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Hantavirus-specific IgA in saliva and viral antigen in the parotid gland in patients with haemorrhagic fever with renal syndrome

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Running head: Hantavirus IgA in human saliva

ABSTRACT

The *Hantavirus* genus comprises rodent borne, zoonotic viruses of the *Bunyaviridae* family that cause haemorrhagic fever with renal syndrome (HFRS) in Eurasia and hantavirus cardiopulmonary syndrome (HCPS) in the Americas. Rodent saliva contains infectious hantavirus and evidence suggests that hantavirus is also shed in human saliva, but person-to-person transmission is rare. In saliva, immunoglobulin (Ig) A is the predominant immunoglobulin class. Secretory IgA serves as an important first line of defence on epithelial surfaces and the binding of secretory IgA to pathogens can inhibit adherence of microorganisms to mucosal cells and neutralize viruses. This study investigated the presence and importance of salivary IgA in relation to viral antigen in the saliva by testing Puumala hantavirus (PUUV) specific IgA, RNA, and antigen in saliva in acutely ill patients with HFRS. In saliva samples, PUUV specific IgA was detected in twelve of 33 (36%) patients with HFRS and twenty (61%) were PUUV RNA positive. There was a statistically significant inverse association between the presence of salivary IgA antibodies and PUUV RNA in the saliva. PUUV-specific IgA in saliva was not found in a long term follow-up, while PUUV IgA in serum was detected in three patients, 28-32 months after the initial study. Notably, both PUUV RNA and PUUV nucleocapsid antigen were detected in endothelial cells within the parotid gland of a deceased patient with HFRS.

Keywords: Puumalavirus, HFRS, HCPS, antibody, zoonosis, transmission

INTRODUCTION

Viruses in the *Hantavirus* genus are rodent borne, zoonotic viruses of the *Bunyaviridae* family and belong to the group of viral haemorrhagic fevers. Typically, hantaviruses cause haemorrhagic fever with renal syndrome (HFRS) in Eurasia and hantavirus cardiopulmonary syndrome (HCPS) in the Americas. HFRS symptoms include fever, renal failure, and hemorraghia and HCPS symptoms include fever and severe pulmonary oedema; however, between 10-30% of HFRS patients caused by *Puumala virus* (PUUV) (genus *Hantavirus* and family *Bunyaviridae*) also display symptoms in the lower respiratory tract [Kanerva et al., 1996; Linderholm et al., 1993; Linderholm et al., 1997]. The hantaviruses are negative stranded RNA- viruses consisting of spherical, enveloped particles 90 to 120 nm in diameter that contain two glycoproteins (Gn and Gc) and enclose three unique negative-stranded RNAs (L, M, and S) associated with the RNA dependent RNA polymerase and the nucleocapsid protein.

Typically, hantavirus infection is the result of inhalation of aerosol containing virus shed in rodent excreta (saliva, urine, and faeces). However, in South America several person-to-person transmissions with *Andes virus* (ANDV) have been noted; the highest risk of contracting hantavirus by person-to-person transmission is attributed to very close contact (sexual relations or intimate kissing) with a person infected with a hantavirus [Ferres et al., 2007; Martinez et al., 2005]. In a previous study, saliva from patients with HFRS was found to contain PUUV RNA [Pettersson et al., 2008]. Similarly, rodent saliva contains infectious hantavirus [Douron et al., 1984; Hardestam et al., 2008a; Padula et al., 2004; St Jeor, 2004]. These observations indicate that human saliva aerosol or droplets could be a route of

person-to-person transmission, yet human saliva from HFRS patients containing PUUV RNA has not been shown hitherto to be infectious [Hardestam et al., 2008b]. The reported possibility of person-to-person transmission with the highly pathogenic ANDV (case-fatality rate up to 40%) raise concerns whether hantaviruses could constitute a risk for further epidemic spread among humans. Consequently, presence of virus in saliva during infection and factors that could enhance or decrease transmission are therefore of utmost interest.

Whole human saliva may inhibit the infectivity of several different viruses *in vitro* [Fox et al., 1988; Hartshorn et al., 2006]. In the *Hantavirus* genus, human saliva inhibited propagation in cell culture for PUUV and *Hantaan virus* (HTNV), while ANDV was less sensitive to the saliva antiviral effect [Hardestam et al., 2009]. Several components of human saliva could be responsible for this inhibition and mucin had some inhibitory effect on HTNV infection *in vitro* [Hardestam et al., 2008b]. Furthermore, presence of hantavirus-specific salivary antibodies in an infected individual could be important for protection against the disease and transmission. In saliva, Immunoglobulin (Ig) A is the predominant immunoglobulin class: it exists as polymeric IgA with a secretory component bound to the Ig molecules. This secretory IgA has increased resistance against proteolytic degradation. Secretory IgA is produced by plasma cells that are concentrated along mucous cell membrane surfaces and the daily production of IgA is greater than that of any other Ig class. The output in most secretions amounts to some 5-8g/day in adults [Russel, 2007]. In serum, IgA constitutes only 10-15% of the total amount of Ig and exists primarily as a monomer. The parotid gland IgA-to-IgG ratio is about 500 times increased compared to that in serum and 83-87% of salivary IgA in whole saliva is polymeric IgA [Brandtzaeg, 2007]. The secretory IgA serves as an

important first line of defence on epithelial surfaces and the binding of secretory IgA to pathogens can inhibit adherence of microorganisms to mucosal cells and neutralize viruses. For instance, specific parotid and salivary IgA can neutralize *Human immunodeficiency virus type 1* (HIV-1) (family *Retroviridae*, subfamily *Orthoretrovirinae*, genus *Lentivirus*) [Devito et al., 2002; Moja et al., 2000].

Only one study has described salivary IgA antibodies against hantavirus where ANDV-specific IgA was detected in saliva (six patients with acute HCPS) [Padula et al., 2000]. In serum, PUUV-specific IgA was shown to have a neutralizing effect against PUUV [de Carvalho Nicacio et al., 2000]. The present study investigated the presence and importance of salivary IgA in relation to virus in saliva by analysing PUUV-specific IgA and viral RNA in saliva in acutely ill patients with HFRS. In addition, viral RNA and antigen were examined in a salivary gland of a fatal case with HFRS.

MATERIALS AND METHODS

Patients and sample collection

In part of a prospective study of patients with HFRS, saliva, plasma, and serum samples were collected from 33 consecutive patients verified by PUUV specific IgM or real-time RT-PCR at the Division of Infectious Diseases at Umeå University Hospital (Umeå, Sweden). The hospital is situated in the endemic area in northern Sweden. Patients were added to the study from January 2007 to February 2009. The patients were 26-82 years (mean 52 years); 21 were female. The sample collection was performed during the acute phase as previously described [Pettersson et al., 2008] and was random with no consideration to time of day or recent food intake. For some patients, additional samples were obtained later in the acute phase.

To study the long-term duration of PUUV-specific IgA, patients were invited for new sampling in June 2010. Twelve patients agreed to participate in the follow-up and samples were collected in the same manner as previously described. Tissue from the parotid salivary gland was sampled at autopsy three days post-mortem from a patient with HFRS who died five days post onset of disease.

All experiments were performed in compliance with relevant laws and institutional guidelines and in accordance with the ethical standards of the Declaration of Helsinki. The project was approved by the Regional Ethics Review Board in Umeå and informed consent was obtained from all patients.

Real-time RT-PCR

RNA from patient saliva, plasma, or tissue was extracted using a QIAamp® Viral RNA kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's instructions. The real-time RT-PCR was performed as previously described [Evander et al., 2007]. Briefly, the RNA was reverse-transcribed followed by a real-time PCR TaqMan® assay in triplets with PUUV-specific primers and probe from the S-segment. Taqman® RNAse P control reagents (Applied Biosystems, Foster City, CA, USA) were used to determine cell numbers in tissue. The real-time PCR was performed using an ABI Prism 7900HT Sequence Detection System 2.0 (Applied Biosystems).

Immunofluorescence assay (IFA)

To detect PUUV-specific IgM and IgG antibodies in serum from patients with HFRS, IFA was performed as described previously [Evander et al., 2007]. Briefly, the samples were diluted in PBS (1:16 for IgM and 1:40 for IgG) and then applied onto spot-slide wells in a moist

chamber with the local strain PUUV Umeå/hu [Johansson et al., 2004] grown in VeroE6 cells as antigen at 37°C over night for IgM analysis and 60 minutes at 20°C for IgG analysis. For IgA analysis, the same antigen was used to detect PUUV-specific IgA antibodies in serum and saliva. The slides were incubated over night with patient saliva (diluted 1:10) and 90 minutes with serum (diluted 1:10 and 1:40) at 37°C and then PUUV-specific IgA antibodies were detected by a polyclonal rabbit anti-human IgA-FITC (F0204, DAKO A/S, Glostrup, Denmark) diluted 1:40 and incubated 60 minutes at 37°C. The salivary IgA results were confirmed by detection of PUUV-specific salivary IgA antibodies using another detecting antibody – the anti-human IgA-FITC (diluted 1:30) (AF010.M, Binding Site ltd, Birmingham, UK).

Immunohistochemistry

The parotid salivary gland was examined for presence of viral antigen using PUUV nucleocapsid protein specific monoclonal antibody (A1C5, Progen Biotechnik GmbH, Heidelberg, Germany). Staining was performed on formalin-fixed paraffin-embedded 4µm-sections that were processed for immunohistochemistry using a biotin, streptavidin, and peroxidase technique visualized with diaminobenzidine. Parotid samples from two non-hantavirus patients were used as negative controls.

Statistical analysis

For statistical calculations and graphs, the SPSS software (SPSS, Inc., Chicago, USA) was used. Associations between parameters were evaluated using the Mann-Whitney U test. P values<0.05 were considered statistically significant.

RESULTS

PUUV-specific RNA in saliva and plasma from acute phase

Of the 33 patients, 20 (61%) had PUUV RNA in their saliva and 27 (82%) in their plasma (Table 1). The mean value of viral copy number in PUUV RNA positive samples was 33,091 copies/ml in saliva and 113,460 copies/ml in plasma, with a wide range between samples (Table 1). Interestingly, PUUV RNA was detected in saliva (as long as 15 days) and plasma (as long as 20 days) after disease onset (Table 1). As expected, in patients where saliva and plasma were collected consecutively, the RNA-levels decreased with time (Table 1).

In patients where plasma and saliva were collected on the same day (for patients with additional samples only one sample pair was evaluated), twelve of 19 sample pairs were PUUV RNA positive in both saliva and plasma, three were negative in both, four were only positive in plasma, and no sample was only positive in saliva (Table 1 and Table 2). There was a significant association between presence of PUUV RNA in saliva and plasma samples, and the RNA levels in plasma were significantly higher in RNA positive than in RNA negative saliva (P=0.010, Mann-Whitney U test).

PUUV-specific antibodies in serum from acute phase

PUUV-specific IgA, IgM or IgG were found in the sera from all patients at first visit. The only patient with a negative IgA serum (no. 27, Table 1) was IgG and IgM positive in the serum and IgA negative in the saliva. Interestingly, this patient had been sick for six days and had an extremely high viral load in the serum, 1.38 x 10^6 copies/ml. Three patients were only weakly positive for PUUV-specific IgA in serum (no. 20, 28 and 30, Table 1). Notably, the only patients with negative IgM and IgG in the serum were found in this group (no. 28 and 20

respectively) and all three were IgA negative in the saliva. The sample with negative IgG (no. 20, Table 1) was collected only three days after disease onset and had high viral copy number in the plasma. After two additional days, the patient was positive for IgG in serum. The patient with serum negative for PUUV IgM (no. 28) was positive for PUUV RNA both in plasma and saliva (Table 1).

PUUV-specific antibodies in saliva from acute phase

PUUV-specific IgA in the saliva was detected in eleven patients at the initial visit (Table 1) and in one additional patient two days later (no. 12, Table 1). In total, twelve of 33 patients with HFRS had salivary IgA and there was a statistically significant inverse association (*P*=0.009, Mann-Whitney U test) between presence of salivary IgA antibodies and PUUV RNA in the saliva samples (Fig. 1). Of the eleven patients with HFRS with salivary IgA antibodies in their initial sample, seven were negative for PUUV RNA in the saliva (Table 1). In contrast, 16 of 20 patients with HFRS who were positive for PUUV RNA in their initial saliva sample had no detectable PUUV-specific salivary IgA. Only five of 33 patients had both PUUV-specific IgA and PUUV RNA in one of their saliva samples (Table 1). In two of these patients, consecutive samples were collected. For patient 5, PUUV RNA in disappeared from the saliva four days later, while patient 12 had no detectable salivary IgA at the initial visit, but IgA was present in the patient's saliva two and four days later concomitant with a decrease and final disappearance of PUUV RNA in the patient's saliva (Table 1).

Long-term follow-up of PUUV-specific IgA antibodies in saliva and serum

To study the long-term duration of PUUV-specific IgA, patients were invited for a follow-up sampling between 17 months and 32 months after falling ill with HFRS. Twelve patients

agreed to give a follow-up saliva and serum sample. All subjects were negative for IgM and positive for IgG at this time. One patient (no. 13, Table 1) was positive for PUUV IgA in the serum 32 months after the first sample, and two patients (no. 5 and 22, Table 1) were weakly positive for IgA in their serum samples after 29 months and 28 months, respectively. These three patients were all negative for IgM and positive for IgG at follow-up. The remaining patients were negative for IgA in their sera. All patients were negative for IgA in the saliva. No PCR was performed in the follow-up samples.

PUUV IgA and RNA in serum and saliva in relation to airway symptoms, treatment, and radiological findings

In addition to the typical symptoms and clinical signs of HFRS, 22 (67%) patients had respiratory tract symptoms. Perhaps, PUUV RNA and PUUV-specific IgA originated from the lungs through coughing and may be more abundant in individuals where lungs and airways are affected. Seventeen of the 22 HFRS patients with respiratory tract symptoms had dyspnea and 13 were coughing. Eight of the patients were treated with oxygen due to low oxygen-saturation in the blood. A chest x-ray was performed on 12 patients; nine of these were pathological (including infiltrates and/or pleural fluid) and three were normal. However, no statistical significant associations (Mann-Whitney U test) were found between presence of PUUV-specific IgA or PUUV RNA in their saliva or plasma and symptoms, treatment, and radiological findings (data not shown).

Presence of PUUV antigen and RNA in a human salivary gland

To further investigate whether PUUV RNA present in the saliva could originate from the salivary glands, a parotid salivary gland sample from a patient with HFRS was analysed for

presence of PUUV RNA and PUUV antigen using a monoclonal antibody (A1C5) specific for the PUUV nucleocapsid protein. Using immunohistochemistry, the PUUV nucleocapsid antigen was detected in the capillary endothelium and within mononuclear cells (Fig. 2). Using real-time RT-PCR, 19 PUUV RNA copies/10,000 cells were demonstrated in the salivary gland sample and 18,000 PUUV RNA copies/ml in the tracheal secretions. Unfortunately, no saliva had been collected from this patient. Parotid samples from two non-hantavirus patients were both negative for nucleocapsid antigen (data not shown).

DISCUSSION

Can hantavirus in human saliva be infectious, and if not, why? In the studied patients with HFRS, both PUUV RNA and nucleocapsid antigen were found in the saliva and salivary gland, respectively. Presence of hantavirus in saliva could potentially promote transmission unless salivary components inhibit this infectivity route.

Both PUUV RNA and nucleocapsid antigen were detected in a human parotid gland. The viral antigen was demonstrated in the capillary endothelium and within mononuclear cells; however, no staining was found within the secretory epithelium. The findings indicate that virus found in saliva can be the result of shedding from the salivary gland. Presence of hantavirus antigen in the human salivary glands has not previously been published, but hantavirus antigen in rodent and mouse salivary glands has been demonstrated [Botten et al., 2000; Compton et al., 2004; Lee et al., 1981]. Hantavirus is infectious in rodent saliva and experimental studies with sigmodontine rodents hosting ANDV have investigated the hypothesis that saliva is one of the sources of infection within reservoir populations [Padula

et al., 2004]. Person-person transmission of ANDV has been suggested to be mediated by close contact, such as having a sexual relations or engaging in intimate kissing with a person infected with hantavirus [Ferres et al., 2007; Martinez et al., 2005]. One of the studies suggests that the unique route of transmission of ANDV is by means of small-particle infectious saliva or respiratory aerosols during the close contact between both persons [Martinez et al., 2005] .For other hantaviruses, this type of transmission has not been demonstrated and, except for ANDV, whole human saliva has an inhibitory effect on several hantaviruses *in vitro* [Hardestam et al., 2009]. Hantavirus-specific antibodies could also play a role in decreasing the possibility for transmission during an infection.

In the study, the presence of secretory IgA antibodies in the saliva of a patient with HFRS was significantly inversely associated with the presence of PUUV RNA in saliva, suggesting that the virus could be contagious before appearance of salivary IgA. Increased titers of IgG antibodies in the serum of patients with HFRS coincide with the disappearance of PUUV RNA in the plasma [Evander et al., 2007] and similar kinetics of the virus-antibody relationship may occur in the human oral cavity. For other viruses, it has been shown that secretory IgA protects against infection [Belec et al., 2003; Renegar et al., 2004]. Hantavirus-specific IgA in saliva could potentially provide protection against person-to-person transmission. For HIV-1, specific salivary IgA neutralises infection *in vitro* and mucosal IgA seems to be more efficient than serum IgA [Moja et al., 2000], which could be explained by the polymeric structure of the mucosal secretory IgA [Hocini et al., 1997]. In addition, the antibody function of secretory IgA is most likely enhanced by the high level of cross-reacting activity detected in human secretions [Ma et al., 1998]. Only one study has described detection of salivary IgA antibodies in hantavirus disease; the study used ANDV nucleoprotein expressed in

Escherichia coli as antigen in an ELISA [Padula et al., 2000]. In that report, ANDV-specific salivary IgA was detected in six patients with HCPS up to a month after onset of symptoms [Padula et al., 2000]. Similarly, the present study found one patient with HFRS who displayed IgA antibodies in the saliva 32 days after disease onset. Furthermore, PUUV RNA was detected in the saliva and plasma from patients with HFRS more than two weeks after disease onset, a finding that is also similar to previous reports on PUUV RNA in plasma [Evander et al., 2007; Saksida et al., 2008]. A decreased nucleocapsid-specific serum IgA response in convalescent and late-convalescent-phase sera has been observed in serum from patients with HFRS [de Carvalho Nicacio et al., 2000; Elgh et al., 1998; Groen et al., 1994; Meisel et al., 2006; Padula et al., 2004], a finding also similar to the present study; however, serum IgA could be detected in convalescent sera for up to ten years [de Carvalho Nicacio et al., 2000]. The possibility of boosting with hantavirus during the follow-up period cannot be excluded. In the present study, samples from 12 patients with HFRS more than two years after disease were all negative for PUUV-specific IgA in their saliva, while three patients were still IgA positive in sera.

In the acute phase, all patients but one had serum IgA while the proportion of detectable IgA in saliva was lower. PUUV-specific IgA directed both against hantavirus nucleocapsid and glycoproteins was detected since the antigen in the IFA consisted of PUUV-infected cells. The conjugate used to reveal IgA allowed recognition of all potential molecular forms of PUUV-specific IgA antibodies – e.g., monomeric, dimeric, and secretory IgA – but close to 90% of salivary IgA in whole saliva is polymeric IgA [Brandtzaeg, 2007]. In most samples, IgA was not detectable in saliva until the PUUV-RNA was cleared, which is in contrast to serum, where

IgA was detected regardless whether PUUV-RNA was present or not. Complexes in the saliva, including PUUV-specific IgA and PUUV particles, might cause a negative IFA result. In the oral cavity, secretory IgA is known to exist in complex with salivary agglutinin (agglutinin gp-340; [Ligtenberg et al., 2004] and mucin [Biesbrock et al., 1991; Wickstrom et al., 2000] and they inhibit infection by aggregation with virus [Habte et al., 2006; Hartshorn et al., 2006]. Further studies that optimize hantavirus IgA detection in salivary samples could increase the sensitivity of IgA detection. In the present study, PUUV RNA was detected both in saliva and plasma and virus RNA in saliva was associated with viremia in patients with HFRS, indicating that analysis of oral fluid could be useful for molecular investigation during outbreaks. Saliva is an attractive non-invasive sample for diagnostics and epidemiological studies and oral fluid samples have been shown to be useful for many infectious agents such as the early detection of HIV [Nugent et al., 2009]. Determination of specific viral nucleic acids and antibodies in saliva samples makes this test even more useful for isolated communities and it has been suggested as a sampling technique for some native populations in South America who, due to cultural reasons, do not always accept venepuncture [Padula et al., 2000]. The direct detection and identification of many viruses in saliva by PCR could become a standard method and numerous studies on different viruses have been reported [Corstjens and Malamud, 2008]. Detection of hantavirus-specific secretory IgA is non-invasive technique and has a promising future as a diagnostic tool. In addition, hantavirus-specific secretory IgA could provide insight into the dynamics of hantavirus infection in relation to the host immune response. Here, PUUV-specific salivary IgA was present in saliva of infected patients and appearance of salivary IgA was associated with the disappearance of the virus.

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FIGURE LEGENDS

Figure 1. Inverse association between presence of IgA antibodies and PUUV RNA in saliva samples (P = 0.009). Mean saliva PUUV RNA copy number was 30,960 copies/ml in patients without PUUV-specific salivary IgA and 2,046 copies/ml in patients with PUUV-specific salivary IgA.

° Outlier, * Extreme outlier

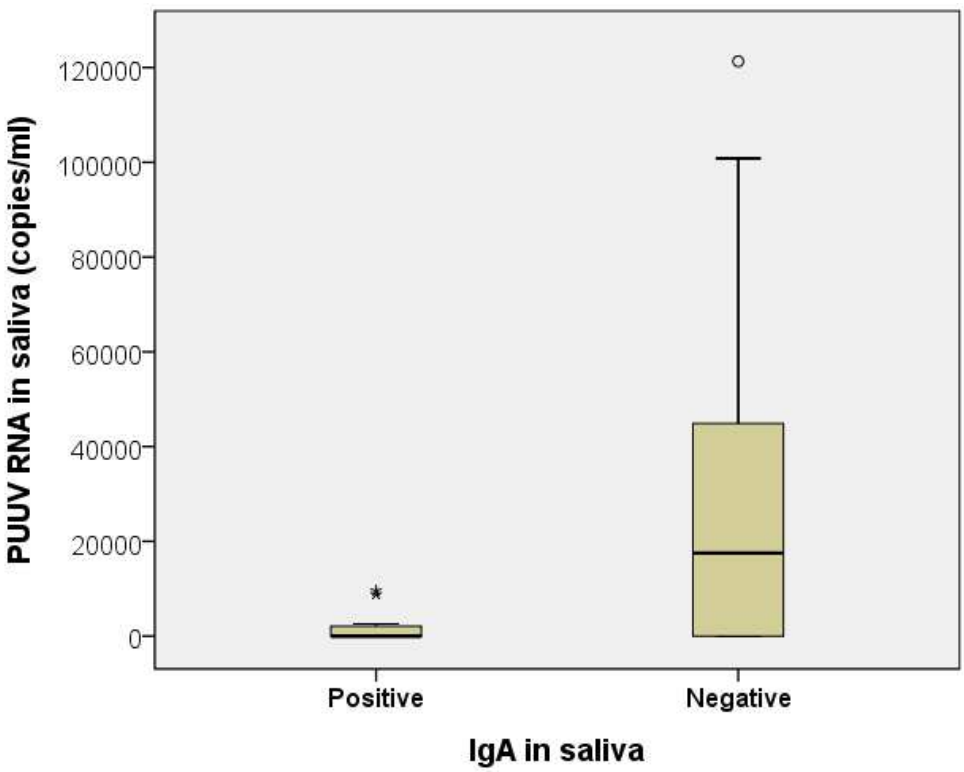
Figure 2. PUUV nucleocapsid antigen in the parotid salivary gland of an HFRS patient. Using PUUV nucleocapsid protein specific monoclonal antibody and immunohistochemistry technique, viral antigen was demonstrated within the capillary endothelium of the organ. Specific punctuate granular staining within the capillary endothelial walls are indicated.

Table 1. PUUV-specific IgA and RNA in HFRS patients.

Patient no.	Day of saliva collection (after disease onset)	PUUV IgA in saliva[a]	PUUV RNA (copies/ml) in saliva	Day of plasma collection (after disease onset)	PUUV RNA (copies/ml) in plasma
1	7	++	0	6	81,330
2	10	++	0	8	0
3	12	++	0	12	5,027
4	9	++	0	8	0
5	6	++	8,810	8	43,013
	10	++	0	11	3,145
6	25	++	0	27	0
	32	++	0	32	0
	52	-	0	52	0
7	10	+	0	10	0
8	7	+	0	7	9,721
	9	-	0	9	0
	11	-	0	11	0
9	9	+	9,582	10	3,724
10	5	+	2,589	5	36,231
11	5	+	1,530	8	3,044
12	7	-	31,284	7	73,291
	9	+	8,142	9	11,809
	11	+	0	11	5,142
13	3	-	pos[b]	3	pos[b]
14	6	-	43,095	6	26,756
15	11	-	57,965	11	64,809
	15	-	14,090	15	0
16	12	-	0	12	0
17	5	-	23,130	7	26,842
18	5	-	100,800	5	48,741
19	9	-	15,195	9	110,454
	11	-	0	11	39,176
	13	-	0	13	17,517
	20	-	0	20	1,801
20	3	-	0	3	149,903
	14	-	0	14	0
21	8	-	83,100	8	20,516
22	9	-	32,594	9	31,411
23	17	-	0	16	0
24	6	-	121,323	5	959,294
25	2	-	66,994	2	117,562
26	5	-	44,898	7	40,427
27	6	-	17,516	5	1,381,413
28	5	-	6,372	5	26,626
29	7	-	3,745	7	189,233
30	5	-	2,163	4	54,315
31	6	-	0	6	1,952
32	6	-	0	7	5,215
33	2	-	0	3	41,271

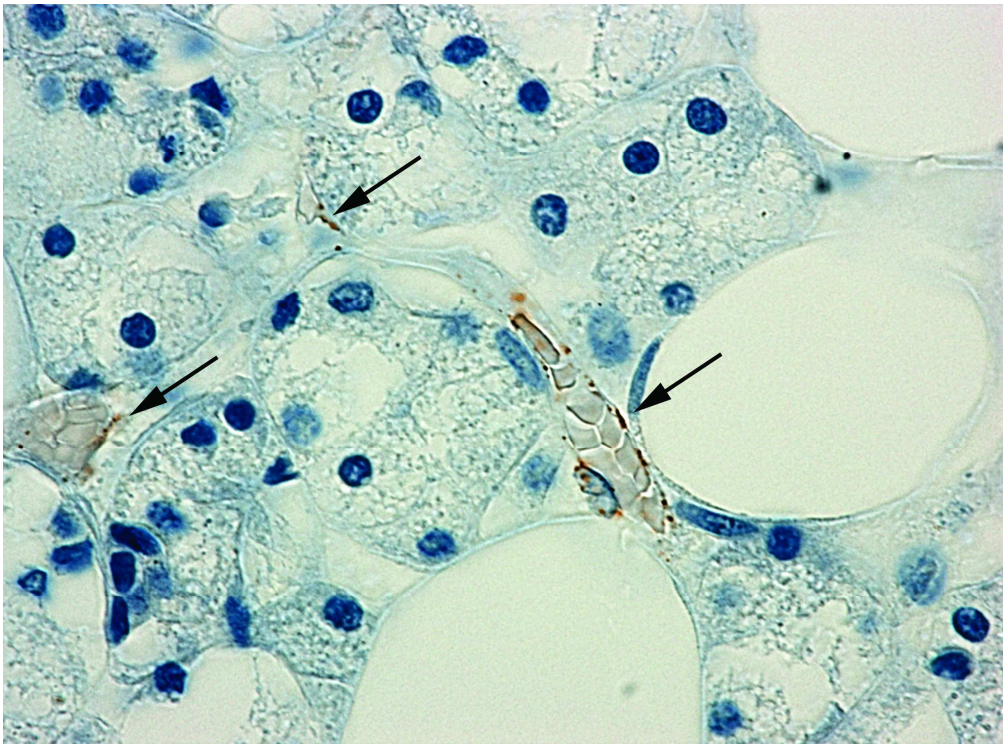
[a] IgA was diluted 1:10 and scored as negative (-), strongly positive (++) or weakly positive (+) by IF.

[b] PUUV-specific PCR on patient 13 was only performed by conventional PCR and scored as positive (pos) or negative.



Inverse association between presence of IgA antibodies and PUUV RNA in saliva samples (P = 0.009). Mean saliva PUUV RNA copy number was 30,960 copies/ml in patients without PUUV-specific salivary IgA and 2,046 copies/ml in patients with PUUV-specific salivary IgA.
° Outlier, * Extreme outlier

166x130mm (96 x 96 DPI)



PUUV nucleocapsid antigen in the parotid salivary gland of an HFRS patient. Using PUUV nucleocapsid protein specific monoclonal antibody and immunohistochemistry technique, viral antigen was demonstrated within the capillary endothelium of the organ. Specific punctuate granular staining within the capillary endothelial walls are indicated.
215x159mm (300 x 300 DPI)