in our selection, as the effect of strain-specific differences in mumps virus pathogenesis on viral load in saliva is unknown. Mumps virus isolates are often used for sequencing of genes other than the SH gene. As mumps virus culturing is prone to mutations, we have sequenced most of our clinical specimens directly, which probably resulted in lower success rates.

This study shows that sequencing of the mumps virus F gene and HN gene additional to the SH gene increases the resolution of the molecular information. In this way, endemic mumps virus circulation can be distinguished from mumps virus importations. The F gene, SH gene and HN gene sequences combined have sufficient variation to identify multiple mumps virus clusters within a mumps virus genotype G outbreak, whereas these clusters could not be identified based on the SH gene sequence alone due to a lack of variation within this gene. Furthermore, epidemiologically linked mumps cases have identical F gene, SH gene and HN gene sequences, which was illustrated by the mumps outbreak in 2015. This latter finding was supported by sequencing data from previous local mumps outbreaks in the Netherlands. All in all, our data show that sequencing of the F gene, SH gene and HN gene combined could serve as a molecular tool during mumps outbreaks to identify transmission chains, even when part of the chain of transmissions is missed. Although this method is more costly than sequencing of the SH gene alone, the identification of new mumps clusters via this method could support public health decisions about implementation and evaluation of outbreak control measures.

## ACKNOWLEDGEMENTS

We want to acknowledge the municipal health services, laboratories and clinicians for sending clinical materials from mumps patients to the national laboratory for genotyping. Furthermore, we thank Daphne Gijselaar for technical assistance.

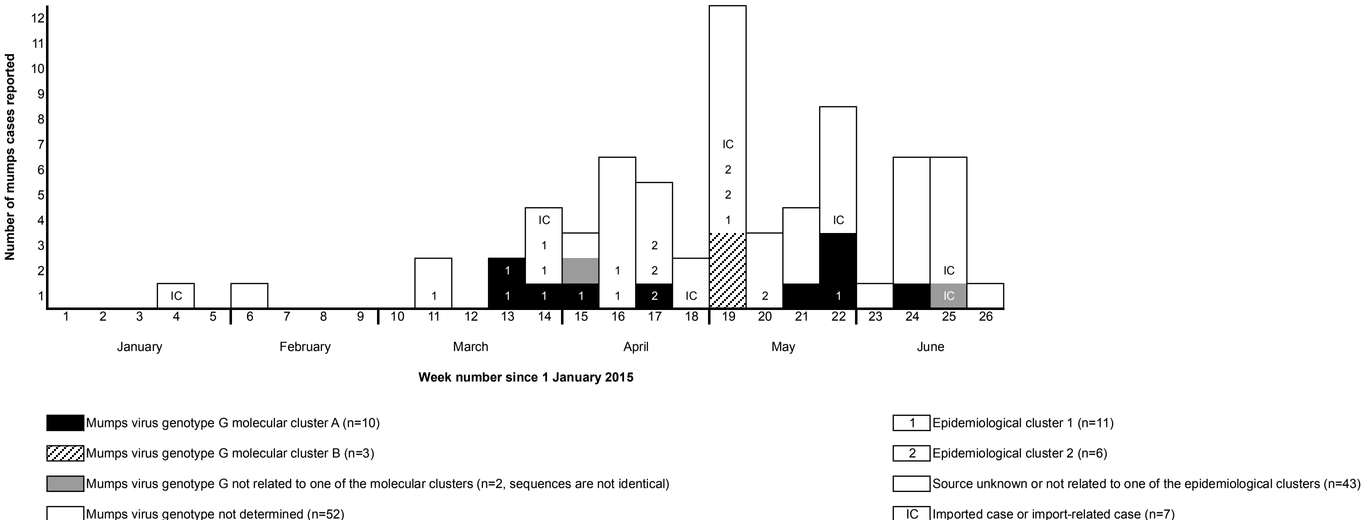


**Figure 3.** Epidemiological curve of the mumps cases reported between January and June 2015 by week of onset disease. Colours indicate the molecular clusters based on the SH gene, F gene and HN gene sequences. Numbers represent the epidemiological clusters. Epidemiological cluster 1 consists of mumps patients who were related to a hockey club. Epidemiological cluster 2 cases were related to a pub and a school in the same geographical area. There was no epidemiological link between cluster 1 and 2, but sequence data have shown that the clusters were linked.

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## SUPPLEMENTARY MATERIAL

**Table 1.** Overview of primers used for sequencing of the F gene, SH gene and HN gene.

| Name | Sequence (5' – 3') | Location |
|---|---|---|
| FW-F4351 | GGAGTCCATTYAGGAAGTCTGCC | 4351-4373 |
| FW-HN6497 G | GAATAATGCCGTTCAATCATGAGAC | 6497-6521 |
| BV6132 SH1 | AGTAGTGTCGATGATCTCAT | 6133-6152 |
| BV2013 SH5 | GATCCTAAGCTTGTTCTGG | 6539-6557 |
| BV6138 SH3 | GTCGATGATCTCATCAGGTAC | 6139-6159 |
| BV2011 SH6 | TCCTAAGCTTGTTCTGGCTTG | 6535-6555 |
| FW-HN1 | CAAGCCAGAACAARCTTAGGA | 6535-6555 |
| RV-HN1 | AGTATCTCATTTAGGCCCG | 8442-8460 |
| FW-HN2 | CCAGAACAARCTTAGGATC | 6539-6557 |
| RV-HN2 | TCATTTAGGCCCGCCATTCT | 8435-8454 |
| RV-HN8482 G | GCTTATATCTAACGATGGGTGAG | 8482-8504 |
| FW-HN6534 | CCAAGCCAGAACAAACTTAGGGTC | 6534-6557 |
| RV-HN8433 | CTCATTTAGGCCCGCCATTCTGG | 8433-8453 |
| FW-HN7172 | GGTAAGACACACTGGTGYTA | 7172-7191 |
| RV-HN7233 | GAAGTATGATCCTTGCAGTTGG | 7212-7233 |
| FW-HN7795 | AGTTGTCCCCTCAAACAATC | 7795-7814 |
| RV-HN7842 | ACTCTTCCTTCTGCACCCAT | 7823-7842 |
| F-FW1A | GTCCATTYAGGAAGTCTGCCTC | 4354-4375 |
| F-RV3A | AGGGTCACGAGACGTTACGAC | 6236-6256 |
| F-FW1B | TYAGGAAGTCTGCCTCAATGAG | 4360-4381 |
| F-RV1B | TTGCTTGTACCGCTATAGCTARC | 5025-5047 |
| F-FW2B | CAAGCACAGACAAATGCACG | 4930-4949 |
| F-RV2B | TTGCCTGCAAGGCATAGTTTTG | 5579-5600 |
| F-FW3B | TGCCAATACAATGAGGCAGAGAG | 5542-5564 |
| F-RV3B | GTCACGAGACGTTACGACCC | 6234-6253 |

# OUTBREAKS IN THE NETHERLANDS

# CHAPTER 5

## Differences among mumps virus surface proteins between genotype G and other genotypes at sites important for immunity and pathogenesis

*In preparation*

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## ABSTRACT

The surface proteins of the mumps virus, the fusion protein (F) and hemagglutinin-neuraminidase protein (HN), are important in mumps pathogenesis and are important targets for the immune response to mumps virus. We compared the variable amino acid positions in the F and HN proteins between the Jeryl Lynn vaccine strains and wild type mumps virus strains, with specific attention for genotype G strains, the most frequently detected mumps genotype in outbreaks in vaccinated communities. Sequence entropy, defined as a measure to compare diversity and the location of the variable sites, showed that the variation for genotype G strains is very specific. Differences between Jeryl Lynn strains (genotype A) and genotype G strains were found in or near known B cell epitopes, and in glycosylation sites, both of which might result in escape from the immune responses induced by the Jeryl Lynn vaccine. Of special interest were amino acid positions 113, 354, 356, 403 and 442 of the HN protein, which were all located in B cell epitope regions and segregated genotype A strains (positions 354, 356 and 442) and genotype G strains (positions 113 and 403). Additional differences between Jeryl Lynn and genotype G strains were found in or near sites that were predicted to play a role in pathogenesis, such as the cleavage site in the F protein and fusion promotion sites, receptor binding sites, neuraminidase activity regions and neurovirulence regions in the HN protein. These differences might contribute to the occurrence of genotype G outbreaks in vaccinated communities.

## IMPORTANCE

Since the introduction of the MMR vaccine in many national immunization programs, mumps incidence decreased drastically. However, during the last decade several mumps genotype G outbreaks occurred among persons who received 2 MMR doses that contain the Jeryl Lynn strains as mumps component. In this study, we showed that the Jeryl Lynn vaccine strains and mumps virus genotype G strains had unique variable sites that were located in regions of the F and HN proteins with important functions in mumps-specific immunity by the host and mumps virus pathogenesis. Although not proven in a biological model, these variable sites might facilitate the breakthrough infections of genotype G mumps virus in persons vaccinated with the Jeryl Lynn strains and should therefore be studied further.

## INTRODUCTION

Mumps is a contagious childhood disease that is caused by the mumps virus of the family *Paramyxoviridae*. The disease is characterized by unilateral or bilateral swelling of the parotid glands, but complications such as orchitis, oophoritis, meningitis, encephalitis, and deafness can occur (1,2). Since the introduction of the measles, mumps and rubella (MMR) vaccine in many national immunization programs, mumps incidence has dramatically decreased. In the last decade however, there were several mumps outbreaks among MMR vaccinated adolescents (3–6).

One hypothesis is that the outbreaks are a result of secondary vaccine failure – waning of the immune response – because most of the outbreaks affected adolescents who received their second MMR dose more than 10 years ago (7–9). Another possibility is that the outbreaks in vaccinated communities result from immune escape. Mumps virus diverged into genetic lineages, called genotypes, as defined based on the nucleotide sequence of the small hydrophobic (SH) gene that encodes a membrane protein. The Jeryl Lynn strains are a mixture of 2 mumps virus strains that both belong to genotype A, whereas most recently detected outbreak strains belong to genotype G (10–13). Although vaccine-induced antibodies neutralize mumps virus genotype G strains, the level of neutralization is lower than for the vaccine strain (14,15). In addition, *in silico* analyses suggest differences among lineages in predicted B cell and T cell epitopes (16,17). However, these studies did not focus on genotype G. Here, we study the differences between the Jeryl Lynn vaccine strains and the currently circulating genotype G strains with a focus on the fusion protein (F) and the haemagglutinin-neuraminidase protein (HN). These are the main surface proteins of the mumps virus and are important for viral entry, fusion and B cell mediated immunological responses. We analysed genomes of mumps virus strains that were isolated from patients in the Netherlands before the introduction of the MMR vaccine into the Dutch National Immunization Program (1957-1982) and from more recent outbreaks. Besides differences in epitope regions and glycosylation sites, which point towards an immunological mismatch, we have also studied differences in regions that could play a role in mumps virus pathogenesis.

## METHODS

### Mumps virus strains obtained from clinical specimens

Mumps virus sequences from 46 clinical isolates obtained between 1957 and 1982 were used. Selection of the mumps virus cultures was based on availability of the isolates. According to the available information from these isolates, mumps virus

had been cultured from oral swabs, nasal swabs and liquor samples. All but 1 of the viruses were isolated from Dutch mumps patients. The non-Dutch isolate was from a patient from Albany, USA. Besides the sequences from the 46 clinical isolates from the pre-vaccination era, mumps virus genotype G sequences from 109 clinical samples obtained between 2004 and 2015 were used. All but 1 of these samples have been described previously (18). Only 1 sample per mumps patient was included. The GenBank accession numbers for these sequences are KJ125045-KJ125051, KJ125053-KJ125059, KJ125061-KJ125067, KU756625-KU756710, KU756712-KU756812, KU756814-KU756914, KU756916-KU756930 and KX136898-KX137038. In accordance with Dutch law, no informed consent was required for this study.

## GenBank sequences

Besides the 46 F and HN gene sequences from mumps virus strains from the pre-vaccination era and the 109 F and HN gene sequences from recent mumps virus genotype G strains, 17 F gene sequences (genotype A and G) and 33 (genotype A and G) HN gene sequences retrieved from GenBank were used for the mapping of all regions of interest. For phylogenetic analysis, SH gene sequences from 78 mumps virus strains were retrieved from GenBank (Supplementary table 1). To identify unique sites in genotypes A and G, an additional 11 F gene sequences and 43 HN gene sequences were retrieved from GenBank (Supplementary table 1). These sequences included 27 mumps virus reference strains as defined by the World Health Organization, including 2 strains per genotype and 3 unclassified strains (19).

## Sequencing and phylogenetic analysis

RNA extraction, cDNA synthesis and sequencing of the F gene, SH gene and HN gene for all genotype G mumps virus strains were performed as described previously (18). For sequencing of the strains from the pre-vaccination era, the same procedures were followed for the sequencing of the F and SH gene (18). For sequencing of the HN gene, cDNA was synthesized with the use of primer FW-HN1 (nt 6535-6555) and thereafter a PCR was performed with the use of primers FW-HN1 and RV-HN1 (nt 8442-8460). The six primers used for sequencing were FW-HN2 (nt 6539-6557), FW-HN7172 (nt 7172-7191), RV-HN7233 (nt 7212-7233), FW-HN7795 (nt 7795-7814), RV-HN7842 (nt 7823-7842) and RV-HN2 (nt 8435-8454) as described previously (20). BioNumerics software version 7.5 (Applied Maths) was used to analyse nucleotide sequences and to recreate a maximum parsimony tree with bootstrap resampling (1000 replicates) based on the SH gene sequences.

**Table 1.** Overview of the important functional regions in the F and HN proteins.

| Protein | Region | Amino acid position* |
|---|---|---|
| F | Glycosylation pattern | 73-75; 182-184; 352-354; 427-429; 433-435; 457-459 |
| F | B cell epitope | 221, 323, 373 |
| F | Fusion promotion | 91; 195; 383 |
| F | Cleavage site | 98-102 |
| F | Neurovirulence | 91 |
| HN | Glycosylation pattern | 127-129; 176-178; 284-286; 329-331; 400-402; 448-450; 464-466; 507-509; 514-516 |
| HN | B cell epitope | 113-130; 199-207; 220-240; 261-296; 327-363; 375-403; 440-443; 533 |
| HN | T cell epitope | 279; 287 |
| HN | Fusion promotion | 82; 89; 96; 98; 102; 104; 111; 118; 222; 226; 228; 230; 567; 571 |
| HN | Receptor binding | 162; 175; 226; 228; 230; 335; 407; 422; 530; 531; 533; 540; 566; 567; 575 |
| HN | Neuraminidase activity | 180; 204; 239; 264; 268; 303; 407; 422; 466; 512; 540; 551; 561 |
| HN | $Ca^{2+}$-binding | 268; 270; 272; 302 |
| HN | Neurovirulence | 360; 466 |

* F protein numbering is based on accession number JN012242 and HN protein numbering is based on accession number ABY81903. Regions and amino acid positions are based on references (17,21–31). Underlined positions indicate glycosylation recognition patterns, for which structural analysis showed burial of N and/or S/T residue, yielding uncertainty of glycosylation.

## Generation of the homology models

Homology models were generated for both the F and HN proteins, as there are no experimentally determined structures of the mumps F and HN proteins available. The consensus sequence was calculated for both the F and HN proteins based on 123 and 196 sequences, respectively, with the cons module of Emboss (32). This consensus sequence was then used in a BLAST (33) search against the PDB (34) to identify available protein structures of homologs for both proteins. Overall, PDB entries 4GIP (35) and 3MAW (36) were used as templates for the F protein in the pre- and postfusion conformation, respectively, as the F protein undergoes a conformational change during the fusion process (37). PDB entry 4GIP contains the structure of the F protein from parainfluenza virus 5 and has an identity and similarity score with the mumps F protein consensus sequence of 49% and 69%, respectively; whereas PDB entry 3MAW contains the structure of the F protein from Newcastle Disease virus and has an identity and similarity score of 35% and 54%, respectively. PDB entry 1Z4V (38) was selected as template for the HN protein. This structure contains the HN protein from parainfluenza virus 5 and has an identity and similarity score with the mumps HN protein consensus sequence of 46% and 66%, respectively. After selection of the templates, FoldX (39) was used to replace the amino acids of the template with the mumps F and HN protein

consensus sequences. Next, an energy minimization was carried out in YASARA with the YASARA force field (40). Finally, the models were evaluated by MolProbity (41).

### Multiple sequence alignment and mapping of the mutations

Multiple sequence alignments for the F and HN protein sequences were performed with the aid of Clustal Omega (42). The sequence variation was subsequently mapped onto the protein structures with the aid of Scop3D, a tool to visualize variation across multiple sequences on the protein structure (43). The entropy, which provides information on the randomness of spread of the observed variation, was also calculated for both proteins with the aid of scop3D (43). F protein numbering is based on accession number JN012242 and HN protein numbering is based on accession number ABY81903.

### Determination and visualization of variable positions and important functional regions

Important functional regions were defined based on literature (Table 1). An N-glycosylation site was defined as the glycosylation recognition pattern N-X-S/T with X being any amino acid except proline. Both the N as well as the S/T residue need to be surface exposed for the site to be able to be glycosylated (44). All regions were subsequently mapped onto the sequences and models of the protein structures for analysis (Figure 1), analysed for diversity, and visualized on the structures of the homology models with the aid of PyMol (PyMOL Version 11r1, Schrödinger LLC). Numbering of the positions is based on the position of the residue in the sequence.

### Calculation of the solvent accessible surface area

To predict accessibility of variable sites, the absolute solvent accessible surface area (aSAS), the area of a residue that is accessible by a water molecule (1.4Å), was calculated with the aid of DSSP (31,32). The relative solvent accessible surface (rSAS) area was then obtained by calculation of the ratio of the aSAS to the aSAS of the residue in the tripeptide G-X-G (33,34). A residue is said to be surface accessible (exposed) if the relative SAS is >25%, otherwise a residue is said to be buried (49,50).

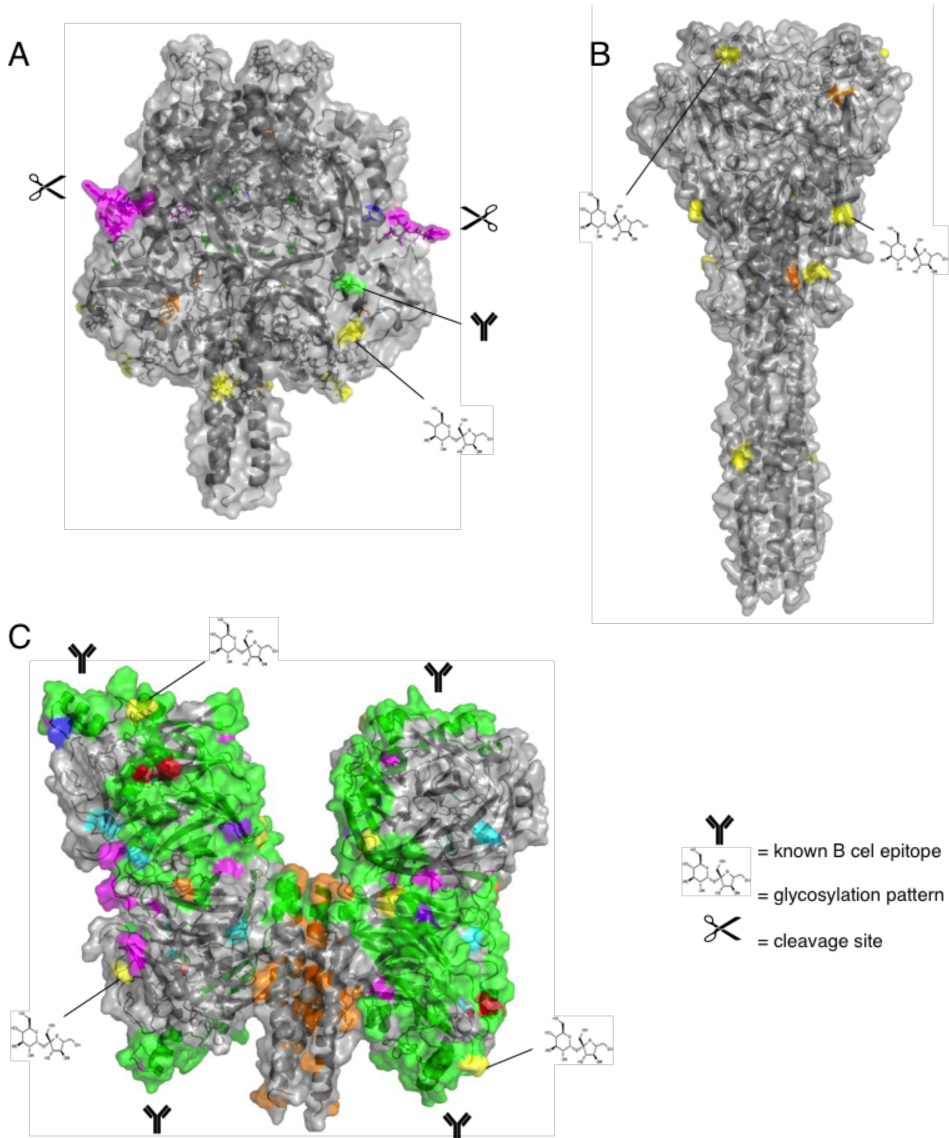


**Figure 1.** Structure of the F and HN protein with the important regions mapped on the structures in different colours. (A, B) F protein with glycosylation sites (yellow), fusion promotion sites (orange), cleavage site (pink), neurovirulence (dark blue) and known B cell epitope regions (green) mapped on the prefusion (A) and postfusion (B) structure. (C) HN protein with glycosylation sites (yellow), fusion promotion sites (orange), receptor binding regions (pink), neuraminidase activity regions (blue), $Ca^{2+}$-binding sites (red), neurovirulence regions (dark blue), known T cell epitope regions (purple) and known B cell epitope regions (green) mapped on the structure.

## Statistical analysis

Fisher's Exact Test was used to find significant differences in the distribution of the variable and non-variable positions in the regions of interest and other regions. Only those positions of the protein present in the structure were taken into account. All p-values <0.05 were considered statistically significant.

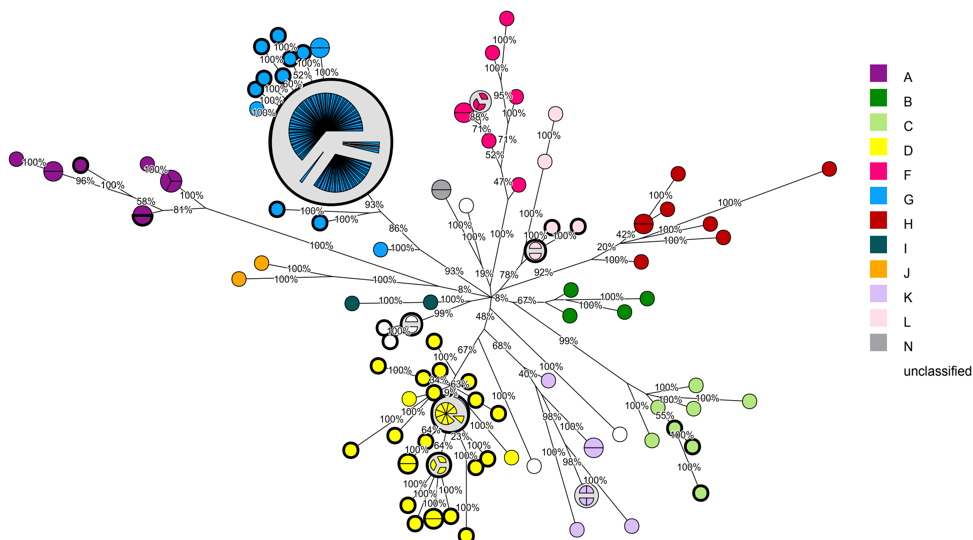


**Figure 2.** Maximum parsimony tree based on SH gene sequences of 46 mumps virus strains from the pre-vaccination era and 109 recent mumps virus strains, all indicated by thick lines. Percentages indicate bootstrap values (1000 replicates). All but one mumps virus strain were from Dutch patients, the non-Dutch strain was isolated from a patient from the USA in 1954. Genotypes are labelled by colour. WHO reference strains (n=27) and other mumps virus sequences retrieved from GenBank (n=51) are included in the phylogenetic tree and are indicated by thin lines.

# RESULTS

## Phylogenetic analysis

A phylogenetic analysis based on the SH gene was performed to determine the genotypes of the mumps virus strains from the pre-vaccination era (n=46) and from recent outbreaks (n=109) (Figure 2). Two strains from the pre-vaccination era belonged to genotype A, including a strain that was identical to MuVi/Boston.USA/0.45 "Enders" and was isolated from a patient in the USA. These strains were isolated in 1954 and 1962, respectively. Three strains from the pre-vaccination era belonged to genotype C and were all isolated in 1980-1981. The majority of the strains from the pre-vaccination era (n=33) belonged to genotype D and were isolated over a longer time period (1961-1982), which

indicated that this genotype circulated during several decades in the Netherlands before the introduction of the MMR vaccine in 1987. Four strains belonged to genotype L and were isolated between 1957 and 1964. The 4 remaining strains from the pre-vaccination era were isolated between 1962 and 1964 and did not cluster with any of the genotypes as defined by the reference strains which suggested a genotype that was not previously described. None of the mumps virus strains from the pre-vaccination era belonged to genotype G.

**Table 2.** Overview of variable sites in the F and HN protein that were unique for either genotype A or genotype G strains.

| | | | Variable positions in proximity of important region§ | | |
|---|---|---|---|---|---|
| Protein | Region† | Variable positions in important region | Prefusion | Postfusion | Positions not in or not close to an important region |
| F | Glycosylation recognition pattern | - | NA | NA | **24*, 49**, 115, 141, 170*, **177**, 238, 273, 274*, **275**, 298, **326***, 330*, **331, 343, 345***, 350*, **360***, 389, 413, 425, **431**, 439*, *479*, ***480****, *488, 492, 530** |
| F | B cell epitope | - | - | NA | |
| F | Cleavage site | 100*, 101 | 96*, 97* | NA | |
| HN | Glycosylation recognition pattern | 129*, 330* | NA | | ***8****, *37, 63**, ***80****, **135,** 153*, 403 |
| HN | B cell epitope | 113*,129*, **205, 224,** 330*, **354*, 356,** 403, **442** | **214*,** 317, **470** | | |
| HN | Fusion promotion | - | 94*, 97*, **106*,** 113*, **224** | | |
| HN | Neuraminidase activity | - | **205, 468, 552** | | |
| HN | Neurovirulence | - | **356, 468,** 477 | | |
| HN | Receptor binding | - | **224, 577*** | | |

Bold numbers indicate positions that are unique for genotype A, all other positions are unique for genotype G. Underlined positions are surface exposed residues (for the F protein: single line indicates an exposed location in the prefusion conformation, double line indicates an exposed location in the postfusion conformation, dashed line indicates an exposed location in both conformations). NA: not applicable.

* Amino acid substitution is non-conservative.

† There are no variable sites in or close to the fusion promotion sites and the neurovirulence sites of the F protein and the Ca$^{2+}$-binding sites, known MHC-II binding sites, and CD4$^{+}$ and CD8$^{+}$ response sites of the HN protein, so these regions are excluded from the table.

§The postfusion structure is only applicable to the F protein.

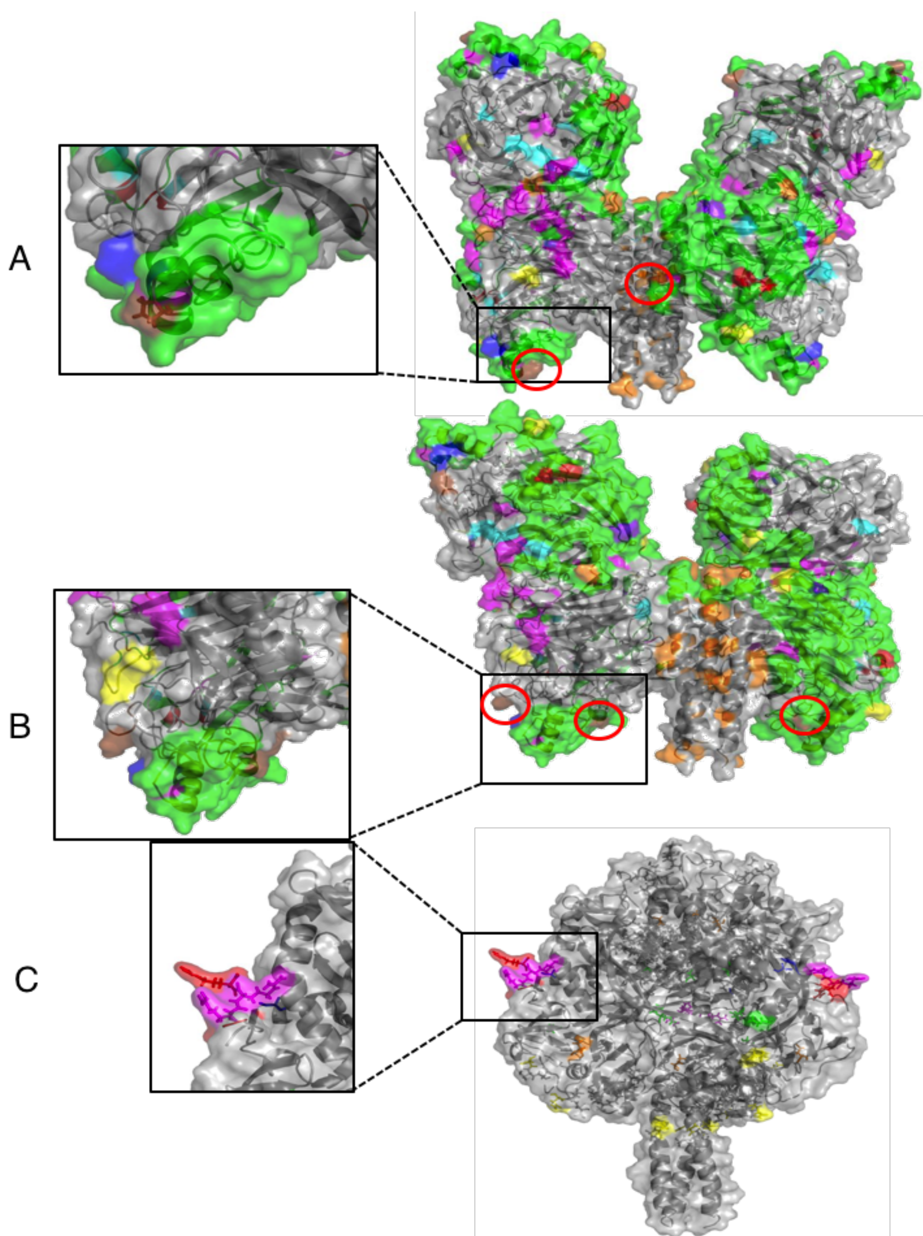


**Figure 3.** Overview of important variable positions on both the HN and F protein as described in literature. (A, B) The HN protein with important regions which are coloured as in Figure 1. The view in (B) is turned over a vertical axis by 180°. (A) Presentation of variable positions 354 and 442 in the Jeryl Lynn vaccine strain (brown positions in red circels), with zoom on position 354. (B) Variable regions 400-402 and 464-466 are shown (brown positions in red circles), with zoom on region 464-466. (C) The F protein with variation of position 96, 97 and 100 (red) at or near the cleavage site (pink).

A

Jeryl Lynn

Position
Accessibility
Sequence
Entropy
Abundance

Genotype G

Sequence
Entropy
Abundance

Accessibility
Entropy
Abundance

0% 25% 50% 75% 100%

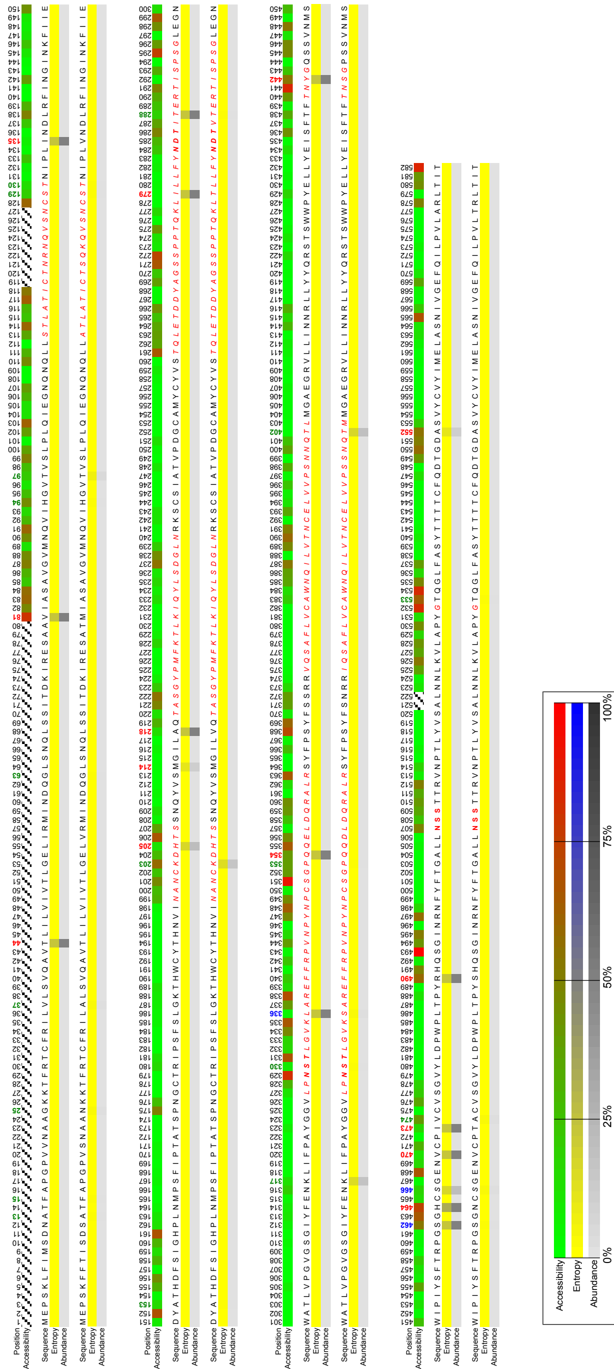


**Figure 4.** Overview of the differences between the Jeryl Lynn strains and genotype G strains. Accessibility, entropy and abundance are expressed as percentages and indicated by colours. Epitope regions (italic) and glycosylation patterns (bold) are indicated on the amino acid sequences. Variable positions for Jeryl Lynn (red), genotype G (green) or both (blue) are indicated on the amino acid positions.

## Analysis of the F protein

F protein sequences were compared between the different mumps virus genotypes using Scop3D to identify sites that were unique for either the Jeryl Lynn vaccine strains or wild type genotype G strains. Out of the 22 variable positions that displayed variation within genotype A, 13 were unique for genotype A (Table 2). None of these positions was in an important functional region (Table 2). With respect to genotype G sequences, 23 out of the 37 variable positions were unique for genotype G, of which positions 100 and 101 were located in the cleavage site (Table 2, Figure 3C). This cleavage site is the site where a protease furin cleaves the protein to expose the fusion peptide that is essential for fusion of the virus and the host cell membrane. Both variable positions were found only once in the same sample, which suggested a random variation that is most likely not relevant. The change of H to Y at position 100 was a non-conservative change, which means that the physicochemical properties of this amino acid were altered. Position 101 showed a conservative change of K to R. Additionally, 2 other variable positions, positions 96 and 97, were located near the cleavage site. The change of G to R was found in 1 genotype G mumps virus sample, whereas the change of S to L was observed in 19 samples. This latter change made the fusion peptide more hydrophobic and this might enhance intercalation of the fusion peptide into the host cell membrane. The changes of positions 96-97 and 100-101 did not occur in the same sample.

To check the evolution within each genotype separately, we also looked at intragenotypic variations. The amino acid sequence variation for the F protein within the Jeryl Lynn vaccine strains alone was 11%-45%, as compared with 30%-40% within genotype G and 30%-36% within the other wild type genotypes analysed. The majority of the variation within all genotype groups was non-conservative (57%-64%). The rather low entropy in the variable positions for the Jeryl Lynn vaccine strains (21%) suggested that the amino acid diversity at the variable positions was limited. However, the overall entropy for the genotype G strains (4%) and other wild type strains (7%) was even lower, which indicated that variation seen for genotype G strains and other wild type strains could be of more significance. The occurrence of variable and non-variable positions in a region of interest or in other regions did not significantly differ within any of the genotypes ($p \geq 0.08$). Furthermore, variable positions did not overlap between the Jeryl Lynn strains and the genotype G strains (data not shown).

## Analysis of the HN protein

Overall, 15 out of 38 variable positions were unique for genotype A. Five of these unique positions were located in known B cell epitope regions (regions 199-207,

220-240, 327-363 and 440-443). Three of these positions were surface exposed: 354, 356 and 442 (rSAS=60%, 40% and 77% respectively, Figures 3A and 4). These 3 positions were variable in 7, 13 and 6 samples, respectively, without co-occurrence. Only position 354 (Q to P) showed non-conservative variation. Furthermore, 10 out of 15 unique positions that displayed variation were located in proximity of an important protein region, such as a fusion promotion site, a receptor binding site, a B cell epitope, a neuraminidase activity site and a neurovirulence region (Table 2). Only 4 positions were surface exposed (Figure 4). Ten of 36 variable positions were unique for genotype G, of which 4 positions were located in an important functional region. Positions 129 and 330 were located in a known B cell epitope region as well as in a glycosylation recognition pattern (known B cell epitope regions 113-170 and 327-363, including the glycosylation recognition patterns at positions 127-129 and 329-331), but each of these positions only showed variation in 1 sample. Two other positions were located only in a known B cell epitope region (regions 113-130 and 375-403). Although neither of these positions was surface exposed, they might still influence the tertiary structure and could therefore be relevant (Figure 4). Four out of 10 unique variable positions were located near an important region: three variable positions were located in proximity of a fusion promotion site and 1 position was located near a known B cell epitope region (Table 2). Structural comparison between genotype A and G showed that the variations in both genotypes occurred in different areas of the protein.

The amino acid variation within the HN protein of the Jeryl Lynn vaccine strains was 9%-45%, as compared with 1%-20% within the genotype G strains and 2%-30% within the other wild type strains. Similar to the variation within the F protein, the majority of the variable positions in the HN protein within all genotype groups was non-conservative (54%-68%). The entropy for the HN protein for each genotype was identical to the entropy for the F protein of the corresponding genotype, which indicated that the amino acid diversity at the variable positions was limited. Furthermore, the entropy for the genotype G strain at glycosylation sites and known B cell epitopes was 1.47% and 4.68% respectively, whereas the entropy for the Jeryl Lynn vaccine strain at these positions was 0% and 21.5% respectively. The distribution of the variable and non-variable positions was not significantly different within any of the genotype groups ($p \geq 0.11$). The overlap of the variable positions between the different genotype groups was equal for all comparisons (data not shown).

Although the predicted glycosylation sites at regions 400-402 and 464-466 were probably not surface exposed, the variation in these regions might be of interest as this variation was unique for either the Jeryl Lynn or genotype G strains. Position 402 was variable within the genotype G strains and the other wild type strains,

whereas this position was fully conserved across the Jeryl Lynn strains, which resulted in loss of the glycosylation recognition pattern in these latter strains (N-Q-T → N-Q-A/M). In addition, this position was also located in a known B cell epitope region, which may influence antibody recognition. The glycosylation recognition pattern at positions 464-466 was only present in the genotype G and other wild type strains, whereas in all Jeryl Lynn strains, variation at position 464 induced loss of this pattern (N-C-S to H/K-C-S/R). Two genotype G strains showed variation at position 466 (S to N). Furthermore, position 464 was located in proximity of a known B cell epitope region and was surface exposed (Figures 3B and 4) When a variable position is located in both a glycosylation recognition pattern and a known B cell epitope, the loss of the glycosylation recognition pattern could change the B cell epitope structure, which could lead to loss of recognition by antibodies that target that B cell epitope. Taken together, these sequence variants might affect mumps virus pathogenesis and could result in escape from the immune responses induced by the Jeryl Lynn vaccine.

## DISCUSSION

In this study, we compared the mumps virus surface proteins of the Jeryl Lynn vaccine strains with genotype G strains and other wild type strains using homology models to determine if strain variation may have been a contributing factor to the occurrence of the recent mumps outbreaks among vaccinated persons. Sequence variation is relatively limited in both the F and HN proteins (about 5% for both the Jeryl Lynn and the genotype G strains and about 11% for the other wild type strains), as compared to the SH protein (11%, 84% and 51% for Jeryl Lynn, genotype G strains and other wild type strains, respectively), which is the most variable protein of the mumps virus (13). The low sequence entropy for the genotype G and other wild type strains in both F and HN proteins indicates that the amino acid diversity at the variable positions is limited in the wild type mumps virus strains, which suggests that only specific changes occur in these latter strains.

We identified 5 variable positions in known B cell epitope regions in the HN protein that might have an effect on the ability of the host to recognize these epitopes, which needs to be validated by functional assays. Although we studied only known B cell epitope regions, it is likely that more B cell epitope regions exist that are currently unknown. We also found a small number of differences in or near other important functional regions such as the cleavage site and fusion related regions of the F protein. These variations are mostly conservative, as they do not tolerate much change due to their primary function of virus-cell fusion. However, overall, the variation seen in the F protein is rather limited and mostly related to regions not associated with functional domains. We did not find any variable sites

in regions important for $Ca^{2+}$-binding, which plays a role in structural stability and may therefore be conserved.

Some of the variable sites described in this study were previously described by others. Variation at positions 279 and 287 (change of I to T and V to I, respectively) of the HN protein has been reported previously, and it was suggested that this variation could lead to a mismatch in $CD4^{+}$ T cell epitopes, which might result in the loss of important T cell help for an immunological recognition and recall (16). Positions 279 and 287 could both individually and additively lead to a mismatch between T cell responses against the Jeryl Lynn vaccine strains and other wild type strains. In a study by Kulkarni-Kale *et al.*, variation at positions 266 and 354 in the HN protein, which are located in a known B cell epitope, was also described (17). Especially position 354 is of interest, because this variable position is unique for genotype A strains in our analysis. This position shows variation in other wild type strains as well, such as the SBL-1 and Kilham strains, which were not included in our analysis (17). Another study by Vaidya *et al*. compared mumps virus genome sequences from 8 Indian mumps cases, including 2 genotype G and 6 genotype C strains, with several vaccine strains, including the Jeryl Lynn strain (51). Their analysis showed that for the HN protein, variation was found in the regions that encompass positions 35-53, 240-245, 265-288, 329-340, 352-360 and 405-410. In all of these regions, except for the region of 240-245, we also found variations for the HN protein, which confirms our results once again. Additionally, we show that most of these regions, except for regions 240-245 and 405-410, are also surface exposed (Figure 4).

To conclude, we found differences in important functional regions in the F and HN proteins between the Jeryl Lynn vaccine strains and mumps virus wild type strains, which include the currently circulating mumps virus genotype G strains. Unique variations for both genotype A and G strains could indicate reduced recognition of genotype G strains by vaccine-induced immune responses. This could contribute to the occurrence of the recent genotype G mumps outbreaks among Jeryl Lynn vaccinated persons. As this study does not provide biological evidence that these regions are indeed responsible for the occurrence of genotype G outbreaks among Jeryl Lynn vaccinated young adults, functional assays need to be set up to confirm these results.

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# SUPPLEMENTARY MATERIAL

**Table 1.** Overview of the GenBank sequences used for phylogenetic analysis and for analysis of the F and HN protein.

| | GenBank accession number | | |
|---|---|---|---|
| Genotype | SH gene | F gene | HN gene |
| A | AF201473, AF338106*, AF345290, FJ211586, FN431985, GU980052*, HQ416906, HQ416907 | AF201473, AF338106*, AF345290, AJ010821, AJ133693, FJ211586, FN431985, HQ416906, HQ416907 | AF201473, AF338106*, AF345290, AY584603, AY584604, FJ211586, FN431985, HQ416906, HQ416907, X93178, X93179, |
| B | AB000388*, AB823535, AB827968, JQ945269* | AB823535, AB827968 | AB823535, AB827968, JQ946041* |
| C | AY669145, EU370206*, JQ034465, JQ034466, JQ945268* | AY669145 | AY669145, JQ034465, JQ034466, JQ999999* |
| D | JQ034452*, JQ945275* | | JQ034464*, JQ946039* |
| F | DQ649478, EU780221*, EU884413, FJ556896, HQ693823, HQ693825, JQ034459, JQ034460, JQ034461, JQ945272* | EU884413, FJ556896 | DQ649478, EU884413, FJ556896, HQ693823, HQ693824, HQ693825, HQ693826, JQ034459, JQ034460, JQ034461, JQ034462, JQ034463*, JQ946034* |
| G | AF280799*, EU370207, EU597478*, JN012242, JN635498, JX287385, JX287387, JX287389, JX287390, JX287391, JX390987, JX390988, JX390989, JX390990, JX390991, JX390992, JX390993, JX390994, JX390995, JX878447, KF481689 | JN012242, JN635498, JX287385, JX287387, JX287389, JX287390, JX287391, KF481689 | EU370207, JN012242, JN635498, JQ946046*, JX287385, JX287387, JX287389, JX287390, JX287391, JX390987, JX390988, JX390989, JX390990, JX390991, JX390992, JX390993, JX390994, JX390995, JX878447, KC852187, KC852188, KF481689 |
| H | AB600843*, AF467767, AY681495, JN687469, JQ388690, JQ388691, JQ945273*, JX287388 | AF467767, AY681495, JQ388690, JQ388691, JX287388 | AF467767, AY681495, JN687469, JQ388690, JQ388691, JQ946035*, JX287388 |
| I | JQ945274*, AY309060* | | JQ946037* |
| J | JQ945271*, AB105475* | | JQ946033*, JQ946044* |
| K | EU082458, JQ945276*, JQ945270*, JX287386, KC921200, KC921201, KC921202, KC921203, KF212191 | JX287386 | JQ946040*, JQ946045*, JX287386, KC921200, KC921201, KX921202, KX921203, KF212191, JF268685 |
| L | AB105483*, AB105480* | | JQ946036*, JQ946043* |
| N | AY508995*, AY685920* | | |

| | |
|---|---|
| Unclassified | AF142774*, AB003415*, AY380077* |

* WHO reference strain.

# VACCINE-INDUCED PROTECTION

# CHAPTER 6

## Mumps serum antibody levels before and after an outbreak to assess infection and immunity in vaccinated students

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# ABSTRACT

## Background

Since 2009, various mumps outbreaks have occurred in the Netherlands, affecting mostly young adults vaccinated against mumps. In this retrospective study, we estimated attack rates for symptomatic and asymptomatic mumps virus infection based on mumps-specific immunoglobulin (Ig)G concentrations in paired blood samples obtained before and after the mumps outbreaks, collected in 2 university cities. We aimed to identify a serological correlate of immune protection and risk factors for mumps virus infection.

## Methods

Mumps-specific IgG levels were measured by Luminex technology in paired pre- and post-outbreak samples from students from Leiden (n=135) and Utrecht (n=619). Persons with a 4-fold increase in mumps IgG concentrations or mumps IgG concentrations >1500 RU/ml were assumed to have had a mumps virus infection.

## Results

Attack rates for symptomatic and asymptomatic mumps virus infection were 2.0% and 3.8%, respectively. Pre-outbreak mumps-specific IgG concentrations were lower among cases than among noncases (p=0.005) despite vaccination history, but no serological cutoff for immune protection could be established. Mumps among housemates was significantly associated with serological evidence for mumps virus infection (odds ratio, 7.25 [95% confidence interval, 3.20–16.40]; p< 0.001).

## Conclusions

Symptomatic and asymptomatic mumps virus infections in vaccinated persons can be identified by retrospective assessment of mumps-specific IgG antibodies in blood samples.

# INTRODUCTION

Since the end of 2009, various mumps outbreaks have occurred in the Netherlands. The outbreaks affected mostly young adults, who had been twice vaccinated with the measles, mumps, and rubella (MMR) vaccine in childhood (1). This phenomenon could be due to waning immunity in this age group, because antibody responses after vaccination last shorter than after natural infection. In the absence of mumps virus circulation, a substantial proportion of persons is seronegative 15 years after the second MMR vaccination (2,3). Furthermore, recent findings suggest that the MMR vaccine is not very effective in eliciting an antibody response of high avidity against mumps compared with measles and rubella (4), which also could explain the poor protection of vaccinated adolescents.

Mumps attack rates above 10% among vaccinated university students have been reported during various recent outbreaks (5,6). Those attack rates were based on a particular setting within a specific time frame, and they are therefore probably higher than overall attack rates in a nationwide outbreak. In contrast, attack rates may be underestimated because calculations are based on self-reporting of mumps symptoms, whereas many mumps virus infections run an asymptomatic course (7–9). In theory, more reliable attack rates could be obtained from measuring mumps-specific immunoglobulin (Ig)G concentrations, because these generally increase after mumps virus infection (10). However, a challenge is the lack of a serological correlate of protection in vaccinated individuals. Only 1 study has shown that pre-outbreak mumps antibody neutralization titers in patients with mumps were lower than in persons who were not infected with mumps virus during the outbreak, but it was not possible to set a cutoff point separating all clinical patients with mumps from nonpatients (3).

In this study, we first measured mumps-specific IgG antibody concentrations in paired pre- and post-outbreak samples from exposed students in 2 Dutch university cities to identify mumps virus infections. In this way, we could calculate the proportions of symptomatic and asymptomatic infections and determine attack rates and risk factors for mumps virus infection, irrespective of clinical outcome. Second, to identify a correlate of protection, mumps-specific IgG concentrations in pre-outbreak samples were compared between infected and non-infected persons.

# METHODS

## Study design

A retrospective study was performed including 2 student cohorts from the cities of Leiden and Utrecht. The study in Leiden served as a pilot for a larger serological study in Utrecht. Questionnaires for both cohorts were comparable and included

questions on MMR vaccination status, mumps history, and possible risk factors such as age, gender, membership of a student association, residence in a student house, number of housemates, and circulation of mumps in the environment. Students were included if pre-outbreak serum samples were available that were collected during their first year of (bio)medical school for posthepatitis B vaccination titer control. After informed consent was obtained from the students, these serum samples were retrospectively tested, along with post-outbreak blood samples collected as described below. Studies were approved by the medical ethics committee of the Leiden University Medical Center and the University Medical Center Utrecht.

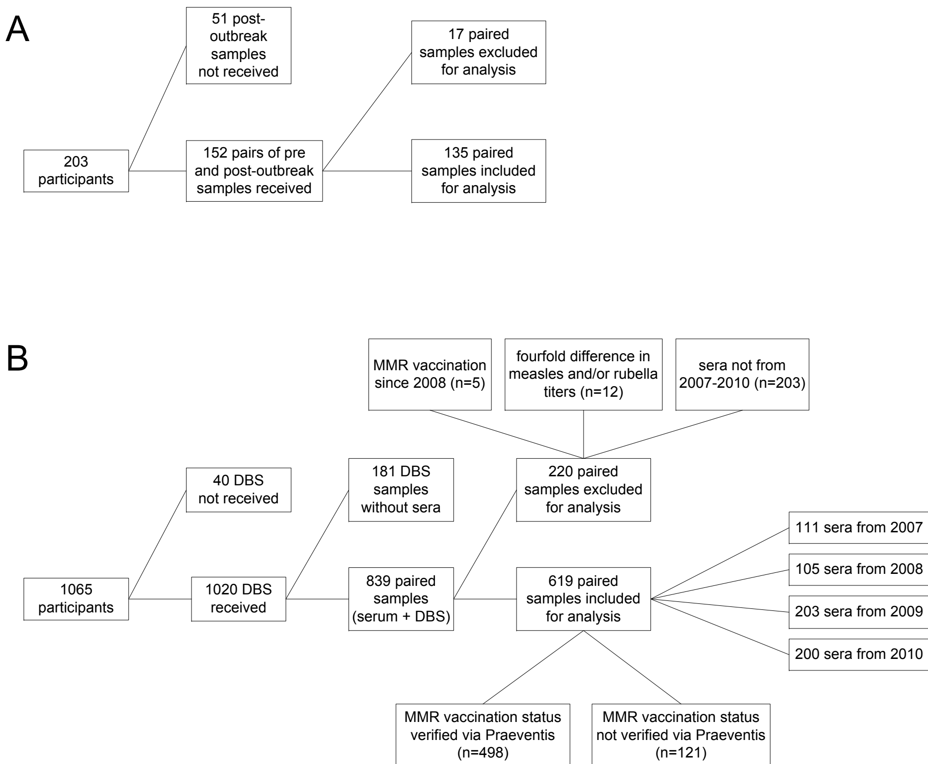


**Figure 1.** A flowchart for inclusion of samples is shown. (A) The flowchart for the Leiden cohort is illustrated. In total, 135 paired samples were included for analysis. The paired samples that were excluded (n=17) were all excluded on the basis of the measles and rubella concentration differences between the pre- and post-outbreak samples. (B) The flowchart of the Utrecht study is shown. In total, samples from 619 persons were included for analysis. Years in the right column are the years in which serum samples were drawn. All dried blot spots (DBS) were obtained between March and June 2012.

### Leiden study

In total, 135 paired pre- and post-outbreak samples from medical students were included (Figure 1A). Pre-outbreak sera were taken between 2008 and 2010. Students were approached directly at the university by a medical team from the academic hospital to participate in this study. They filled out a questionnaire and gave permission to test their pre-outbreak serum sample retrospectively. The post-outbreak sera were taken between January and February 2011.

### Utrecht study

Based on the results from the serological pilot study in Leiden, a larger study was performed among biomedical and medical students in Utrecht. Here, all students received a dried blot spot (DBS) self-sampling kit for post-outbreak sampling and a permission form to check vaccination status in the nationwide vaccination registration system (Praeventis), along with the questionnaire and informed consent form. All DBSs were sampled between March and June 2012. Stored sera from these students dated back to 2007–2012, depending on the year the student enrolled. Based on the reported mumps cases in Utrecht and other parts of the Netherlands in the national mandatory notification system, all sera collected between 2007 and 2010 were considered to be pre-outbreak samples. Using the inclusion criteria described below, samples from 619 students were included for analysis (Figure 1B). The vaccination status provided in the questionnaire was used for analysis, after verification of vaccination history for 498 (80.5%) of these students from the data recorded in Praeventis (data not shown). Of these 498 students, 469 students (94.2%) had received 2 MMR doses, which is in line with national MMR vaccination coverage data (11). When students' vaccination status was not reported and could not be retrieved via Praeventis, they were not included in the analyses restricted to fully vaccinated persons.

### Inclusion and exclusion criteria

In total, 754 of 788 students with pre- and post-outbreak blood samples were included. Besides measurement of the mumps-specific IgG concentration in the sera and DBS samples, IgG concentrations for measles and rubella were measured as external control for antibody concentration fluctuations between samples within a person over time. Persons were excluded for further analyses (n=29) when their ratio of measles and/or rubella IgG concentrations of both samples was at least factor 4 (12). In addition, all persons in the Utrecht study who had received an MMR vaccination since 2008 were excluded (n=5). This latter criterion could not be applied for the Leiden cohort, because data on recent MMR vaccinations were lacking (Figure 1).

## Mumps-specific immunoglobulin G assay

Samples were stored at –20°C until use. For all samples, IgG antibody concentrations for MMR were determined with a fluorescent bead-based multiplex immunoassay using Luminex technology as described previously (13). In short, 5 µL serum was 1:200 diluted in assay buffer (phosphate-buffered saline containing 0.1% Tween-20 and 3% bovine serum albumin). A punch (r=3.175 mm) of each DBS sample was dissolved in 300 µL assay buffer, resulting in a solution comparable to the 1:200 dilution of serum samples. When the 1:200 dilution fell outside the range of the reference serum curve, the results of a 1:4000 dilution were used for analysis.

On each plate, the WHO International Standard Anti Rubella Immunoglobulin RUBI-1-94 (The National Institute for Biological Standards and Control), controls, and blanks were included. The fluorescent intensity of the samples was interpolated in the reference serum curve to obtain antibody concentrations, which were expressed in RIVM units per milliliter (RU/ml) for mumps. The RIVM units for mumps used in this assay were previously standardized against other mumps standards, in which mumps IgG-positive test results were equivalent to values higher than 45 RU/ml (14,15). RUBI-1-94 has a mumps-specific IgG concentration of 4384.512 RU/ml and was selected as alternative serological standard for mumps, thus enabling comparison and bridging of our results to other studies. For measles and rubella, IgG concentrations were expressed as international units per milliliter (IU/ml).

## Definition of mumps virus infection

The period between the 2 blood samples varied between 2 and 5 years. Because no major outbreaks of measles and rubella were reported between 2007 and 2012, most subjects were assumed not to have been exposed to measles or rubella in this time period. Therefore, mumps-specific IgG antibody concentration rises were normalized against the concentration changes for measles and rubella, to correct for possible differences due to quality issues and technical differences related to sample storage and recovery of antibodies from DBS. The mumps-specific IgG concentrations were individually corrected using the average ratios of both measles- and rubella-specific IgG concentrations between the 2 consecutive blood samples.

Two criteria were set for the detection of mumps virus infections. First, a 4-fold increase or more of mumps-specific IgG in the 2 consecutive blood samples, acknowledged as the most specific criterion to confirm mumps virus infection, was used (12). Second, a single-point cutoff criterion was calculated by the use of a receiver operator characteristics (ROC) curve. The positive reference group for this

analysis consisted of laboratory-confirmed mumps cases who had been vaccinated twice with the MMR vaccine in childhood (n=15). These persons were identified through enhanced surveillance of mumps in the Netherlands and were contacted in the context of a medically ethically approved clinical study to collect samples between 6 and 10 months after mumps virus infection. The negative control group consisted of 451 twice MMR vaccinated age-matched individuals (between 18 and 25 years of age) from a large Dutch serosurveillance study in 2006/2007 (16). Persons who fulfilled at least 1 of the 2 serological criteria and had reported clinical mumps in the questionnaire were regarded as symptomatically infected, whereas persons who had not reported clinical mumps in the questionnaire were regarded as asymptomatically infected.

### Statistical analysis

SPSS version 19 and GraphPad Prism version 6 were used for data analyses. The attack rates for symptomatic and asymptomatic mumps virus infection were calculated for the entire outbreak period, assuming that students were exposed since January 2010 and that the exposure period was similar for all students included. Because the time frame and geographic region differed between the 2 student cohorts, attack rates were calculated separately. Distributions of pre-outbreak mumps-specific IgG concentrations in serum samples were compared using the Mann-Whitney U test. Median IgG concentrations, ROC analysis, and mixture modeling were used to identify a correlate of protection against mumps virus infection. For all analyses, p values $<0.05$ were considered as statistically significant. Risk factors in the Leiden and Utrecht student cohorts were compared with multilevel analysis. Thereafter, possible risk factors for symptomatic and asymptomatic mumps virus infection were determined with logistic regression analysis. Factors with a p value $\leq 0.10$ were included in the multivariate analysis to calculate odds ratios (OR) and 95% confidence intervals (CIs).

## RESULTS

### Cohort description

In total, 135 students in Leiden and 619 students in Utrecht were included (Figure 1). The majority of students were female (n=606; 80.4%) and median year of birth was 1989 (interquartile range [IQR], 1988–1990). Of 498 students of whom vaccination status could be checked, 469 (94.2%) had received 2 MMR doses. This is in line with MMR vaccination coverage data in these birth cohorts (11), and it was therefore assumed that most of the students in Leiden and Utrecht with unknown vaccination status were vaccinated twice in childhood according to the

National Immunization Program. Data on vaccination status of the students are shown in Table 1.

**Table 1.** Vaccination status for the 2 separate cohorts and the total cohort

| Cohort description | MMR vaccinations | No. (%) participants |
|---|---|---|
| Leiden* | At least 2x MMR | 47 (34.8) |
| | 1x MMR | 5 (3.7) |
| | Vaccinated, but unknown doses | 76 (56.3) |
| | No MMR | 2 (1.5) |
| | Unknown vaccination status | 5 (3.7) |
| Utrecht† | At least 2x MMR | 534 (86.3) |
| | 1x MMR | 14 (2.3) |
| | Vaccinated, but unknown doses | 52 (8.4) |
| | No MMR | 14 (2.3) |
| | Unknown vaccination status | 5 (0.8) |
| Total | At least 2x MMR | 581 (77.1) |
| | 1x MMR | 19 (2.5) |
| | Vaccinated, but unknown doses | 128 (17.0) |
| | No MMR | 16 (2.1) |
| | Unknown vaccination status | 10 (1.3) |

* Based on self-reported vaccination history. Five students (3.7%) did not know whether they were vaccinated. Seventy-six students (56.3%) indicated that they were vaccinated, but they did not know the number of MMR doses.

† Vaccination status of 121 students (19.5%) could not be verified via Praeventis.

## Identification of Mumps Virus Infections

The median mumps-specific IgG concentrations in the reference group sampled 6–10 months after proven mumps virus infection were 6648 RU/ml (IQR, 5923–8136 RU/ml), whereas the median concentrations in the negative control group were 139 RU/ml (IQR, 82–256 RU/mL). Receiver operator characteristics analysis showed that at 1500 RU/ml, sensitivity and specificity were 100% and 99.6%, respectively, with an area under the curve (AUC) of 0.99 (95% CI, 0.99–1.00; $p<0.001$). From the negative controls, 0.4% of vaccinated persons had a mumps-specific IgG concentration higher than 1500 RU/ml (Figure 2A). The majority of pre-outbreak samples from the Utrecht study cohort had IgG concentrations below 1500 RU/ml, except for 3 students for whom the first serum samples were obtained in 2010 (Figure 2B).

When applying our criteria for infection, defined as a 4-fold or more increase in IgG concentration or a post-outbreak IgG concentration higher than 1500 RU/ml, 44 of 754 students (5.8%) had a mumps virus infection, and 15 of these persons had a symptomatic infection, whereas 29 persons had an asymptomatic infection (Table 2). The cutoff of 1500 RU/ml led to the identification of 4 additional mumps virus infections that did not result in a 4-fold or more increase in IgG concentration.

With respect to symptomatic mumps virus infections, 13 of 15 blood samples fulfilled both serological criteria (Figure 3). However, for asymptomatic mumps virus infections, only 6 persons fulfilled both serological criteria, whereas a 4-fold or higher increase in IgG concentration could be detected in 26 students (Figure 3). This result indicates that the mumps-specific IgG concentrations after symptomatic mumps virus infections are higher than after asymptomatic infections. In addition, 3 persons with asymptomatic mumps virus infection had an IgG concentration in their post-outbreak blood sample above 1500 RU/ml but no 4-fold increase (Figure 3). Of the 25 persons who reported clinical mumps in the questionnaire, samples from 10 persons did not meet the serological criteria. Post-outbreak IgG concentrations in these 10 persons varied between 32 and 787 RU/ml.

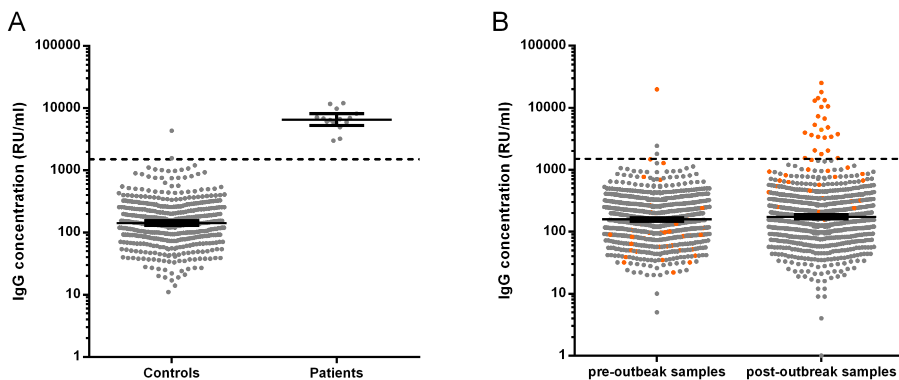


**Figure 2.** The graphic illustrates determination of a cutoff for mumps virus infections. (A) Based on an ROC analysis, a cutoff of 1500 RU/ml (range, 1384-2288 RU/ml) was calculated for mumps virus infection (dashed line). Patient samples were from fully MMR-vaccinated mumps patients, sampled between 6 and 10 months after infection (n=15). For the control group, we used IgG levels from vaccinated age-matched participants in a Dutch national serosurveillance study carried out in 2006/2007 (n=451). (B) The graphic shows mumps-specific IgG concentrations of pre- and post-outbreak samples from participants included in the Utrecht and Leiden cohort (n=754). Orange dots represent the pre- and post-outbreak IgG concentrations in individuals infected with mumps vius (n=44). Dashed line indicates the cutoff of 1500 RU/ml. Median IgG concentrations did not significantly differ between pre-outbreak samples and post-outbreak samples (158 vs 167 RU/ml; p=0.166).

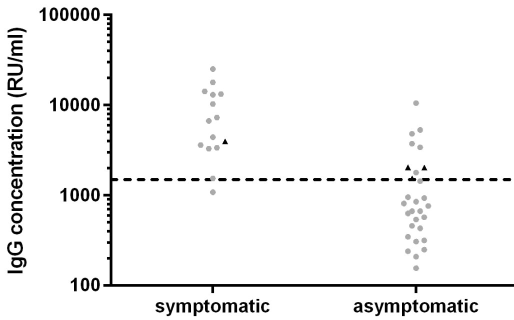


**Figure 3.** Distribution of the post-outbreak mumps-specific IgG concentrations in persons with symptomatic and asymptomatic mumps virus infections from the Leiden cohort (n=135) and Utrecht cohort (n=619). Mumps-specific IgG concentrations were higher in the post-outbreak samples of persons with a symptomatic mumps virus infection compared with persons with an asymptomatic mumps virus infection. Grey dots represent post-outbreak samples with a 4-fold or more increase in IgG concentrations. Black triangles represent post-outbreak samples with no 4-fold increase in IgG concentration. Dashed line indicates the single-point cutoff at 1500 RU/ml.

**Table 2.** Attack rates for symptomatic and asymptomatic mumps virus infection for the 2 separate cohorts and the total cohort, stratified by vaccination status*

| | | No. (%) mumps virus infections | | |
|---|---|---|---|---|
| Cohort description | No. participants | Symptomatic | Asymptomatic | Total |
| Leiden† | | | | |
| At least 2x MMR | 47 | 2 (4.3) | 0 (0.0) | 2 (4.3) |
| At least 1x MMR | 128 | 3 (2.3) | 4 (3.1) | 7 (5.5) |
| All students | 135 | 3 (2.2) | 5 (3.7) | 8 (5.9) |
| Utrecht§ | | | | |
| At least 2x MMR | 534 | 11 (2.1) | 19 (3.6) | 30 (5.6) |
| At least 1x MMR | 600 | 12 (2.0) | 22 (3.7) | 34 (5.7) |
| All students | 619 | 12 (1.9) | 24 (3.9) | 36 (5.8) |
| Total | | | | |
| At least 2x MMR | 581 | 13 (2.2) | 19 (3.3) | 32 (5.5) |
| At least 1x MMR | 728 | 15 (2.1) | 26 (3.6) | 41 (5.6) |
| All students | 754 | 15 (2.0) | 29 (3.8) | 44 (5.8) |

* Mumps virus infections were defined as either a 4-fold increase or more in mumps-specific IgG concentrations in the 2 consecutive blood samples or an IgG concentration higher than 1500 RU/ml in the post-outbreak sample.

† Based on self-reported vaccination history. Five students (3.7%) did not know whether they were vaccinated. Seventy-six students (56.3%) indicated that they were vaccinated, but they did not know the number of MMR doses.

§ Vaccination status of 121 students (19.5%) could not be verified via Praeventis.

## Attack rates

Eight students from the Leiden cohort (n=135) had serological evidence for mumps virus infection, resulting in an attack rate of 5.9% (Table 2). Three of those students had symptomatic mumps (attack rate 2.2%), diagnosed by a physician in 1 case. In the Utrecht cohort, 36 of the 619 students had a mumps virus infection during the outbreak based on their IgG concentrations, resulting in an attack rate of 5.8% (Table 2). Twelve of these 36 students (attack rate 1.9%) had a symptomatic mumps virus infection according to the questionnaires, and 6 of these were diagnosed by a physician. Attack rates in students who had received at least 2 MMR doses (n=534) were comparable with the total Utrecht cohort (Table 2).

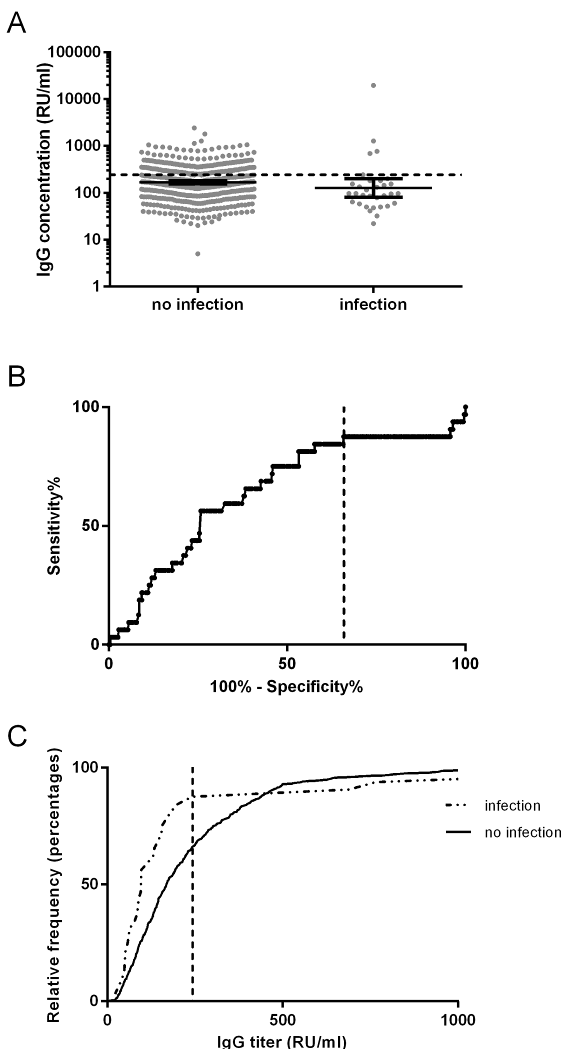


**Figure 4.** Comparison of pre-outbreak antibody levels between persons who were infected with mumps during the outbreak and persons who were not infected. All persons from the Leiden and Utrecht cohort with at least 2 MMR vaccinations were included (n=571). (A) Distribution of pre-outbreak mumps-specific IgG concentrations in persons with and without a mumps virus infection. Median IgG concentrations were lower in infected persons (p=0.005). Sensitivity and specificity were 87.5% and 34.1%, respectively, with a cutoff at 243 RU/mL (dashed line). (B) ROC analysis of the mumps-specific IgG pre-outbreak concentrations of persons with and without a mumps virus infection. Dashed line indicates the cutoff at 243 RU/mL. (C) Relative frequency distribution of pre-outbreak mumps-specific IgG concentrations in persons with and without a mumps virus infection. Dashed line indicates the cutoff at 243 RU/ml.

### Correlate of protection for vaccinated students

To determine whether individuals with low mumps-specific IgG concentrations have an increased risk for mumps virus infection, serological data from pre-outbreak samples from Utrecht (2007–2010) and Leiden (2008–2010) were merged for all persons who had received 2 MMR doses (n=571). Thirty-two persons (5.6%) had been infected with mumps virus based on serological analysis. Sera from the others (n=539) were considered negative controls. Median mumps-specific IgG concentrations in the pre-outbreak sera of the infected students were significantly lower than median concentrations in the control group (97 RU/ml [IQR, 59–175 RU/ml] vs 169 RU/ml [IQR, 94–304 RU/ml]; p=0.005; Figure 4A). A ROC analysis showed an AUC of 0.65 (95% CI, 0.54–0.75). However, no clear pre-outbreak cutoff could be identified that separated infected persons from noninfected persons. Mixture modeling did not substantiate this difference. The cutoff value that discriminated best between the pre-outbreak IgG concentrations from infected and noninfected persons was 243 RU/ml, resulting in a sensitivity and specificity of 87.5% (95% CI, 71.0%–96.5%) and 34.1% (95% CI, 30.1%–38.3%), respectively. However, specificity percentages have to be interpreted with caution, because probably not all persons were exposed to mumps (Figures 4B and C). Among persons exposed to mumps, the IgG concentrations between infected and noninfected persons overlapped as well (data not shown), which indicates that pre-outbreak IgG concentrations are not the only protective factor against mumps virus infection. There was no significant difference in pre-outbreak concentrations between persons with symptomatic and asymptomatic infection (data not shown).

### Risk factors for mumps virus infection

Risk factors for mumps virus infection were determined from analysis of the questionnaire responses. Questionnaires used in Leiden and Utrecht were comparable, and because multilevel analysis did not result in significant differences between the 2 student cohorts, the data were merged in logistic regression analysis. The risk factor significantly associated with mumps virus infection in both univariate and multivariate analyses was circulation of mumps among housemates (OR, 7.25 [95% CI, 3.20–16.40]; p<0.001) (Table 3).

## DISCUSSION

This study has shown that serological analysis can be used to define mumps virus infection in vaccinated persons during outbreak situations with high sensitivity and specificity. Approximately two thirds of these serologically confirmed mumps virus infections were asymptomatic, judged from the fact that those persons had not

reported clinical mumps in the questionnaire. This percentage is comparable to the percentage asymptomatic mumps virus infections estimated previously (8). Besides the 4-fold increase in mumps-specific IgG concentrations that serves as the gold standard in serological studies, we have added a single-point cutoff value of 1500 RU/ml to discriminate antibodies acquired through vaccination from antibodies induced by mumps virus infection. Although this cutoff value is very conservative, some individuals classified as being infected with mumps virus would have been missed on the basis of solely a 4-fold increase in mumps-specific IgG concentration. When less conservative serological criteria were applied, more asymptomatic infections compared with symptomatic infections were identified, thereby changing the ratio between symptomatic and asymptomatic infections (data not shown). In total, 10 clinical mumps cases could not be confirmed as such based on our serological approach. Because these cases had indicated in the questionnaire that mumps was not laboratory confirmed by either polymerase chain reaction or IgM serology during period of disease, it is possible that the symptoms were not caused by a mumps virus infection. Four of these mumps cases were diagnosed by a physician, but no further information was provided regarding whether samples of these 4 cases tested negative for infection or whether there were no laboratory tests performed. The other 6 mumps cases were not diagnosed by a physician and therefore no laboratory tests were performed.

Attack rate calculations were based on the assumption that mumps had not circulated among these cohorts before January 2010. However, it cannot be excluded that some students had been exposed to mumps earlier. Mumps outbreaks have occurred in the Netherlands in 2004 at an international university of hospitality management and between 2007 and 2009 in an orthodox religious community with low vaccination coverage (17,18). Still, the latter outbreak involved another age group and genotype mumps virus (D), and surveillance data showed no evidence for previous mumps virus infections in our study cohort. Three persons in the Utrecht cohort had pre-outbreak IgG concentrations higher than 1500 RU/ml, and they potentially had a mumps virus infection before the pre-outbreak serum was drawn. Sera from these 3 persons had been banked at the beginning of 2010.

In a previous study, no cutoff point could separate all mumps patients from nonpatients based on pre-outbreak mumps neutralization titers (3). In this study, a potential explanation for the lack of a cutoff is that it remains unknown who was exposed, and lack of exposure in part of the study population will result in an underestimation of the specificity. Furthermore, the Luminex assay uses purified whole-virus antigens, and therefore the assay will also detect nonneutralizing IgG antibodies, which do not prevent the virus from entering the cells (19). The failure to define a specific concentration of mumps-specific antibodies that is protective

against mumps virus infection suggests that effective protection against mumps virus infection is governed by host immune mechanisms other than IgG concentrations in serum.

Median IgG concentrations in pre-outbreak samples from persons who became infected with mumps after serum sampling were 97 RU/ml. This concentration is higher than the 45 RU/ml, which is used as a measure to confer immune protection (13–15). When applying these cutoffs to mumps-specific IgG concentrations from 451 vaccinated age-matched individuals included in a large Dutch serosurveillance study, the IgG concentration was below 97 RU/ml in 147 persons (32.6%) and below 45 RU/ml in only 42 (9.3%) persons (16).

To conclude, serological analysis enabled us to calculate attack rates for both symptomatic and asymptomatic mumps virus infection and to determine risk factors for mumps virus infection. This study shows the usefulness of serological analysis in addition to questionnaires and the possibility to retrospectively identify mumps virus infections based on mumps-specific IgG concentrations in paired pre- and post-outbreak samples.

**Table 3.** Univariate and multivariate analysis of risk factors for mumps virus infection*

| | Univariate analysis results | | Multivariate analysis results | | | |
|---|---|---|---|---|---|---|
| Characteristic | OR (95% CI) | p value | OR (95% CI) | p value | OR (95%CI) | p value |
| Year of birth | | 0.501 | | 0.377 | | 0.166 |
| 1958-1985 | 0.00 | 0.979 | 0.00 | 0.978 | 0.00 | 0.977 |
| 1986-1988 | 0.35 (0.09-1.35) | 0.127 | 0.29 (0.07-1.15) | 0.079 | 0.20 (0.05-0.81) | 0.024 |
| 1989-1991 | 0.45 (0.13-1.61) | 0.218 | 0.35 (0.10-1.29) | 0.114 | 0.28 (0.08-1.05) | 0.058 |
| 1992-1994 | Ref | - | Ref | - | Ref | - |
| Gender | | | | | | |
| Female | Ref | - | Ref | - | Ref | - |
| Male | 1.22 (0.59-2.53) | 0.594 | 1.37 (0.65-2.88) | 0.409 | 1.49 (0.69-3.22) | 0.309 |
| MMR vaccination | | | | | | |
| No | Ref | - | | | | |
| ≥ 1 dose | 0.96 (0.12-7.38) | 0.965 | | | | |
| Membership of student association | | | | | | |
| No | Ref | - | Ref | - | Ref | - |
| Yes | 2.18 (1.06-4.49) | 0.034 | 1.78 (0.84-3.76 | 0.130 | 1.53 (0.71-3.32) | 0.277 |
| Living in a student house | | | | | | |
| No | Ref | - | Ref | - | Ref | - |
| Yes | 2.42 (1.11-5.28) | 0.027 | 2.04 (0.91-4.61) | 0.085 | 1.56 (0.66-3.66) | 0.311 |
| No. housemates | | 0.319 | | | | |
| 1-3 | Ref | - | | | | |
| 4-6 | 0.98 (0.39-2.42) | 0.959 | | | | |
| 7-10 | 1.68 (0.61-4.65) | 0.319 | | | | |
| 11-19 | 2.46 (0.81-7.50) | 0.115 | | | | |
| 20 or more | 2.34 (0.45-12.10) | 0.311 | | | | |
| Circulation of mumps in the environment | | <0.001 | | | | <0.001 |
| No | Ref | - | | | Ref | - |
| Yes, in the social environment | 1.35 (0.61-2.99) | 0.463 | | | 1.11 (0.49-2.51) | 0.808 |
| Yes, among housemates | 8.49 (3.95-18.20) | <0.001 | | | 7.25 (3.20-16.40) | <0.001 |

* All persons with a 4-fold or more increase in mumps-specific IgG concentration or pre-outbreak IgG concentration ≥ 1500 RU/ml were considered to have had a mumps virus infection (n=44).

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## POTENTIAL CONFLICTS OF INTEREST

All authors: No reported conflicts.

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# VACCINE-INDUCED PROTECTION

# CHAPTER 7

## Mumps-specific cross-neutralization by MMR vaccine-induced antibodies predicts protection against mumps virus infection

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# ABSTRACT

## Background

Similar to other recent mumps genotype G outbreaks worldwide, most mumps patients during the recent mumps genotype G outbreaks in the Netherlands had received 2 doses of measles, mumps and rubella (MMR) vaccine during childhood. Here, we investigate the capacity of vaccine-induced antibodies to neutralize wild type mumps virus strains, including mumps virus genotype G.

## Methods

In this study, we tested 105 pre-outbreak serum samples from students who had received 2 MMR vaccine doses and who had no mumps virus infection (n=76), symptomatic mumps virus infection (n=10) or asymptomatic mumps virus infection (n=19) during the mumps outbreaks. In all samples, mumps-specific IgG concentrations were measured by multiplex immunoassay and neutralization titers were measured against the Jeryl Lynn vaccine strain and against wild type genotype G and genotype D mumps virus strains.

## Results

The correlation between mumps-specific IgG concentrations and neutralization titers against Jeryl Lynn was poor, which suggests that IgG concentrations do not adequately represent immunological protection against mumps virus infection by antibody neutralization. Pre-outbreak neutralization titers in infected persons were significantly lower against genotype G than against the vaccine strain. Furthermore, antibody neutralization of wild type mumps virus genotype G and genotype D was significantly reduced in pre-outbreak samples from infected persons as compared with non-infected persons. No statistically significant difference was found for the vaccine strain. The sensitivity/specificity ratio was largest for neutralization of the genotype G strain as compared with the genotype D strain and the vaccine strain.

## Conclusions

The reduced neutralization of wild type mumps virus strains in MMR vaccinated persons prior to infection indicates that pre-outbreak mumps virus neutralization is partly strain-specific and that neutralization differs between infected and non-infected persons. Therefore, we recommend the use of wild type mumps virus neutralization assays as preferred tool for surveillance of protection against mumps virus infection.

## INTRODUCTION

At the end of 2009, large mumps outbreaks started in the Netherlands and continued for 3 years, followed by some years with smaller and more local mumps outbreaks (1,2). Most of the patients were young adults who had received 2 measles, mumps, and rubella (MMR) vaccinations during childhood at 14 months and 9 years of age, according to the Dutch national immunization program (3,4). Similar to other recent mumps outbreaks among MMR vaccinated persons worldwide, the mumps outbreaks in the Netherlands were dominated by mumps genotype G virus strains, whereas the Jeryl Lynn mumps strain of the MMR vaccine belongs to genotype A. It has been shown previously that vaccinated persons develop sufficient neutralizing antibodies against wild type mumps genotype G virus, although the neutralization capacity is lower against the wild type strain as compared with the vaccine strain (5,6). However, no correlate of protection has been defined so far that distinguishes MMR vaccinated persons with sufficient immunological protection from those who are not protected against mumps virus infection.

Here, we aimed to investigate the capacity of vaccine-induced antibodies to neutralize various mumps virus strains and to establish a correlate of protection based on pre-outbreak neutralizing antibody titers. Pre-outbreak serum samples were selected from MMR vaccinated students who were infected with mumps virus during the outbreak and these samples were compared with samples from non-infected students. The correlation between mumps-specific IgG concentrations and functional antibodies against the Jeryl Lynn vaccine strain was determined. Furthermore, we studied strain-specific neutralization between the Jeryl Lynn vaccine strain, mumps virus genotype G, which circulated in the Netherlands during the recent mumps outbreaks among vaccinated persons, and mumps virus genotype D, which caused a mumps outbreak among unvaccinated children in 2007-2009 in the Netherlands (7). We investigated pre-outbreak samples from both persons with symptomatic and asymptomatic infections to determine if reduced neutralization of wild type strains affects the occurrence of clinical mumps in mumps virus infected persons.

## METHODS

### Study subjects and pre-outbreak serum samples

All pre-outbreak sera used in this study were samples from a medically ethically approved serological study previously described (NL38042.041.11) (8). This retrospective study was performed among students from the city of Utrecht in 2012. Besides a self-sampled dried blot spot sample and a filled out questionnaire

concerning MMR vaccination history, risk factors and mumps symptoms, a serum sample was retrospectively obtained from each student. In addition, MMR vaccination history was verified for 80.5% of the students from the data recorded in the nationwide vaccination registration system (Praeventis) (8). In total, 619 pre-outbreak serum samples collected between 2007 and 2010 were obtained and included for the initial analysis to identify mumps virus infections based on mumps-specific antibody concentrations (8). For this study, a selection was made consisting of 105 pre-outbreak serum samples from persons who had received 2 MMR doses and who had symptomatic mumps virus infection, asymptomatic mumps virus infection or no mumps virus infection during the mumps outbreaks that followed. The serological criteria for mumps virus infection were a fourfold increase in IgG concentration or a single-point cutoff at 1500 RU/ml (8). Persons with symptomatic mumps virus infection fulfilled at least one of these serological criteria and indicated in the questionnaire that they developed mumps symptoms during the period 2009-2012 (n=10). Persons with asymptomatic mumps virus infection fulfilled also at least one of the serological criteria, but they did not report any mumps symptoms in the questionnaire (n=19). Non-infected persons did not fulfill any of the serological criteria for mumps virus infection and did not report mumps symptoms (n=76). The 105 pre-outbreak serum samples selected for this study included all available samples from infected persons that fulfilled the criteria in this study and a selection of the samples from non-infected persons. The geometric mean IgG concentrations in the selected pre-outbreak samples from persons without a mumps virus infection did not differ from the total cohort from the initial study (192 RU/ml versus 171 RU/ml; p=0.618) (8).

## Cell culturing and mumps virus isolation

Vero cells (Monkey African Green Kidney, ECACC) were cultured at 37°C in 5% $CO_2$ in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% fetal calf serum (FCS), penicillin, streptomycin, and L-glutamine. For inoculation of the Jeryl Lynn mumps virus strain, DMEM supplemented with 2% FCS, penicillin, streptomycin, and L-glutamine was used. The same medium composition was used for isolation of wild type mumps virus strains, with the addition of nystatin. Virus culturing was performed at 36°C in 5% $CO_2$. Mumps virus Jeryl Lynn strain was seeded on Vero cells and passaged 2 times before harvesting. Mumps virus strains belonging to genotype G (MuVi/Utrecht.NLD/40.10) and genotype D (MuVi/Sint Philipsland.NLD/02.08) were isolated from laboratory-confirmed mumps virus-positive throat swabs and were passaged 2 times and 4 times respectively before harvesting. Virus stocks were stored at -80°C until use. Aliquots were made from all virus stocks to avoid multiple freeze-thaw cycles.

## Multiplex IgG immunoassay

Mumps-specific IgG concentrations were measured with a fluorescent bead-based multiplex immunoassay (MIA) using Luminex technology as described previously (9). Purified Jeryl Lynn antigen was coupled to carboxylated beads for detection of mumps-specific IgG. Samples were 1:200 diluted in assay buffer (phosphate buffered saline (PBS) containing 0.1% Tween-20 and 3% bovine serum albumin). On each plate, the WHO International Standard Anti Rubella Immunoglobulin RUBI-1-94 (The National Institute for Biological Standards and Control), controls and blanks were included. Antibody concentrations were expressed in local (RIVM) units per milliliter (RU/ml) and were based on the fluorescent intensity of the reference serum curve RUBI-1-94, which has a mumps-specific IgG concentrations of 4384.512 RU/ml and was selected as alternative serological standard for mumps to enable comparison and bridging of our results to other studies (8). When the 1:200 sample dilution fell outside the range of the reference serum curve, further dilutions up to 1:50,000 were used for analysis.

## Focus-reduction neutralization test (FRNT)

Neutralization by vaccine-induced antibodies was tested by FRNT, partly based on the protocol described by Vaidya *et al.* (10). Neutralization tests were performed in 96-wells plates. Fourfold dilutions were made in DMEM supplemented with 2% FCS, penicillin, streptomycin, and L-glutamine. Viruses and samples were mixed and incubated for 2 hours at 37°C. Medium was removed from pre-cultured Vero cells and 50 *μ*l of virus mixture was added to each well. Plates were incubated for 4 hours at 36°C, before the mixture was removed from the Vero cells and 200 *μ*l of 0.8% carboxymethylcellulose in DMEM was added to each well. Plates were incubated for 40 hours at 36°C with 5% $CO_2$ before they were washed with PBS and thereafter fixed with a mixture of aceton and methanol (2:3). After 10 minutes, plates were washed with ice cold PBS, and then incubated with block buffer (PBS containing 1% bovine serum albumin) for 30 minutes at 36°C. Anti-mumps nucleoprotein antibody (7B10, Abcam) was diluted in block buffer (1:3000) and 100 *μ*l was added to each well. After incubation for one hour at 36°C, plates were washed 3 times with PBS containing 0.1% Tween-20 (PBST) before 100 *μ*l of goat-anti-mouse IgG-HRP (DAKO) diluted in block buffer (1:2000) was added to each well and plates were incubated for one hour at 36°C. Plates were washed 3 times with PBST and wells were stained with 50 *μ*l of True Blue peroxidase substrate (Kirkegaard & Perry Laboratories). The numbers of plaques were counted and the 50% neutralizing dose ($ND_{50}$) of each sample was calculated with the Kärber formula, using the serum dilution factor as calculated from the virus mixture (10).

The WHO international standard RUBI-1-94 was used as positive control in each assay run. For a valid assay, the titer of RUBI-1-94 was required to be within 2 standard deviations of its cumulative historical mean value. Furthermore, assays were only valid if the mean plaque number of the virus controls was in the range of 20-80 and cell controls had no plaques. If assays did not meet the validation criteria, samples were retested. Only samples with at least two valid results per virus strain in separate runs were used for analyses. To correct for inter-assay differences, we normalized the $ND_{50}$ values by multiplying the raw $ND_{50}$ titers by the RUBI-1-94 factor, which was defined as the cumulative historical mean value of RUBI-1-94 divided by the measured $ND_{50}$ of RUBI-1-94.

### Statistical analysis

Geometric mean (with 95% confidence interval [CI]) IgG concentrations and neutralization titers were compared between infected and non-infected persons using the Kolmogorov-Smirnov test. Correlations between IgG concentrations and neutralization titers were computed using nonlinear regression with the Spearman correlation coefficient. For comparison of neutralization titers against different mumps virus strains, the Friedman test was used. With a receiver operator characteristics (ROC) analysis, the area under the curve (AUC) with 95% confidence limits was calculated for mumps-specific IgG concentrations and neutralizing antibodies against the different mumps virus strains to determine a correlate of immune protection. The DeLong test was used for comparison of AUCs, using the genotype G neutralization titer as reference (11). All analyses were performed using SPSS version 22, SAS version 9 and GraphPad Prism version 6. P values <0.05 were considered as statistically significant.

## RESULTS

### Mumps-specific IgG concentrations

Since mumps-specific IgG concentrations are widely used to define levels of mumps immunity, mumps-specific IgG concentrations were studied in pre-outbreak serum samples from infected and non-infected persons. Mumps-specific IgG concentrations were measured by MIA using purified Jeryl Lynn as antigen and these results were compared with neutralization titers against the same mumps virus Jeryl Lynn strain. In this way, we could study the correlation between mumps-specific IgG concentrations and neutralization titers.

Mumps-specific IgG concentrations were significantly lower in pre-outbreak samples from infected persons than in samples from non-infected persons (122 RU/ml (86-173 RU/ml) versus 192 RU/ml (161-228 RU.ml); p=0.008). The Jeryl Lynn-specific neutralization titer in pre-outbreak samples from infected and non-

infected persons was $ND_{50}$ 67 (47-95) and $ND_{50}$ 115 (87-152), respectively ($p=0.078$). The correlation between IgG concentrations and neutralization titers against Jeryl Lynn in pre-outbreak samples was poor for both the infected and non-infected persons ($r^2=0.017$ and $r^2=0.165$, respectively) (Figure 1). There was no difference in mumps-specific IgG concentrations and neutralization titers between symptomatic and asymptomatic mumps virus infections.

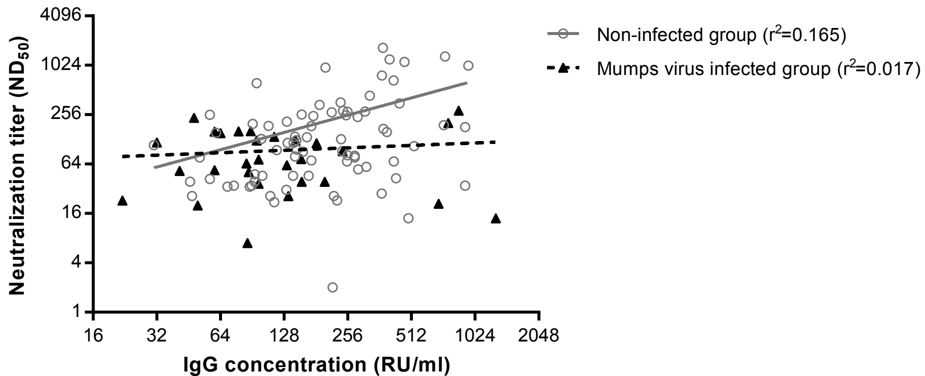


**Figure 1.** Correlation between mumps-specific IgG concentrations and neutralization titers against Jeryl Lynn in pre-outbreak samples from vaccinated students (n=105). Lines represent the best fit lines with nonlinear regression.

## Strain-specific neutralization in pre-outbreak samples

To investigate if antigenic differences between the vaccine strain and wild type strains resulted in reduced neutralization of the wild type strains, we measured virus neutralization in pre-outbreak serum samples against the Jeryl Lynn vaccine strain as well as against wild type genotype G and genotype D strains. In pre-outbreak samples from persons without a mumps virus infection, neutralization titers against the different mumps virus strains did not significantly differ ($p>0.105$) (Figure 2A). For infected persons pre-outbreak neutralization titers against genotype G were significantly lower than neutralization titers against Jeryl Lynn ($p=0.009$) (Figures 2A and 3). When the mumps virus infected group was further divided into symptomatic and asymptomatic mumps virus infections, only in the asymptomatic group neutralization titers against genotype G were significantly lower than neutralization titers against Jeryl Lynn ($p=0.036$) (Figure 2B). The lack of significant results in the symptomatic group may be due to the low number of symptomatic infections in our study cohort.

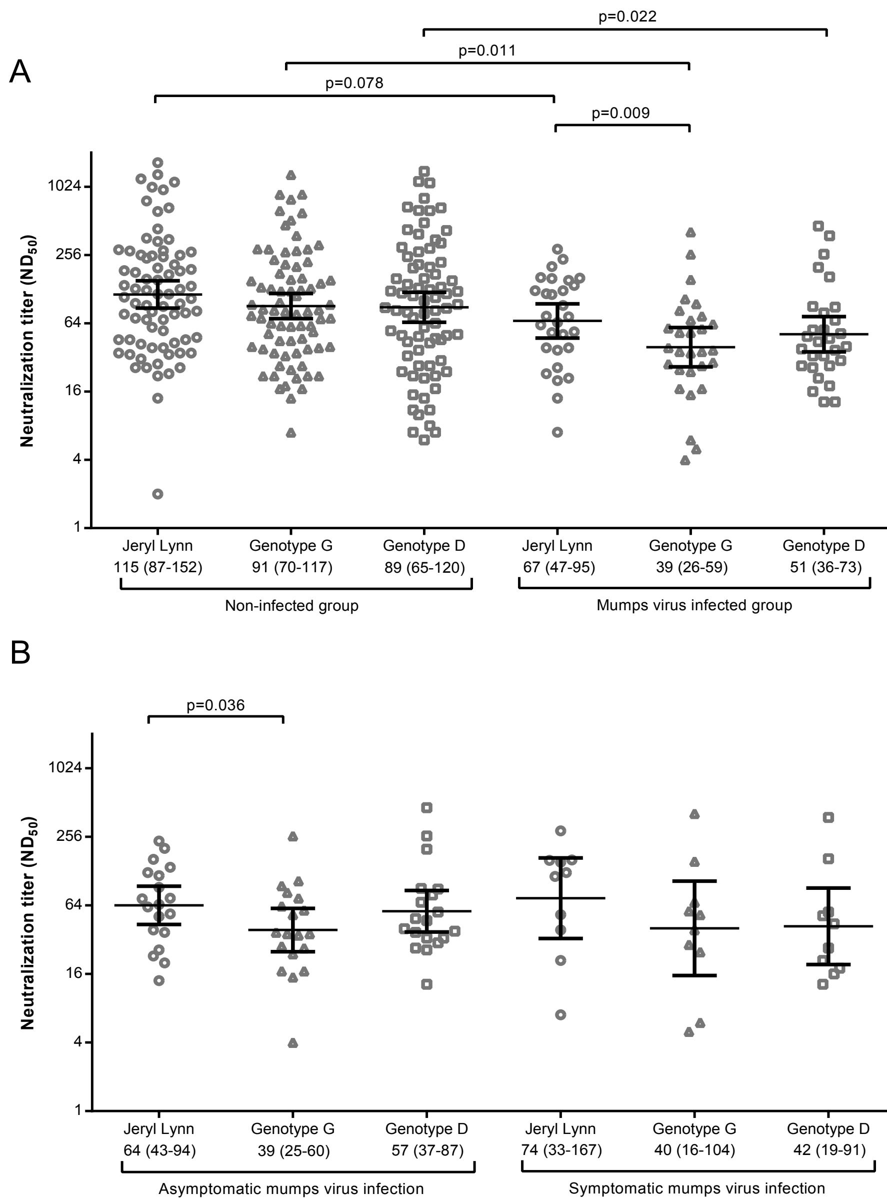


**Figure 2.** Neutralization titers against the different mumps virus strains. P values <0.10 are shown above the graphs. Geometric mean neutralization titers with 95% confidence interval are shown on the X axes. A) Neutralization titers in persons with or without a mumps virus infection. B) Neutralization titers in mumps virus infected persons, further differentiated into symptomatic and asymptomatic mumps virus infections.

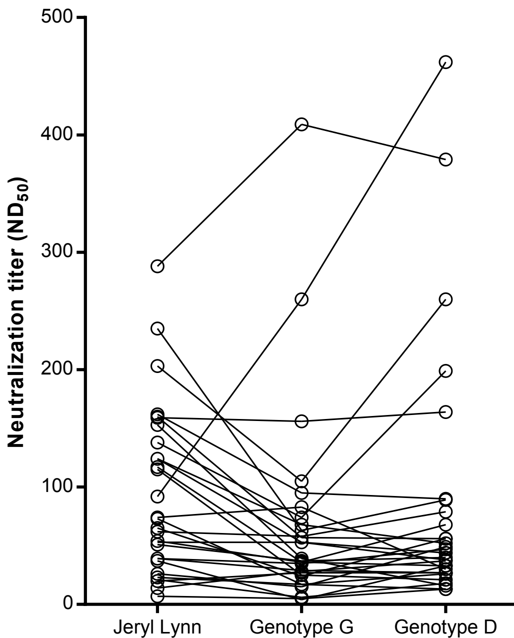


**Figure 3** Paired neutralization titers against the different mumps virus strains in pre-outbreak samples from infected persons. Neutralization titers were significantly lower against the wild type genotype G strain than against the Jeryl Lynn vaccine strain (p=0.009). There were no significant differences between the wild type genotype D strain and any of the other strains.

## Correlate of protection

To establish a correlate of protection based on mumps-specific neutralizing antibodies, pre-outbreak neutralization titers were compared between infected and non-infected persons. The difference in pre-outbreak neutralization titers against Jeryl Lynn between infected and non-infected persons was not significant (p=0.078) (Figure 2A). However, neutralization titers against the wild type mumps virus genotype G and genotype D strains were significantly lower in infected persons than in non-infected persons (p=0.011 and p=0.022, respectively) (Figure 2A), which suggests that the capacity of vaccine-induced antibodies to neutralize wild type strains is a more appropriate marker for protection. The difference in neutralization titers between symptomatic and asymptomatic infections was not significant (Figure 2B).

A ROC analysis with the 3 different mumps virus strains to compare sensitivity/specificity ratios resulted in an AUC of 0.703 (0.595-0.812) for genotype G, which is larger than the AUCs for Jeryl Lynn (0.628 (0.516-0.741); p=0.071) and for genotype D (0.638 (0.526-0.750); p=0.090), although the differences were not statistically significant (Figure 4). A ROC analysis based on the IgG concentrations resulted in an AUC of 0.689 (0.564-0.813), which is also not significantly different from the AUC for genotype G (p=0.841) (Figure 4). No cutoff could be established that separated all mumps virus infections from all non-infections. For mumps-specific IgG concentrations, the cutoff at 243 RU/ml, as published previously, resulted in a sensitivity of 86.21% and a specificity of 39.47% when applied to the pre-outbreak samples from this study (8). When this sensitivity of 86.21% was used to calculate a cutoff for the neutralization titers, the cutoff for Jeryl Lynn-specific neutralization was at $ND_{50}$ 167 (89.66% sensitivity and 38.16% specificity),

which was comparable with the cutoff for genotype D-specific neutralization ($ND_{50}$ 169 with 86.21% sensitivity and 30.26% specificity). The cutoff value for genotype G-specific neutralization was lower ($ND_{50}$ 97 with 86.21% sensitivity and 43.42% specificity). These data indicate that the sensitivity/specificity ratio was most optimal for neutralizing antibody titers against the mumps virus genotype G strain, although differences were not statistically significant. Furthermore, the specificity at these cutoffs was highest for genotype G-specific neutralization.

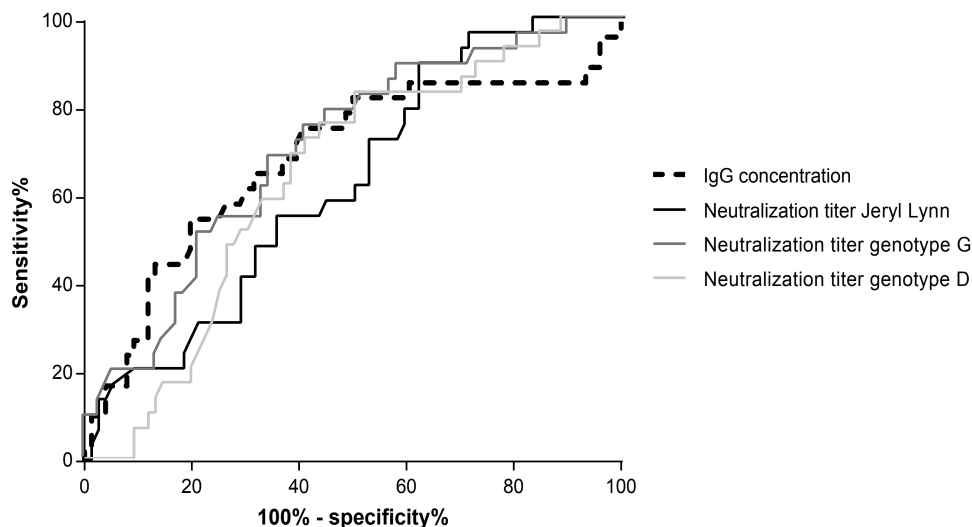


**Figure 4** Receiver operator characteristics analysis based on IgG concentrations and neutralization titers against Jeryl Lynn, genotype G and genotype D mumps virus strains.

## DISCUSSION

In pre-outbreak serum samples from mumps virus infected persons who had received 2 MMR doses, neutralization titers against mumps virus genotype G were lower than against the Jeryl Lynn strain, which suggests that neutralization by vaccine-induced antibodies is partly strain-specific. This difference in neutralization between the vaccine strain and a wild type mumps virus genotype G strain was previously described by others (5,6). Contrary to those previous studies, we only observed this difference in pre-outbreak serum samples from infected persons. Furthermore, in our study only neutralization titers against the wild type genotype G and genotype D mumps viruses differed between infected and non-infected persons, whereas in one of the previous studies the neutralization titers against Jeryl Lynn were lower for infected persons than for non-infected persons (5).

In our study, both symptomatic and asymptomatic mumps virus infections were identified using reported symptoms as well as mumps-specific IgG concentrations as criteria for mumps virus infection. In this way, asymptomatic mumps virus infections could be identified and these were excluded from the non-infected group. Inclusion of asymptomatically infected persons in the non-infected group could affect the results. This might explain the difference in results between our study and previous studies in which pre-outbreak neutralization titers were compared between mumps patients and non-patients. In spite of the use of mumps-specific IgG concentrations as criteria for mumps virus infection, it cannot be excluded that some students had been exposed to mumps earlier. The high neutralization titers against the wild type viruses in 2 pre-outbreak samples from infected students suggest that these persons may have been previously infected with mumps virus, although surveillance data showed no evidence for previous mumps virus infections in our study cohort (Figure 3).

The poor correlation between mumps-specific IgG concentrations and neutralization titers observed in our study confirmed results from other studies (10,12–14). The MIA that was used to measure the mumps-specific IgG concentrations is based on purified Jeryl Lynn virus and measures the total amount of mumps-specific IgG present in the sample. In contrast, the neutralization assay was used to measure virus neutralizing antibodies, which may be an important immunological mechanism in protection against mumps virus infection (15,16). ROC analysis showed that the sensitivity/specificity ratio is largest for genotype G-specific neutralization titers. Although no cutoff value for pre-outbreak neutralization titers could be identified that separates all infected persons from non-infected persons, our data suggest that genotype G-specific neutralization is a more adequate serological marker for protection against mumps virus infection, as most of the recent mumps outbreaks among MMR vaccinated persons were caused by mumps virus genotype G strains.

The results from this study suggest that antigenic differences between the mumps virus vaccine strain and wild type strains affect mumps-specific humoral immune responses in MMR vaccinated persons. Two mumps virus membrane proteins that play a major role in virus neutralization are the fusion (F) protein and the haemagglutinin-neuraminidase (HN) protein (17–19). As was hypothesized previously, the difference in neutralization between Jeryl Lynn and the wild type strains may result from antigenic differences in the neutralizing epitopes of the F and HN proteins (6). Because of the potential biological relevance of mumps virus neutralization and the difference in strain-specific neutralization between infected and non-infected persons, we recommend the use of wild type mumps virus neutralization assays as preferred tool for surveillance of protection against mumps virus infection.

## ACKNOWLEDGEMENTS

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# VACCINE-INDUCED PROTECTION

# CHAPTER 8

## Severity of mumps disease is related to MMR vaccination status and viral shedding

*Vaccine* 2016 34(16):1868-1873

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# ABSTRACT

## Background

During recent years, various mumps outbreaks have occurred among measles, mumps, and rubella (MMR) vaccinated persons in various countries worldwide, including the Netherlands. We studied mumps virus shedding in MMR vaccinated and unvaccinated mumps patients and related these findings to clinical data.

## Methods

In this study, we included 1112 mumps patients of whom diagnostic samples were tested positive in our laboratory between 1 January 2007 and 31 December 2014. We compared mumps virus shedding and severity of disease between patients who had received 2 doses of MMR (n=592) and unvaccinated mumps patients (n=195). Mumps virus shedding in saliva and urine specimens was measured by qPCR. Severity of disease was studied in a subset of patients with clinical data available.

## Results

Mumps patients who had received 2 MMR doses shed less often mumps virus in their urine than unvaccinated patients. Salivary viral loads were higher at day of onset of disease in twice MMR vaccinated patients with viruria than in twice MMR vaccinated patients without viruria. However, salivary viral loads did not significantly differ between patients who had received 2 MMR doses and unvaccinated patients. Bilateral parotitis and orchitis were less often reported in patients who had received 2 MMR doses than in unvaccinated patients. Furthermore, the prevalence of bilateral parotitis and orchitis was higher among twice MMR vaccinated patients with viruria than among twice MMR vaccinated patients without viruria.

## Conclusions

MMR vaccination was associated with less severe disease among mumps patients. Systemic spread of virus was associated with more severe disease. The elevated salivary viral loads in patients with systemic mumps disease suggest that these patients pose a higher risk for mumps virus transmission. Our study contributes to the understanding of mumps virus pathogenesis and shows the protective effect of MMR vaccination on severity of disease.

## INTRODUCTION

Mumps is characterized by parotitis, usually bilateral. However, at least one third of the mumps virus infections are asymptomatic or cause non-specific symptoms such as fever, headache, malaise and myalgia without parotitis (1–4). The most common complication is orchitis, which occurred in up to 12.7% of the post-pubertal males with mumps reported during the recent mumps outbreaks (5–8). The Netherlands began measles, mumps, and rubella (MMR) vaccination in 1987. The vaccine, containing the Jeryl Lynn mumps virus strain, is administered via the national immunization program in a 2-dose schedule at 14 months and 9 years of age. During recent years mumps outbreaks have occurred among MMR vaccinated persons in the Netherlands as well as in other countries worldwide (9–12). The majority of mumps patients in the Netherlands were between 18 and 25 years of age and had received 2 MMR doses (7). After a mumps outbreak among unvaccinated persons caused by mumps virus genotype D in 2007–2009, the predominant mumps virus genotype during the recent mumps outbreaks from 2009 onwards was genotype G (12,13).

The incubation period of mumps varies between 15 and 24 days, with a median of 19 days (14). The virus has been isolated from mumps patients as early as 7 days before up to 8 days after onset of symptoms. However, virus shedding decreases rapidly after onset of symptoms (15,16). When the virus spreads from the respiratory tract through the body, systemic mumps virus infection can occur. Infection of the kidneys results in viruria, which can last for up to 14 days after onset of symptoms (17). Viruria occurs less often in MMR vaccinated mumps patients than in unvaccinated patients (18–20).

It has been shown previously in a selection of children with mumps that viral loads in saliva are lower in MMR vaccinated mumps patients than in unvaccinated patients, suggesting a lower risk for mumps virus transmission via salivary droplets (20). However, contrary to the adult mumps patients during the recent outbreaks, the vaccinated mumps patients in that study were children who had mumps shortly after the second MMR dose, since the median time between onset of disease and second MMR vaccination was 4 years. Secondly, there are indications that MMR vaccination reduces the risk for mumps-associated complications, such as orchitis (7,8). These findings suggest that although MMR vaccination does not fully protect against mumps virus infection, it may prevent virus transmission and complications.

We set out to study this hypothesis by assessing the association between mumps symptoms and viral shedding in saliva and urine in both patients who had received 2 MMR doses and unvaccinated mumps patients. Since salivary droplets are an important mode of transmission, elevated salivary viral loads are probably

associated with an increased risk for mumps virus transmission (16). On the other hand, mumps virus shedding in urine indicates systemic infection and may therefore be associated with more severe symptoms. Here, we have studied the association between mumps symptoms such as parotitis, fever and orchitis and viral shedding in saliva and urine to better understand the pathogenesis of mumps in both MMR vaccinated and unvaccinated patients.

## METHODS

### Clinical samples and patient data

Since December 2008, mumps is a notifiable disease in the Netherlands. Mumps surveillance is carried out by the National Institute for Public Health and the Environment (RIVM) in collaboration with municipal health services and peripheral laboratories. The majority of samples from suspected mumps cases sent to the RIVM laboratory for mumps virus testing between 1 January 2007 and 31 December 2014 were saliva samples, urine specimens and throat swabs, which were tested for the presence of mumps virus RNA by quantitative PCR (qPCR). All laboratory-confirmed cases were reported to the national registration system for notifiable diseases in the Netherlands (Osiris). Data from the laboratory database were linked to the notification database by matching patients by date of onset of disease, postal code, gender and year of birth. In this way, a database was created that included patient characteristics, test results and epidemiological data. Information on MMR vaccination status and clinical symptoms was primarily retrieved from Osiris. If this information was missing in Osiris, the information from the laboratory database was used. Only samples from unvaccinated and from twice MMR vaccinated persons were included in this study, since 2 MMR vaccine doses are offered by the national immunization program in the Netherlands. In accordance with Dutch law, no informed consent was required for this study.

### RNA extraction and cDNA synthesis

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. 20 µl of RNA was transcribed into cDNA with 200 U MuLV reverse transcriptase (Invitrogen), 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 of random hexameres (Invitrogen). 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.

## Quantitative PCR

Until March 2010, the SH gene was used as target for the qPCR. For all samples tested from then onwards, the mumps F gene was used as target. For analysis of viral loads, only quantitative results of the qPCR using the F gene as target were used. The qPCR was performed with either the LightCycler 2.0 (until April 2011) or the LightCycler 480 (Roche Diagnostics). In each run, mumps virus Jeryl Lynn was included as a positive control with a viral load of 3540 plaque forming units per milliliter (pfu/ml) for quantification of the PCR results.

## Statistical analysis

SPSS version 22 and GraphPad Prism version 6.04 were used for data analyses. Median age in the unvaccinated and twice MMR vaccinated groups was compared using the Mann–Whitney test. Viral loads were log(10) transformed and analysed using the student's *t*-test. Trends over time were performed using linear regression. We assessed differences in clinical symptoms and virus shedding between vaccinated and unvaccinated patients by using logistic regression. All *p* values below 0.05 were considered statistically significant.

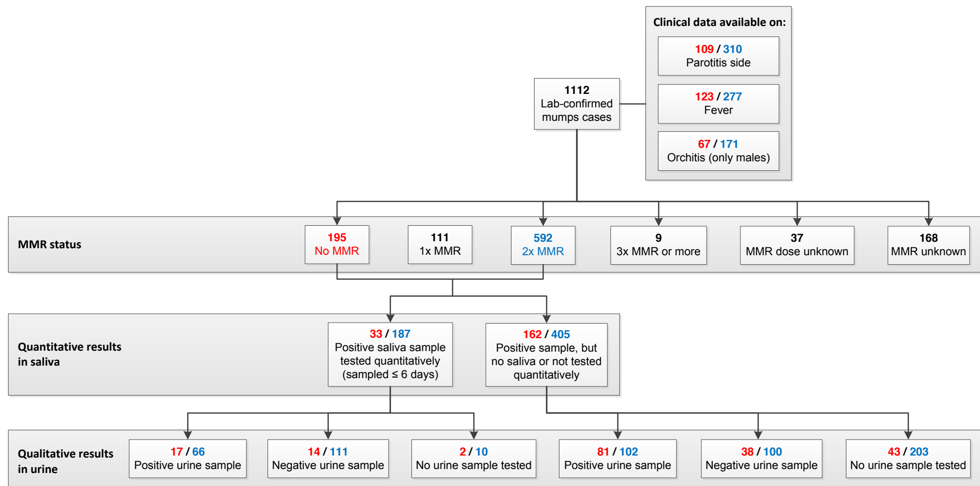



**Figure 1.** Flowchart of saliva samples and urine samples used for analyses from unvaccinated (red) and twice MMR vaccinated (blue) mumps patients. All samples were obtained between 1 January 2007 and 31 December 2014. Clinical information about mumps-specific symptoms and complications was available for a subset of the mumps patients, as indicated in the right upper box. In total, 33 saliva samples from unvaccinated patients and 187 saliva samples from twice MMR vaccinated patients were included for quantitative analysis. Urine samples from 150 unvaccinated patients and 379 twice MMR vaccinated patients were included for qualitative analysis. Of the urine samples, 31 samples were from unvaccinated patients and 177 samples were from twice MMR vaccinated patients of whom also a saliva samples was tested quantitatively.

# RESULTS

## Mumps diagnostics

From 1 January 2007 until 31 December 2014, 1112 suspected mumps cases were tested positive by PCR at the RIVM (Figure 1). The median age in this group was 22 years (IQR 20–24 years), and of the 1099 patients of whom sex was known, 637 were males (58.0%). Of the 907 patients of whom vaccination status was known, 195 patients (21.5%) were unvaccinated, 111 patients (12.2%) had received 1 MMR dose, 592 patients (65.3%) had received 2 MMR doses, and 9 patients (1.0%) had received 3 or more MMR doses. The median age and the sex distribution of the patients did not differ between the unvaccinated patients and the patients who had received 2 MMR doses (p=0.799 and p=0.754, respectively). For quantitative analyses, only samples up to day 6 after onset of disease were included due to the low number of samples available after this period.

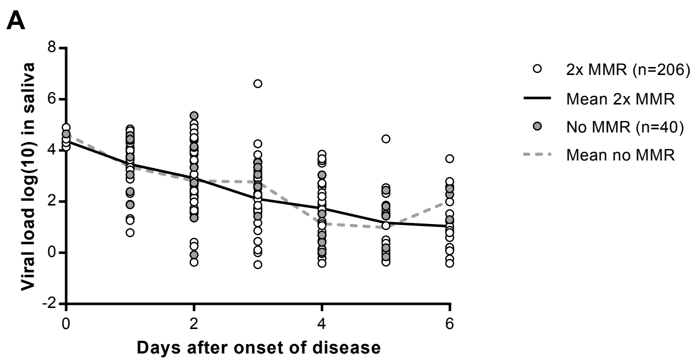
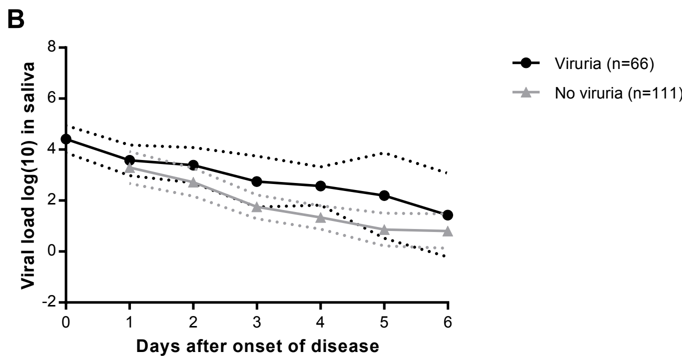
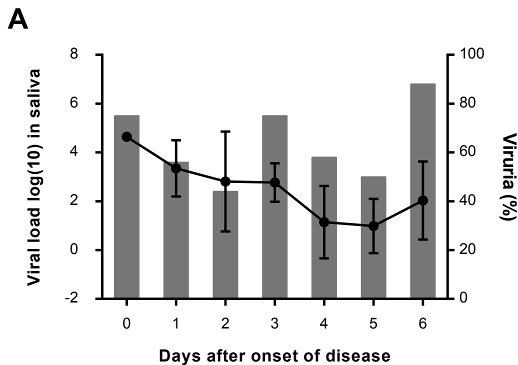



**Figure 2.** Mean viral loads in saliva by day after onset of disease. (A) Viral loads in saliva in twice MMR vaccinated patients and unvaccinated patients. Each circle represents one patient; (B) mean viral loads in saliva from twice MMR vaccinated patients with and without viruria. Dotted lines represent 95% confidence interval.



## Viral load in saliva and urine specimens

Viral load in saliva was highest at day of onset of disease (3.96 ± 0.19 pfu/ml for patients who had received 2 MMR doses and 3.86 ± 0.46 pfu/ml for unvaccinated patients; p=0.821) and declined within the first 6 days after onset of disease with a viral decay of 0.544 ± 0.056 pfu/ml per day for patients who had received 2 MMR doses and 0.50 ± 0.13 pfu/ml per day for unvaccinated patients (p=0.731) (Figure 2A). Salivary viral load at day of onset of disease was higher for twice MMR vaccinated mumps patients with viruria than for twice MMR vaccinated patients without viruria (4.13 ± 0.28 pfu/ml versus 3.68 ± 0.27 pfu/ml) (p<0.001), but the decline after onset of disease did not significantly differ between patients with viruria and patients without viruria (0.42 ± 0.09 pfu/ml per day versus 0.55 ± 0.08 pfu/ml per day; p=0.262) (Figure 2B). The percentage of mumps patients with viruria during the first 6 days after onset of disease was higher among unvaccinated patients than among patients who had received 2 MMR doses (62.1% versus 38.9%; p=0.002) (Figure 3). Too few samples were available from unvaccinated mumps patients to determine the correlation between salivary viral loads and viruria.



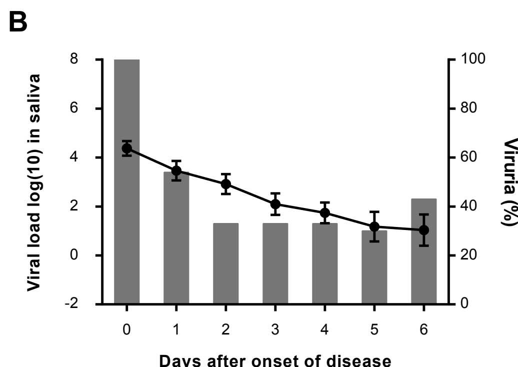




**Figure 3.** Mean viral loads in saliva and frequency of viruria. Mean salivary viral loads are indicated by black circles with error bars showing the 95% confidence interval of the mean. Frequency of viruria is shown by gray bars. (A) Salivary viral loads and frequency of viruria in unvaccinated patients; (B) salivary viral loads and frequency of viruria in twice MMR vaccinated patients.

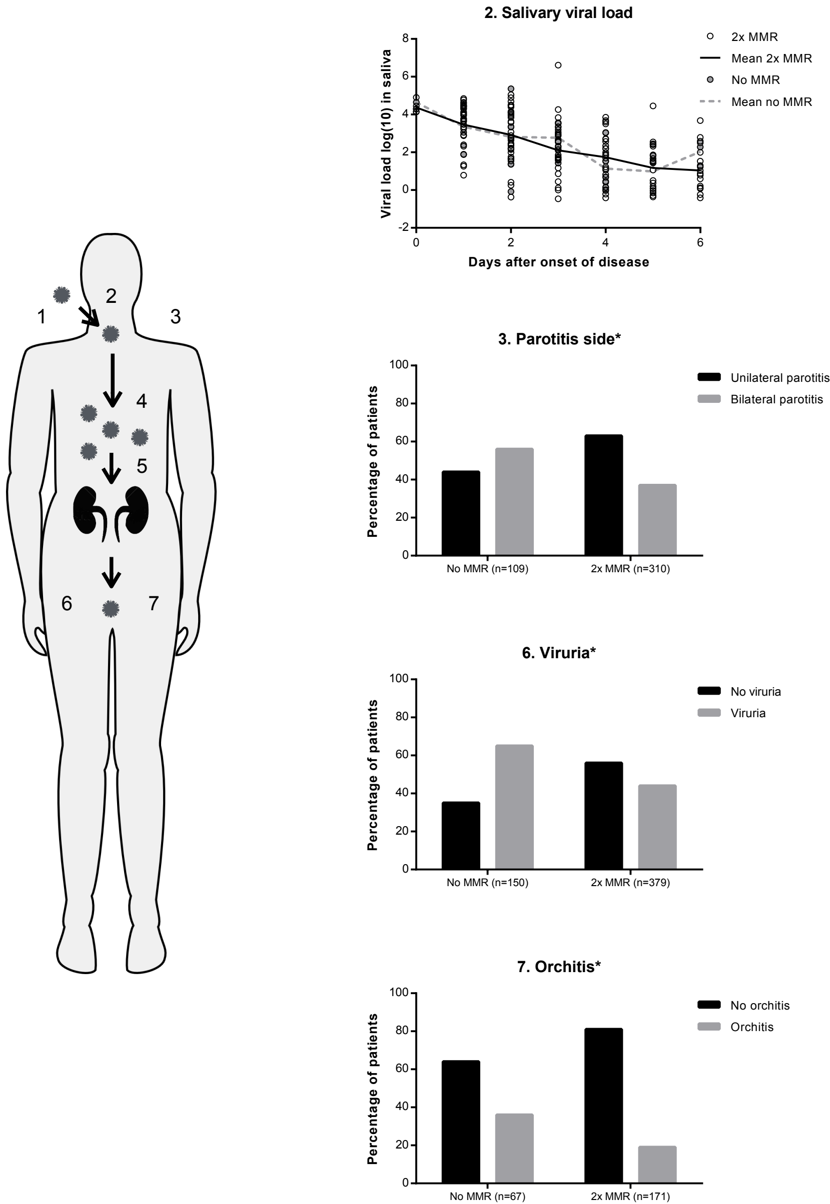



**Figure 4.** Model for mumps virus pathogenesis in MMR vaccinated and unvaccinated mumps patients. Salivary viral loads (2) did not differ between twice MMR vaccinated and unvaccinated patients, whereas bilateral parotitis (3), viruria (6) and orchitis (7) occurred less often in twice MMR vaccinated mumps patients. These findings indicate that the risk for a systemic mumps virus infection (4), which can result in infection of organs (5), viruria (6) and orchitis (7), is lower in MMR vaccinated patients than in unvaccinated patients. 1: viral entry; 2: replication in the upper respiratory tract; 3: parotitis; 4: systemic infection; 5: infection of the organs; 6: viruria; 7: orchitis. * $p \leq 0.005$.

### Clinical symptoms

Bilateral parotitis was more often reported among unvaccinated patients than among patients who had received 2 MMR doses (56.0% versus 37.1%; $p<0.001$) (Figure 4). With respect to patients who had received 2 MMR doses, bilateral parotitis was more frequent in those with viruria than in those without viruria (54.1% versus 27.3%; $p<0.001$). Among unvaccinated patients, there was no difference in the frequency of bilateral parotitis between those with viruria and those without viruria (62.1% versus 52.8%; $p=0.374$). There was no association between the viral load in saliva per day after onset of disease and the occurrence of bilateral parotitis in patients who had received 2 MMR doses ($p=0.308$).

Fever was reported in 68.3% of the unvaccinated patients and in 64.6% of the patients who had received 2 MMR doses ($p=0.475$). There was no association between fever and viruria, irrespective of vaccination status ($p\geq0.243$). Furthermore, no correlation was found between salivary viral load per day after onset of disease and fever in patients who had received 2 MMR doses ($p=0.590$).

Among males, the prevalence of orchitis was lower in patients who had received 2 MMR doses than in unvaccinated patients (18.7% versus 35.8%; $p=0.005$) (Figure 4). A positive association was found between the occurrence of orchitis and mumps virus shedding in urine. Orchitis was reported in 22.9% of the twice MMR vaccinated male patients who shed virus in urine as compared with 2.7% of the twice MMR vaccinated male patients without mumps virus in their urine ($p<0.001$). Unvaccinated male patients with viruria had also more often orchitis than unvaccinated male patients without viruria (46.7% versus 6.7%; $p=0.005$). Too few saliva samples were available from mumps patients with orchitis to determine the correlation between salivary viral load and orchitis.

## DISCUSSION

Our results indicate that mumps virus detection in urine is a marker for severity of disease, as shown here for the occurrence of orchitis and bilateral parotitis. The observation that mumps patients who had received 2 MMR doses shed less often mumps virus in their urine and have less often bilateral parotitis or orchitis than unvaccinated patients supports previous notions that although the vaccine does not fully protect against mumps virus infection, MMR vaccination has a protective effect on severity of mumps disease (7,8) (Figure 4). Furthermore, we found a positive correlation between viruria and salivary viral loads at day of onset of disease in mumps patients who had received 2 MMR doses, showing that mumps patients with a systemic mumps virus infection have an increased risk for transmitting virus via salivary droplets.

The percentage of orchitis cases captured in our patient selection was higher than the percentages reported in other studies (5–7,21,22). The most likely reason for this difference is a bias in the reporting of orchitis cases in our study. Firstly, it is probable that information from many males without orchitis was not filled out on the patients' forms, whereas probably most of the orchitis cases among male patients were explicitly reported on the forms. Secondly, it may be that samples from orchitis patients were more often sent in for laboratory testing whereas mumps patients without orchitis were more often reported based on solely an epidemiological link to a confirmed mumps case or were not reported at all. However, this bias does not influence the effect of vaccination status and viruria on orchitis, since these factors are not expected to influence reporting and sampling of orchitis cases.

As the knowledge on mumps virus pathogenesis and the immunological factors involved in clearance of the virus is limited, the mechanism behind the positive correlation found in this study between salivary viral loads and viruria is unknown. Our findings that mumps patients who had received 2 MMR doses have less often viruria, bilateral parotitis and orchitis indicate that immunological factors play an important role in mumps virus pathogenesis and that vaccine-induced immunity limits mumps virus spread. Whether the spread of mumps virus through the body is limited by local immune responses in the upper respiratory tract or by other immunological factors needs to be determined. Our study contributes to the understanding of mumps virus pathogenesis; a correlation between mumps virus shedding and severity of disease is shown here in both vaccinated and unvaccinated mumps patients. Furthermore, these results underline the importance of urine samples in mumps diagnostics, because they can serve as positive prognostic markers for severity of disease.

## ACKNOWLEDGEMENTS

We acknowledge the municipal health services, laboratories and clinicians for reporting mumps cases and sending clinical materials of mumps patients to the national laboratory for genotyping. We thank Jan van de Kassteele for assistance with statistical analyses.

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# IV

# DISCUSSION

# CHAPTER 9

## General discussion

Partially based on:
Mumps virus pathogenesis: Insights and knowledge gaps
*Hum Vaccin Immunother* 2016 [Epub ahead of print]

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## SUMMARY

The recent mumps outbreaks among MMR vaccinated persons in various countries worldwide have raised questions about MMR vaccine failure, vaccine-induced protection and mumps virus pathogenesis. This thesis contributes to the insights into possible causes for the recent mumps outbreaks among MMR vaccinated persons and provides an overview of my research on sequencing as a tool to support outbreak control measures. The results presented in this thesis could therefore be used for public health decision making about future mumps outbreaks.

In **Part II** we focused on the recent mumps outbreaks in the Netherlands. In **Chapter 2**, we analysed the epidemiology of a nationwide mumps epidemic. Our data showed a slight shift in age and geographic distribution throughout the outbreak seasons, which may reflect increased immunity among students as a result of exposure to circulating mumps virus. Furthermore, we found that a history of MMR vaccination significantly reduced the risk for complications. Besides the SH gene, which is used for mumps virus genotyping, the F gene and HN gene were sequenced (**Chapter 3**). Phylogenetic analysis showed that the genetic diversity of the mumps virus strains during this period was a result of separate introductions of mumps virus rather than ongoing transmission and evolution. Moreover, combining F, SH and HN gene sequences enabled us to study mumps virus transmission during local mumps outbreaks, even in the absence of an epidemiological link (**Chapter 4**). In **Chapter 5**, the mumps virus surface proteins were analysed to study differences between mumps virus genotype G strains and other mumps virus genotypes, including the vaccine strain that belongs to genotype A. We have identified multiple variable sites in the mumps virus surface proteins of the Jeryl Lynn vaccine strain and genotype G strains which could affect mumps virus immunity and pathogenesis, and therefore may contribute to the occurrence of mumps virus genotype G outbreaks among MMR vaccinated persons.

In **Part III** we concentrated on MMR vaccine-induced immunity and the protective effect of MMR vaccination on severity of disease. In **Chapter 6**, mumps-specific IgG concentrations were measured in paired pre- and post-outbreak samples from MMR vaccinated students to study attack rates and risk factors for mumps virus infection. According to our serological criteria, 5.8% of the participants had a mumps virus infection. Two-thirds of the mumps virus infections in this group were asymptomatic. Circulation of mumps among housemates was a significant risk factor for mumps virus infection, which confirms the theory that close contact environments increase the risk for mumps (1). Pre-outbreak mumps-specific IgG concentrations were lower for infected persons than for non-infected persons, although no cutoff point could separate all mumps virus infections from

non-infections. We studied vaccine-induced humoral immune responses further using the neutralization assay (**Chapter 7**). The results indicate that strain-specific neutralization differs between pre-outbreak samples from infected and non-infected persons. Furthermore, our data suggest that genotype G-specific neutralization assays are preferred as tool for surveillance of protection against mumps virus infection. Besides the effect of MMR vaccination on humoral immune responses, we investigated the protective effect of MMR vaccination on severity of disease in mumps patients (**Chapter 8**). MMR vaccination was associated with less severe disease among mumps patients and with less mumps virus shedding in urine, which is an indicator for systemic spread. Furthermore, patients with systemic disease showed elevated salivary viral loads, which suggests that these patients pose a higher risk for mumps virus transmission. This study does not only show the protective effect of MMR vaccination on severity of disease, but also contributes to the understanding of mumps virus pathogenesis.

## MUMPS SURVEILLANCE

Mumps was a notifiable disease in the Netherlands until 1999 and was made notifiable again in December 2008 (2). However, mumps cases during the recent outbreak seasons were probably underreported. Since mumps symptoms are often mild and treatment options are limited, it is likely that not all mumps patients are diagnosed and reported (3). Furthermore, many mumps virus infections, especially among MMR vaccinated persons, are asymptomatic or cause non-specific symptoms (**Chapter 6**). Underreporting of the number of mumps cases and the lack of data about asymptomatic mumps virus infections interfere with an adequate mumps virus surveillance based on epidemiological data, because transmission events may be missed. Molecular surveillance could serve to overcome these difficulties, as this method enables the identification of outbreak clusters in the absence of an epidemiological link.

Routine mumps surveillance provides knowledge about the geographic distribution of mumps virus genotypes (4,5). Mumps virus genotyping – typically based on the SH gene – may therefore be a useful method to distinguish imported mumps cases from endemically circulating mumps virus strains, but the molecular resolution is not sufficient to study mumps virus transmission during an outbreak season. Sequencing of the F gene and HN gene in addition to the SH gene increases the molecular resolution and can therefore be used as a tool to study mumps virus transmission during outbreaks (**Chapter 4**). The F gene and the HN gene encode surface proteins that play a role in mumps virus pathogenesis and may be less conserved than the SH gene (6–10). By increasing the targets for genotyping, the size and spread of mumps outbreak clusters can be estimated

more precisely, which supports public health decisions about implementation and evaluation of outbreak measures. However, it is recommended that complete mumps virus genomes are sequenced from a selection of mumps viruses to confirm that the F gene and HN gene are the most appropriate genes to study mumps virus transmission within mumps virus genotype G outbreaks. In addition, the molecular information available from mumps virus strains circulating in other countries is still limited, and therefore we cannot always distinguish endemically circulating mumps virus strains from strains that were imported multiple times. For example, the number of reported mumps cases since 2009 strongly fluctuated and during some months the number of reported cases was too low for endemic circulation. Based on these data we expect that some strains, despite identical F, SH and HN gene sequences, were imported multiple times.

## MUMPS VIRUS PATHOGENESIS

The understanding of mumps virus pathogenesis and the role of immunity is limited. Analysis of saliva and urine specimens from twice MMR vaccinated mumps patients in relation to clinical data shows that mumps virus shedding in urine is positively associated with high salivary viral loads at day of onset of disease and with the occurrence of bilateral parotitis and orchitis (**Chapter 8**). Because viruria is a consequence of infection of the kidneys, mumps virus shedding in urine indicates systemic mumps virus infection (11). However, it remains unclear how the virus reaches organs such as the kidneys, because the virus has only been sporadically detected in blood during infection (12–14). Results presented in this thesis show that immunological factors play an important role in the development of clinical mumps and severity of disease. MMR vaccination provides protection against clinical mumps, because 66% of the mumps virus infections among MMR vaccinated persons were asymptomatic (**Chapter 6**), compared with 30-40% of the mumps virus infections among unvaccinated persons in previous studies (15,16). Furthermore, MMR vaccination has a protective effect on the development of bilateral parotitis and orchitis and on shedding of mumps virus in urine (**Chapter 8**). Once the virus has entered the body via the upper respiratory tract, vaccine-induced adaptive immune responses seem to prevent mumps virus spread, although it is not clear which immune responses are essential for protection against mumps virus infection. T cell immunity plays a major role in control and clearance of respiratory syncytial virus (RSV) and measles virus infections and may therefore also be involved in the resolution of mumps virus infection (17,18). Furthermore, the level of mumps virus exposure probably determines whether the vaccine-induced immunological responses are sufficient to prevent mumps virus infection, as there is no clear cutoff for pre-outbreak serum antibody titers between

infected and non-infected persons (**Chapters 6 and 7**) and various mumps outbreaks among MMR vaccinated persons occurred in high exposure settings.

## VACCINE-INDUCED PROTECTION AGAINST MUMPS

As primary vaccine failure is unlikely, the recent mumps outbreaks are probably caused by a combination of waning immune responses and a mismatch in epitope regions between the vaccine strain and the circulating wild type strains. The recent mumps outbreaks among MMR vaccinated persons were caused by mumps virus genotype G strains, whereas the Jeryl Lynn vaccine strain belongs to genotype A. Comparison of the mumps virus surface proteins showed that the Jeryl Lynn vaccine strain and mumps virus genotype G strains have unique variable sites that are located in regions of the F and HN proteins with important biological functions (**Chapter 5**). Especially amino acid positions 113, 354, 356, 403 and 442 in the HN protein are of interest, because these positions are located in predicted B cell epitope regions. These positions differ between the Jeryl Lynn vaccine strain and genotype G strains and may therefore cause reduced neutralization of circulating wild type mumps virus strains by vaccine-induced antibodies (**Chapter 7**). *In vitro* studies are required to confirm the importance of these predicted epitopes in vaccine-induced protection against mumps virus wild type strains.

It has previously been shown that mumps-specific humoral immune responses wane after MMR vaccination (19). This may contribute to the occurrence of the recent mumps outbreaks, as vaccine-induced humoral immune responses are lower in pre-outbreak serum samples from infected persons than in samples from non-infected persons (**Chapters 6 and 7**). Other immunological mechanisms, such as mucosal and cellular immune responses, could also be involved in vaccine-induced protection. No studies have been performed so far on the protective effect of vaccine-induced mucosal immunity on mumps virus infection, although mucosal immunity might play an important role in mumps virus pathogenesis. Parenteral vaccination could induce mucosal immunty via transudation of plasma antibodies (20). However, parenteral vaccination may be less effective in inducing mucosal immune responses in the absence of circulating wild type mumps virus, as was shown for the inactivated polio vaccine (21). Mumps virus infection starts in the upper respiratory tract, including infection of the salivary glands, which results in parotitis. The virus can spread further through the body, but systemic infection does not occur in all patients. Neutralization of the virus in the upper respiratory tract by mucosal antibodies might limit mumps virus spread, as was shown for measles virus: IgA can effectively inhibit virus replication in polarized epithelial cells by intracellular neutralization (22). Furthermore, tissue-resident memory T cells might prevent viral entry (23). Therefore, it is important to study the mucosal

immune responses induced by MMR vaccination further, especially with respect to its potentially protective role during mumps virus infection. Moreover, mumps vaccination induces strong T cell responses, but understanding of the role of T cell immunity in protection against mumps is limited and additional research could provide new insights into vaccine-induced protection.

Another research topic that deserves further study is the potential cross-immunity resulting from infection with other paramyxoviruses. Mumps-specific IgG responses induced by MMR vaccination are enhanced in persons who had a prior infection by RSV or parainfluenza virus (24). Furthermore, T cell responses against RSV and mumps virus seem to cross-react (25). Many children are infected by RSV prior to their first MMR vaccine dose at 14 months of age, and it is hypothesized that T cells induced by RSV could influence the development of the mumps-specific immune responses upon MMR vaccination (25). However, it is unknown whether this potential cross-immunity between paramyxoviruses has a beneficial effect on the long term and which epitope regions are responsible for cross-immunity.

## RISK FOR FUTURE MUMPS OUTBREAKS IN THE NETHERLANDS

In the pre-vaccination era, mumps had an inter-epidemic period of approximately 3 years (26,27). Before introduction of the MMR vaccine, it was predicted that a vaccination coverage of 75% would lengthen the inter-epidemic period up to 25 years after introduction of the MMR vaccine (26). Although the vaccination coverage in the Netherlands is higher, this prediction proved to be true, given the mumps outbreaks among vaccinated persons that started to occur in 2009. After the mumps outbreaks in 2009-2012, the number of reported mumps cases decreased. Some small mumps outbreaks occurred in 2013-2015, but these outbreaks were limited to a particular geographic region in the Netherlands, whereas the mumps outbreaks during 2009-2012 were nationwide (**Chapter 4**). Since 2013 various stand-alone mumps cases were reported without further transmission of the virus, which is probably a result of increased herd protection. Contrary to the predicted inter-epidemic period of 25 years after introduction of the MMR vaccine on population level, the inter-epidemic period in certain subpopulations such as students may be shorter. Students account for up to 26% of the population in some of the university cities (28,29). In the absence of nationwide mumps outbreaks during the last 3 years, the herd protection among students has decreased again. In addition, the decrease in this subpopulation is expected to be larger than the decrease on population level, because most students who had a mumps virus infection during the last mumps outbreak graduated and students who entered university after 2012 were probably not

exposed to mumps during the outbreaks in 2009-2012. The absence of nationwide mumps outbreaks during the last 3 years may therefore be sufficient to increase the risk for mumps outbreaks among specific risk groups in the upcoming years.

To explore the risk for future mumps outbreaks, it is essential to estimate herd protection levels. Since mumps is probably underreported because of asymptomatic infections or reluctance to seek care, the actual number of mumps cases during the mumps outbreaks in the Netherlands is expected to be much higher than the number of mumps cases reported. This complicates estimations about potential increases in herd protection levels as a result of mumps virus circulation. The latest nationwide seroprevalence study (Pienter3 study) could give insights into the effect of the mumps outbreaks on mumps seroprevalence. Data from the Pienter3 study could be compared with data from the Pienter2 study, that was performed before the start of the mumps outbreaks among MMR vaccinated persons, to study changes in seroprevalence as a result of mumps virus circulation (30). In this way, the risk for future mumps outbreaks in the Netherlands could be predicted for specific subpopulations based on seroprevalence data.

The recent mumps outbreaks among MMR vaccinated persons were caused by mumps virus genotype G strains, whereas the genotypes circulating in the pre-vaccination era were more diverse (**Chapter 5**). Genotype G strains have some unique variable sites, including 5 sites that are located in B cell epitope regions in the HN protein (**Chapter 5**). This might explain the occurrence of mumps genotype G outbreaks among MMR vaccinated persons. It is therefore likely that future mumps outbreaks will also be caused by mumps virus strains with epitope regions that differ from the vaccine strain.

## PREVENTION OF MUMPS OUTBREAKS IN THE FUTURE

Because of the risk for future mumps outbreaks among MMR vaccinated persons, it is important to develop preventive measures. One relatively easy way to control mumps outbreaks is to prevent mumps virus transmission via its major routes, but these are not exactly known. Indirect contact may be an important mode of transmission for respiratory viruses, such as rhinovirus and RSV (31,32). RSV, a virus that also belongs to the *Paramyxoviridae* family, survives for more than 5 hours on impervious surfaces (31). There are several indications that mumps virus could also be transmitted via indirect contact, although transmission studies during future mumps outbreaks are required to confirm this theory. As mumps among housemates is a risk factor for mumps virus infection, mumps virus may be transmitted via shared glasses and cutlery (**Chapter 6**). Secondly, glasses could also be an important transmission route of mumps virus in parties and pubs. Furthermore, during some local mumps outbreaks in the Netherlands, mumps virus

transmission within sport teams was reported. Given the salivary shedding, transmission within sport teams may occur via shared drinking bottles. As mumps runs an asymptomatic course in the majority of infections and the virus can be transmitted before onset of symptoms, the whole population at risk in a mumps outbreak setting should be informed about risk factors for mumps virus transmission and ways to prevent further transmission.

The mismatch in epitope regions between the vaccine strain and circulating wild type strains suggests that future mumps outbreaks could be prevented by an improved mumps vaccine (**Chapter 5**). Although a new mumps vaccine with improved protection against the currently circulating wild type mumps virus strains is desirable, the safety of the current mumps vaccines and the relatively low burden of disease make it unlikely that this will be developed in the forthcoming years. Furthermore, new mumps vaccines created via conventional vaccinology will probably not be developed as long as the vaccine-induced immune responses that are important for protection against mumps virus infection are unclear. A vaccine development approach based on vaccinomics in combination with reverse vaccinology can tackle this issue. Vaccinomics is a useful method to discover the human immune responses and the changes in responses that occur upon vaccination (33). With this approach, human clinical trials could be conducted to improve the understanding of MMR vaccine-induced immune responses and to define more appropriate correlates of protection for mumps. Reverse vaccinology allows the identification of new protein antigens using genomic information (34). This approach, that has proven successful for meningococcus B vaccine design, enables the development of a new mumps vaccine that induces a protective immune response against all relevant wild type strains based on mumps virus genome sequences from all wild type mumps virus strains (35). Nevertheless, the development of a new mumps vaccine will be a costly and lengthy project.

Without changing the current mumps vaccine, waning mumps-specific immune responses could be boosted by a third MMR vaccination. Alternatively, the second MMR vaccination could be administered at an older age, but this is not preferred because a prolonged time period between first and second MMR vaccine dose increases the risk for mumps outbreaks among primary school children. A third MMR vaccination could be administered either at a specific age via the NIP or as an outbreak control method. If the MMR vaccine is administered via the NIP, the vaccine must elicit immunological responses that last long enough to provide protection during potential future mumps outbreaks. One year after third MMR vaccine administration, 3.1% of the subjects are seronegative or low-seropositive, as compared with 6.6% of the subjects at baseline (36). Furthermore, the shift in mumps-specific neutralization titers is minimal (36). These small effects of a third MMR vaccination on the long term suggest that routine MMR3 vaccination via the

NIP would not be cost-effective. An alternative would be to use a third MMR vaccine as an outbreak control method, where MMR vaccine is only administered during mumps outbreaks to the population at risk to prevent further spread of the virus. In this strategy, the vaccine has to elicit protective immunological responses during a relatively short period. Offering a third MMR vaccination in a mumps outbreak setting seems to decrease the number of mumps cases substantially (37,38). However, in the published reports, it is difficult to rule out that other factors, such as increased herd protection due to high attack rates, contribute to the reduction in mumps cases. The effect of a third MMR vaccine dose on both humoral and cellular immune responses should therefore be studied in depth. It was shown previously that seronegative persons have detectable mumps-specific IgG concentrations for at least 2-3 months after third MMR vaccine administration (39). These data, in combination with the data from the intervention study, suggest that offering a third MMR vaccination during mumps outbreaks is probably an effective method to control a mumps outbreak.

## CONCLUDING REMARKS

In conclusion, the evidence described in this thesis suggests that recent mumps outbreaks among MMR vaccinated persons are probably a result of both a mismatch in epitope regions and waning immune responses. Since the mumps-specific immune responses induced by a third MMR vaccine dose seem to last for a short period and development of a new mumps virus vaccine in the forthcoming years is unlikely, the preferred mumps outbreak control measure is to offer a third MMR vaccine dose during mumps outbreaks among MMR vaccinated persons. Additionally, informing the public about potential mumps virus transmission routes and preventive measures could reduce mumps virus transmission, although the mumps virus transmission routes are yet not fully understood. Further investigation of mumps virus transmission, pathogenesis and protective immune responses is required to better control as well as prevent future mumps outbreaks. Moreover, molecular surveillance remains important to quickly identify mumps outbreaks and to measure the effect of public health decisions.

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# APPENDIX

Nederlandse samenvatting
About the author
Dankwoord

## NEDERLANDSE SAMENVATTING

Bof is een infectieziekte die wordt gekenmerkt door ontsteking van de speekselklier (parotitis) en meestal mild verloopt. Bij sommige patiënten veroorzaakt het bofvirus echter complicaties, waaronder hersenvliesontsteking (meningitis), hersenontsteking (encefalitis), zaadbalontsteking (orchitis), eierstokontsteking (oöforitis), borstontsteking (mastitis), ontsteking van de alvleesklier (pancreatitis) en doofheid. De invoering van het BMR-vaccin (bof, mazelen, rodehond) in het Rijksvaccinatieprogramma in 1987 heeft de ziektelast van bof sterk teruggedrongen. In het afgelopen decennium zijn er wereldwijd echter verscheidene bofuitbraken onder BMR-gevaccineerde personen geweest, onder meer in Nederland. Tijdens deze bofuitbraken waren de meeste bofpatiënten jong volwassenen. De eerste bofuitbraak in Nederland onder BMR-gevaccineerde personen vond plaats in 2004 op de hotelschool in Den Haag. Na deze uitbraak volgde er een uitbraakcluster in Brabant, dat net als het cluster in Den Haag door genotype G bofvirus werd veroorzaakt. In 2007-2009 vond er een genotype D bofuitbraak plaats onder bevindelijk gereformeerde groeperingen met een lage vaccinatiegraad. De grootste genotype G bofuitbraak in Nederland begon in 2009 en breidde zich snel uit na een groot studentenfeest in februari 2010, resulterend in een landelijke uitbraak die tot in 2012 aanhield. Sinds 2012 zijn er alleen nog enkele lokale bofuitbraken geweest.

Het bofvirus is een RNA virus en behoort tot de *Paramyxovirus* familie. Het virale genoom bestaat uit 7 genen, die coderen voor 7 structurele en 2 niet-structurele eiwitten. Het hemagglutinine-neuraminidase (HN) gen en het fusie (F) gen coderen voor twee oppervlakte-eiwitten die belangrijk zijn in de pathogenese van het virus. Het HN eiwit bindt aan siaalzuur en faciliteert op deze manier de toegang tot de gastheercel. Ook maakt het HN eiwit de verspreiding van bofvirus mogelijk en wordt het fusieproces door dit eiwit bevorderd. Het F eiwit faciliteert de membraanfusie en de productie van viruspartikels. Het HN eiwit en het F eiwit zijn een belangrijk doelwit van de bof-specifieke immuunresponsen. Het ‘small hydrophobic’ (SH) gen wordt gebruikt om de bofvirussen te classificeren in 12 genotypen. De bofvirusstam (‘Jeryl Lynn’) die in het Nederlandse BMR-vaccin zit behoort tot genotype A, terwijl de recente bofuitbraken onder BMR-gevaccineerde personen door genotype G bofvirussen werden veroorzaakt.

Na besmetting met het bofvirus kan het 2 tot 4 weken duren voor de eerste symptomen ontstaan. Het virus kan echter wel al worden overgedragen voordat de eerste symptomen zichtbaar zijn, waardoor het lastig is om transmissie van het virus in kaart te brengen. Het bofvirus dringt de epitheelcellen in de luchtwegen binnen, maar het is onduidelijk hoe het virus zich daarna verder

verspreidt. In bofpatiënten zijn zowel humorale als cellulaire immuunresponsen gemeten, maar de precieze rol van deze responsen in bescherming tegen bof is niet bekend. Mogelijk helpt het V eiwit van het bofvirus om te ontsnappen aan de bof-specifieke immuunresponsen. Een deel van de bofpatiënten scheidt ook bofvirus via de urine uit, een gevolg van verspreiding van het virus naar de nieren. Het is echter niet bekend hoe het virus in de nieren terecht komt. Ook over het ontstaan van parotitits en orchitis is weinig bekend. Er zijn daarom meer studies noodzakelijk om de gebrekkige kennis over bofviruspathogenese en de rol van het immuunsysteem tijdens een bofvirusinfectie te vergroten.

De recente genotype G bofuitbraken onder BMR-gevaccineerde personen roepen de vraag op of het huidige vaccin wel voldoende kruisbescherming biedt tegen genotype G bofvirussen. De bofuitbraken zijn in ieder geval geen gevolg van primair vaccinfalen aangezien bof-specifieke immuunresponsen zijn aangetoond in BMR-gevaccineerde bofpatiënten voorafgaand aan een bofvirusinfectie. Secundair vaccinfalen speelt mogelijk ook een rol in het ontstaan van de recente bofuitbraken. De meeste patiënten tijdens deze bofuitbraken waren 18 tot 25 jaar oud, wat betekent dat deze leeftijdsgroep het eerste cohort is dat het BMR-vaccin toegediend heeft gekregen. Waarschijnlijk zijn deze personen tot het begin van de landelijke bofuitbraken in 2009 niet blootgesteld geweest aan het bofvirus, waardoor de immuunresponsen na de tweede BMR-vaccinatie op 9-jarige leeftijd geleidelijk zijn afgenomen. Ook het sociale gedrag van studenten speelt naar verwachting een rol in transmissie van het virus tijdens de recente uitbraken. Studenten leven over het algemeen dicht op elkaar, waardoor het risico op bofvirustransmissie groter is. Daarnaast zijn bepaalde evenementen, waaronder studentenfeesten, in verband gebracht met bofuitbraken. Verdere studies naar bofvirustransmissie zijn nodig om factoren die een rol spelen in transmissie van het virus tijdens uitbraken te identificeren en om in de toekomst preventieve maatregelen te kunnen nemen.

In dit promotieonderzoek zijn de verschillende factoren die mogelijk hebben bijgedragen aan het ontstaan van de recente bofuitbraken onder BMR-gevaccineerde personen onderzocht, om zo BMR-vaccinatie te kunnen evalueren in het licht van de recente bofuitbraken. Verder is er een moleculaire tool ontwikkeld om uitbraakclusters te identificeren en bofvirustransmissie tijdens uitbraken op deze manier in kaart te brengen. Ten slotte is onderzocht of BMR-vaccinatie bescherming biedt tegen het ontwikkelen van een bofvirusinfectie en of vaccinatie een beschermend effect heeft op het ziektebeloop.

In **Deel II** ligt de focus op de recente bofuitbraken en de circulerende bofvirussen in Nederland. In **Hoofdstuk 2** is de epidemiologie van de landelijke bofuitbraken onderzocht. Gedurende de uitbraakseizoenen veranderden de

leeftijdsverdeling en geografische verdeling. Tijdens het eerste uitbraakseizoen hadden voornamelijk studenten bof, maar in de daaropvolgende seizoenen waren bofpatiënten steeds vaker niet-studenten. Dit komt waarschijnlijk door verhoogde immuniteit na blootstelling aan het virus. Verder blijkt dat BMR-vaccinatie het risico op complicaties verkleint.

De RNA sequenties van bofvirussen uit de recente bofuitbraken in Nederland zijn geanalyseerd om uitbraakclusters en nieuwe introducties van het virus te onderzoeken (**Hoofdstuk 3**). Analyses op basis van het SH gen toonden aan dat twee genotype G varianten domineerden tijdens de uitbraakseizoenen 2009-2012. De eerste variant werd voornamelijk in het eerste seizoen gevonden, terwijl de meeste bofvirussen vanaf het eind van het eerste uitbraakseizoen tot de tweede variant behoorden. Ook bleek orchitis vaker voor te komen onder mannen met deze tweede virusvariant. Omdat er geen epidemiologische factoren zijn gevonden die deze verschillen kunnen verklaren, is het waarschijnlijk dat er virologische verschillen tussen deze twee virusvarianten zijn.

Vanwege de kleine variatie in het SH gen binnen een genotype is dit gen niet geschikt om transmissie van het virus in kaart te brengen. Daarom hebben we naast het SH gen ook het F gen en het HN gen geanalyseerd. De genetische diversiteit tijdens de recente bofuitbraken blijkt niet het gevolg te zijn van evolutie, maar van meerdere introducties van het virus. Het combineren van de moleculaire informatie van het F gen, SH gen en HN gen maakt het mogelijk om clusters te identificeren en bofvirustransmissie in kaart te brengen, ook in gevallen waarbij een epidemiologische link ontbreekt (**Hoofdstuk 4**). Op deze manier kunnen nieuwe bofuitbraken sneller worden ontdekt en kan het effect van eventuele maatregelen op virustransmissie tijdens een uitbraak gemeten worden.

In **Hoofdstuk 5** zijn de sequenties van het F gen en het HN gen gebruikt om de oppervlakte-eiwitten van het bofvirus te onderzoeken om zo een mogelijke verklaring te vinden voor de verschillen tussen genotype G bofvirussen en andere bofvirussen, waaronder de Jeryl Lynn vaccinstam die tot genotype A behoort. We hebben meerdere posities gevonden die verschillen tussen genotype G bofvirussen en de Jeryl Lynn vaccin stam. Met name 5 variabele posities in het HN eiwit zijn interessant, omdat deze posities in regio's liggen die door ihet immuunsysteem worden herkend. Variatie in deze regio's kan daarom wellicht zorgen voor een lagere immuunrespons tegen genotype G bofvirussen.

In **Deel III** is het effect van BMR-vaccinatie op het ontwikkelen van een bofvirusinfectie en op het ziektebeloop geëvalueerd. In **Hoofdstuk 6** zijn bofvirusinfecties retrospectief geïdentificeerd in een groep BMR-gevaccineerde studenten op basis van de immunoglobuline (Ig)G concentraties in gepaarde

bloedmonsters die voor en na de bofuitbraak zijn afgenomen. Door het gebruik van een vragenlijst kon onderscheid worden gemaakt tussen symptomatische en asymptomatische infecties en konden risicofactoren worden onderzocht. Volgens onze serologische criteria had 5,8% van de studenten in deze groep een bofvirusinfectie en was twee derde van deze infecties asymptomatisch. Verder bleek het circuleren van bof onder huisgenoten een risicofactor voor bofvirusinfectie te zijn. Voor de bofuitbraak waren de bof-specifieke IgG concentraties lager in monsters van personen met een bofvirusinfectie tijdens de uitbraak dan in monters van personen zonder bofvirusinfectie.

De immunologische responsen in deze groep BMR-gevaccineerde studenten zijn verder onderzocht met behulp van de neutralisatietest in de bloedmonsters die voor de bofuitbraak zijn afgenomen (**Hoofdstuk 7**). In de monsters is de virusneutralisatie gemeten tegen de Jeryl Lynn vaccinstam en tegen wildtype genotype G en genotype D bofvirussen. De stam-specificiteit van de virusneutralisatie verschilt tussen geïnfecteerde en niet-geïnfecteerde personen. Verder blijkt dat de verschillen tussen geïnfecteerde en niet-geïnfecteerde personen het grootste zijn voor de virusneutralisatie tegen genotype G bofvirus, hetgeen aantoont dat serologische bescherming tegen bofvirusinfectie het beste kan worden gemonitord met behulp van genotype G-specifieke neutralisatietesten in plaats van neutralisatietesten met andere bofvirussen of IgG testen.

Naast het effect van BMR-vaccinatie op de humorale immuunresponsen hebben we het effect van BMR-vaccinatie op het ziektebeloop in bofpatiënten onderzocht (**Hoofdstuk 8**). BMR-gevaccineerde bofpatiënten hadden over het algemeen een milder ziektebeloop en scheidden minder vaak virus uit in urine – een indicator voor een systemische infectie – dan ongevaccineerde bofpatiënten. Daarnaast hadden patiënten met een systemische infectie verhoogde virusuitscheiding in speeksel, wat suggereert dat deze patiënten een hoger risico op transmissie van het virus vormen. Deze studie toont daarmee niet alleen het beschermende effect van BMR-vaccinatie op ziektebeloop aan, maar draagt ook bij aan de huidige kennis over bofviruspathogenese.

Ten slotte zijn in **Deel IV** de belangrijkste bevindingen van dit proefschrift samengevat en zijn de risico's op toekomstige bofuitbraken in Nederland en preventieve maatregelen om toekomstige uitbraken te voorkomen bediscussieerd in relatie tot de huidige inzichten (**Hoofdstuk 9**). De recente bofuitbraken onder BMR-gevaccineerde personen zijn waarschijnlijk een gevolg van een combinatie van secundair vaccinfalen en verschillen in epitoopregio's tussen de vaccinstam en de circulerende wildtype bofvirussen. Daarnaast vergroot het sociale gedrag van studenten waarschijnlijk het risico op bofvirustransmissie. Omdat de groep studenten elk jaar wordt aangevuld met

nieuwe studenten en er de laatste 3 jaren geen grote landelijke bofuitbraken zijn geweest, is de immuniteit in deze subpopulatie naar verwachting sterk afgenomen. Hierdoor is er een risico op nieuwe bofuitbraken de komende jaren.

Gezien de effectiviteit en veiligheid van het huidige vaccin, het relatief milde ziektebeloop, de beperkte kennis over bofviruspathogenese en de hoge kosten voor het ontwikkelen van een nieuw vaccin is de kans klein dat er de komende jaren een nieuw bofvaccin ontwikkeld zal worden. Een alternatief is om een derde BMR-vaccinatie toe te dienen. Ondanks de kortdurende immunologische responsen zou een derde BMR-vaccinatie tijdens bofuitbraken kunnen worden toegediend om tijdelijk de immuniteit te verhogen en op deze manier bofvirustransmissie te stoppen. Onderzoek naar de immuunresponsen na een derde BMR-vaccinatie is daarom belangrijk. Bovendien kan het informeren van burgers over mogelijke transmissieroutes en over preventieve maatregelen helpen om bofvirustransmissie tijdens uitbraken te stoppen. Een mogelijke transmissieroute is indirect contact via bijvoorbeeld glazen of bidons. Het is echter noodzakelijk dat er meer onderzoek naar bofvirustransmissie wordt gedaan, aangezien de transmissieroutes en de rol van asymptomatische infecties in de verspreiding van het virus tijdens de huidige uitbraken onvoldoende duidelijk zijn. Ook zijn verdere studies naar bofviruspathogenese en de immunologische responsen die bescherming bieden tegen bofvirusinfectie nodig om toekomstige bofuitbraken te voorkomen en transmissie te beperken. Daarnaast blijft moleculaire surveillance belangrijk teneinde snel bofuitbraken te kunnen identificeren en het effect van publieke gezondheidsmaatregelen te kunnen meten.

# ABOUT THE AUTHOR

## Curriculum vitae

Sigrid Gouma was born on 30 March 1989 in Amstelveen, the Netherlands. After she finished high school St. Ignatiusgymnasium in Amsterdam in 2007, she started her Bachelor of Science study at University College Maastricht with a strong focus on courses related to the field of medical microbiology, immunology and neuroscience. She graduated in 2010 and continued with a Master of Science study in Infection and Immunity at Utrecht University. During her first internship she developed a quantitative hepatitis B surface antigen (HBsAg) assay to monitor chronic hepatitis B patients at the department of Medical Microbiology at University Medical Center Utrecht, under supervision of Dr. Greet Boland. Her second internship focused on the development of a recombinant BCG vaccine at the Vaccinology department at the National Institute for Public Health and the Environment (RIVM) under supervision of Dr. Peter van der Ley and Dr. Arjen Sloots. After graduation in 2012, she immediately started as a PhD student at the RIVM and the Erasmus Medical Center under supervision of Prof. dr. Marion Koopmans and Dr. Rob van Binnendijk. In her PhD research, she studied the causes of the recent mumps outbreaks among measles, mumps, and rubella (MMR) vaccinated persons in the Netherlands.

## PhD portfolio

| | |
|---|---|
| Name: | Sigrid Gouma |
| Research group: | Department of Viroscience |
| Research school: | Molecular Medicine Postgraduate School |
| PhD period: | 2012-2016 |
| Promotor: | Prof.dr. M.P.G. Koopmans |
| Co-promotor: | Dr. R.S. van Binnendijk |

**EDUCATION**

| | |
|---|---|
| 2012-2016 | PhD position at the National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands & Erasmus MC, Rotterdam, the Netherlands |
| 2010-2012 | Master of Science in Infection and Immunity at Utrecht University, Utrecht, the Netherlands |
| 2007-2010 | Bachelor of Science with Honours at University College Maastricht, Maastricht, the Netherlands |

**COURSES**

| | |
|---|---|
| 2016 | Vaccinology course, Institut Pasteur, Paris, France |
| 2015 | Course in Good Clinical Practice, RIVM, Bilthoven, the Netherlands |
| 2014 | Immunology course, HKU-Pasteur, Hong Kong, China |
| 2014 | Virology course, Erasmus MC, Rotterdam, the Netherlands |
| 2014 | Course in scientific integrity, Erasmus MC, Rotterdam, the Netherlands |
| 2014 | Biomedical English writing course for MSc and PhD students, Erasmus MC, Rotterdam, the Netherlands |
| 2014 | Workshop on Excel 2010, Erasmus MC, Rotterdam, the Netherlands |
| 2013 | Course in programming in R, RIVM, Bilthoven, the Netherlands |
| 2013 | Course in research management for PhD students, Erasmus MC, Rotterdam, the Netherlands |
| 2013 | Workshop on Photoshop and Illustrator CS5, Erasmus MC, Rotterdam, the Netherlands |
| 2012 | Bioinformatics course, RIVM, Bilthoven, the Netherlands |
| 2012 | Introductory course on statistics & survival analysis, Erasmus MC, Rotterdam, the Netherlands |
| 2012 | Course in analysis of serological data, RIVM, Bilthoven, the Netherlands |

## SCIENTIFIC CONFERENCES AND MEETINGS

| | |
|---|---|
| 2015 | 18th Annual Meeting ESCV, Edinburgh, United Kingdom (poster presentation) |
| 2015 | Scientific Spring Meeting NVMM 2015, Papendal, the Netherlands (oral presentation) |
| 2014 | National Immunization Program Research Day 2014, Bilthoven, the Netherlands (oral presentation) |
| 2014 | 17th Annual Meeting ESCV, Prague, Czech Republic (oral presentation) |
| 2014 | 24th European Congress of Clinical Microbiology and Infectious Diseases, Barcelona, Spain (poster presentation) |
| 2014 | Scientific Spring Meeting NVMM 2014, Papendal, the Netherlands (poster presentation) |
| 2014 | 9th Conference Louis Pasteur Emerging Infectious Diseases, Paris, France (poster presentation) |
| 2013 | National Immunization Program Research Day 2013, Bilthoven, the Netherlands (oral presentation) |
| 2013 | 5th European Congress of Virology (ECV), Lyon, France (oral presentation) |
| 2013 | Scientific Spring Meeting NVMM 2013, Papendal, the Netherlands (poster presentation) |

## TEACHING ACTVITIES

| | |
|---|---|
| 2014 | Supervision of HLO student |
| 2014 | Lecturing Erasmus MC summerschool |

## OTHER ACTIVITIES

| | |
|---|---|
| 2014-2016 | Board member of PhD Netwerk RIVM (Proneri) |

## List of publications

**Gouma S**, Cremer J, Parkkali S, Veldhuijzen I, van Binnendijk RS, Koopmans MPG. Mumps virus F gene and HN gene sequencing as a molecular tool to study mumps virus transmission. *Infect Genet Evol* 2016 45:145-150.

**Gouma S**, Koopmans MPG, van Binnendijk RS. Mumps virus pathogenesis: Insights and knowledge gaps. *Hum Vaccin Immunother* 2016 [Epub ahead of print].

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Sane J, **Gouma S**, Koopmans MPG, de Melker HE, Swaan C, van Binnendijk RS, Hahné SJM. Epidemic of mumps among vaccinated persons, The Netherlands, 2009-2012. *Emerg Infect Dis* 2014 20(4):643-8.

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