

Quantification of HIV-1 viral load in the fluid of ranulas in HIV- positive patients

Kabunda Syebele^a. Dip.Med., Dip.Dent., Dip. OMFSurg., Dip.Odont(in Oral Surg), Kurt-
W Bütow^b. MCHD(in OMFSurg), DrMedDent, PhD, DSc, FCMFOS, Lynne Webber^c.
MBChB, M Med Path (Virol) DTH, Samuel O. Manda^d. MSc (Stats), PhD (Stats)

a. Medical Officer / Lecturer, Department of Maxillo-Facial and Oral Surgery - University of Pretoria -
South Africa.

b. Professor/Chief Specialist, Head of Department of Maxillo-Ffacial and Oral Surgery - University of
Pretoria - South Africa.

c. Senior Lecturer/Chief Specialist, Head of Department of Medical Virology – University of Pretoria –
South Africa.

d. Senior Specialist Statistician, Biostatistics Unit, South African Medical Research Council, Pretoria,
South Africa.

Corresponding address: *Dr Kabunda Syebele*

Department of Maxillo-Facial & oral surgery

University of Pretoria

P O Box 1266

Pretoria 0001

South Africa

E-mail: kabunda.syebele@up.ac.za

Cell. +27824144130(H), Tel. +27123192551 (W), Fax +27123192173 / 2 (W)

Abstract

Objective. The aim of this study was to detect and quantify the HIV-1 viral load in the ranula fluid of HIV-positive patients.

Materials and methods. Fourteen HIV-positive patients presenting with ranulas were prospectively evaluated. The viral load in the ranula fluid was quantified, and the results were correlated to CD4⁺ cell count and viral load in the patient's blood.

The NucliSens EasyQ system (NucliSens Easymag, Biomerieux), which is based on nucleic acid sequence based amplification (NASBA) and real-time detection applying molecular beacons, was used for the quantitative detection of viral RNA in the fluid.

Results. Various concentrations of HIV-1 viral RNA, ranging from 25 to 1600000 copies/ml were present in the fluid. However, no significant statistical correlation could be established with either CD4⁺ cell count, or the viral load in patients' blood.

Conclusion. Various concentrations of HIV-1 RNA were found in ranula fluid, which appears to serve as a viral reservoir in HIV-infected patients.

Keywords: Ranulas – Viral load - HIV-1

Introduction

Human immunodeficiency virus (HIV-1)-related salivary gland diseases (HIV-SGD) clinical manifestations that are not necessarily exclusive to HIV infection but that are frequently observed in association with HIV infection.¹⁻⁵ The enlargement of parotid salivary glands, especially the cystic type, is one such clinical manifestations. Parotid

enlargement might be considered as a presumptive sign of an underlying HIV infection, and it is good practice to request an HIV test on the basis of parotid gland enlargement.

Oral mucocele and ranula, both salivary gland lesions, are increasingly observed in association with HIV infection and may be included in the HIV-SGD group.⁶⁻⁹ There is currently a lack of evidence to support any direct link between HIV infection with oral mucocele or ranula.

Ranula is defined as an oral mucocele that arises from the floor of the mouth with a typical histopathological presentation of “mucus extravasation phenomenon pseudocyst”. The mucocele can also be described as a simple one when it develops in the floor of the mouth superiorly to the mylohyoid muscle, or as a plunging ranula as it extends inferiorly, through the muscle, into submandibular and/or cervical spaces.

Uccini and co-workers have reported the presence of high concentration of HIV-1 RNA and p24 antigen in the fluid from parotid gland cysts in HIV-positive patients.^{10,11} No publication to date has reported the presence or concentration of HIV-1 RNA in ranula fluid. There is no existing report that compares the level of viral load in the ranula fluid with the viral load and circulating CD4⁺ cell in the blood of the same patient.

The aim of this study is to detect and quantify the level of viral load in the ranula fluid in HIV-positive patients, and to compare this parameter with the viral load and CD4⁺ cell count.

Materials and methods

This study was based on 54 patients with oral mucoceles and/or ranulas referred to a tertiary department of Maxillo-Facial and Oral Surgery. Patients with ranulas were divided into two categories: simple or plunging. HIV-negative patients and those with

history of previous and recent operation or fine needle aspiration (FNA) of the lesion were excluded. The HIV status of patients was determined at the first consultation, if it was not known, and the history of any treatment with highly active antiretroviral (HAART) recorded.

The following laboratory tests were performed: HIV test (HIV-1 antigen p24 / antibody) combination assay (Abbott Diagnostic Division, Wiesbaden, Germany), CD4⁺ cell count profile (Beckman-Coulter FC500, Beckman-Coulter), measurement of viral load in the blood, and viral load in the cystic fluid. The results for viral load in the ranula fluid were expressed as copies/ml and were correlated with the circulating viral load and CD4⁺ cell count in the blood. The FNA was performed to collect fluid samples. An intravenous injection (I.V.I.) catheter (Jelco[®] - Johnson & Johnson) No. 20 or No. 22 was used for the procedure. Special attention was paid in order not to contaminate the fluid with blood during this process of FNA, by selecting the most translucent overlying mucosa on the ranula. The metallic component of the I.V.I. catheter was immediately withdrawn just after penetration of the cystic wall. The atraumatic and plastic component of the catheter was left in place and used for aspiration of the ranula fluid (Fig. 1). The fluid thus collected had to display a clear yellow to amber color, without any macroscopic trace of blood contamination (Fig. 2). Only fourteen fluid samples from HIV-positive patients, satisfied the following selection criteria: no recent operation, no recent FNA, no trace of macroscopic blood contamination. Therefore, only these specimens were finally considered and prospectively enrolled for the purpose of this study. The fluid was then immediately sent to the laboratory of the Department of Medical Virology, in a plain tube.

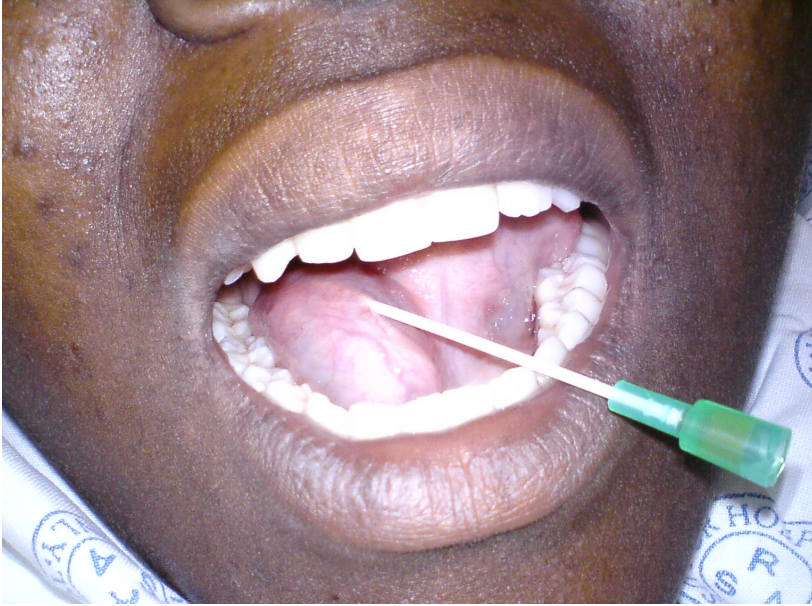


Fig.1. The metallic component of the I.V.I. catheter has been withdrawn, and only the atraumatic plastic component is left in place during FNA procedure in patient 13.



Fig.2. The macroscopic appearance of the ranula fluid sample after FNA. There is no macroscopic blood staining.

Biopsy of the cystic lesion, which is part of the standard protocol, was also performed to ascertain the extravasation phenomenon or retention nature of the cystic lesion. The biopsy was either incisional or excisional.

Quantification of viral load

The Nuclisen EasyQ assay (Biomérieux), which is based on nucleic acid sequence based amplification (NASBA) and real-time detection using molecular beacons for the quantitative detection of RNA in human samples, was used.

The three technologies used in the Nuclisen EasyQ system were the following:

- the Boom method for nucleic acid release and isolation;
- NASBA technology for amplification of RNA, and
- real-time detection of amplifications using fluorescent molecular beacons.

These technologies were performed in two steps:

- nucleic acid release and isolation and
- NASBA amplification and real-time detection.

The Boom method is a solid phase based nucleic acid extraction method involving silica particles as the solid-phase component. This method can be used to isolate nucleic acids from a variety of human fluid specimens other than conventional cerebrospinal and blood samples. The Boom method also has a broad range of specimen input ranges and can thus detect nucleic acid material within normal, high and low volume human samples. The NASBA technology is based on primer extension using the coordinated activities of three enzymes. Real-time detection using molecular beacons is based on the measurement of the time-related increase in fluorescent signal and only occurs after specific binding of the molecular beacons to the amplicon (amplified portion of the nucleic acid).

Measurement and interpretation of the signals was performed using the EasyQ analyzer. The detection of the signal (value) was considered as positive and non detection of the signal was considered as negative. Discordant samples, with viral load in the fluid higher than the viral load in the blood, were analyzed in duplicate. Negative controls were not used, because HIV-negative patients were excluded from the study.

Statistical analysis

As a preliminary analysis, description of the 54 patients were summarized using frequency and percentages for age group and HIV status. Paired t-test or its non-parametric equivalent was used to test for equality of viral load or CD4⁺ cell count in ranula fluid and blood. Pair-wise association between viral load sources and CD4⁺ cell count was estimated using Pearson or Spearman correlation coefficients depending on the shape of the distribution. A p-value less than 0.05 were used to indicate significant results.

Ethical considerations

This study was conducted in accordance with all regulations and guidelines governing research and HIV/AIDS management in South Africa. The protocol for this study and the informed consent documents were reviewed and approved by the Faculty of Health Sciences Research Ethics committee, University of Pretoria.

Results

The distribution of patients with ranulas, according to HIV status and age was determined (Table 1). The majority of patients with ranulas, 36 out of 54 (66.7%), were in the group of HIV-positive patients. Table 2 displays the results for 14 HIV-positive patients,

Table 1: Distribution of patients with ranula according to HIV status and age groups.

Age	0 -10	11- 20	21 - 30	31 - 40	41 - 50	TOTAL
<i>HIV (+)</i>	11	7	8	8	2	36 (66.7%)
<i>HIV (-)</i>	6	11	1	0	0	18 (33.3%)
TOTAL	17	18	9	8	2	54 (100%)

Table 2: Epidemiological and clinical main variables of 14 HIV-positive patients.

No	AGE	SEXE M / F	VIRAL LOAD IN BLOOD (Copies / ml)	VIRAL LOAD IN FLUID (Copies / ml)	CD4 ⁺ Cell IN BLOOD (x10 ⁶ /l)	RANULA: SIMPLE OR PLUNGING	BIOPSY RESULTS
1	6	F	<25	74000	521	Simple	MEP
2	4	F	190	<25	821	Simple	MEP
3	6	M	290	<25	400	Simple	MEP
4	20	F	950	6300	518	Plunging	MEP
5	20	F	1900	26000	121	Simple	MEP
6	27	F	5500	640	302	Plunging	MEP
7	42	F	8300	1000	339	Plunging	MEP
8	39	F	17000	1600000	54	Simple	MEP
9	29	F	20000	110000	207	Plunging	MEP
10	31	F	24000	81	92	Plunging	MEP
11	39	M	46000	230000	121	Plunging	MEP
12	13	M	73000	2900	34	Plunging	MEP
13	23	F	130000	34000	324	Simple	MEP
14	26	F	190000	26000	231	Plunging	MEP

*MEP=Mucus extravasation phenomenon

including the following variables: viral load in the blood, viral load in the ranula fluid and the CD4⁺ cell count in the blood.

Thirteen out of 14 patients were not informed about their HIV status, and were diagnosed HIV positive after consulting for ranula as main complaint. Therefore, these patients were not under any HAART medication. Patient No 9 was the only known HIV positive, and she was on HAART (d4T+3TC+EFV) at the time of first consultation. Patient No 10 was 7 months pregnant. No other significant oral or systemic co-morbidity was reported.

All fluid samples tested positive, and demonstrated different titer values ranging from undetectable (<25 copies/ml) to high concentration values (1600000 copies/ml). Six out of 14 ranula fluid specimens had a higher titer of HIV-1 RNA than the corresponding patient blood. All the lesions were diagnosed as “mucus extravasation phenomenon pseudocyst”.

There was a positive linear correlation between the viral load in the blood and viral load in the fluid (Spearman test, $\rho=0.440$), and between the CD4⁺ cell count and the viral load in the fluid (Spearman test, $\rho=0.421$), but both were not statistically significant. There were no statistical differences between simple and plunging ranulas with regard to the level of viral load in the cystic lesion.

Discussion

Human immunodeficiency virus-related salivary gland disease (HIV-SGD) terminology describes a group of intra- and extra-oral manifestations that are frequently observed in association with the HIV infection. This association does not imply a specific etiological link between HIV and those clinical manifestations. The majority of HIV-SGD, i.e.,

enlargement of the parotid salivary gland, had been described long before the advent of HIV, in immunologically competent patients.^{12,13} Sjögren's syndrome and lymphoepithelial cyst of the parotid gland in HIV-negative patients are associated with parotid enlargement and/or xerostomia. However, the frequent observation of these conditions, in association with HIV infection, has led to the concept of HIV-SGD. The presence of the HIV-1 virus in parotid tissue, and the detection of high levels of viral particles in the fluid from parotid cysts in HIV-positive patients, has contributed to the concept of HIV-SGD.^{10,11} Finally, description of the diffuse infiltrative lymphocytosis syndrome (DILS) in various organs including parotid and minor salivary glands, in HIV-positive patients, was a cornerstone in defining the HIV-SGD.¹⁴⁻¹⁶ DILS is characterized by a persistent increase of circulating and visceral CD8⁺ lymphocytic infiltration. It has been reported to play an important role in the physiopathogenesis of BLEC.^{14,15} The HIV-related parotid cyst, commonly described as "benign lymphoepithelial cyst" of the parotid gland has, therefore, been accepted as the typical example of HIV-SGD.

The inclusion of ranula in the HIV-SGD category remains controversial. The exact prevalence of ranula in HIV-positive patients is not known. However, there are several reports of ranula observed in association with HIV.⁶⁻⁹ Chidzonga⁶ specifically reported a ranula incidence of 88.5% in HIV-positive patients, from 38 cases of ranula. In another small sample, Butt⁸ and co-workers found an incidence of 67.9% in HIV-positive patients among 28 cases of ranula. This study found 66.7% of HIV-positive patients from 54 cases of ranula. This result is in line with other reports concerning the occurrence of ranula in HIV-positive patients.

This study demonstrated the presence and quantified various titers of HIV-1 RNA in the fluid from ranulas. All ranula fluid samples from HIV-positive patients tested positive. The viral load quantified in ranulas fluid in this study ranged from undetectable (<25 copies/ml) to very high concentration (160000000 copies/ml). The relevance of this latter finding remains unclear and is the subject of ongoing research. The HIV-1 virus has, indeed, been identified in several other human fluids aside from the blood, e.g., saliva, breast milk, urine, and semen.^{17,18} In this study special steps were taken to minimize blood contamination during sample collection through FNA. The discordance between the concomitant HIV-1 RNA values in the blood and in the ranula fluid, respectively, remains to be elucidated. Various studies that have compared HIV-1 RNA values in blood and other body fluids have reported different results, depending on the method used.¹⁹ Uccini et al^{10,11} has detected 3 times more (3 to 15 ng/ml) HIV-1 p24 protein in the parotid BLEC fluid, whereas levels were undetectable in the peripheral blood of the same patients. The author postulated that the metaplastic squamous epithelium of benign lymphoepithelial cystic lesions of the parotid gland recruits HIV-1-infected cells from the underlying lymphoid tissue and favors their discharge into the cystic cavity. It was also suggested that the BLEC of parotid gland was a viral reservoir in HIV-1-infected patients.

The presence of HIV-1 virus in the normal saliva could also explain the presence of HIV-1 virus in the extravasated mucus from the ranula. However, one would expect reduced viral load in the ranula fluid than in the concomitant patient's blood. Indeed, the viral load in normal saliva is reported to be minimal compared to the corresponding viremia.^{20,21} In this study it was found that 6 of the 14 specimens had HIV-1 RNA

copies higher than their respective blood samples. This latter finding is of utmost importance because it might imply one of the following: either there is a genuine high concentration of HIV-1 virus in some cases of ranula (viral reservoir), or it is a case of blood contamination during FNA. The contamination theory seems unlikely, given the strict selection criteria implemented (clear fluid only). Furthermore it is difficult to explain the very high concentrations observed in some ranula fluid samples (1600000 copies/ml). Even if the fluid aspirated was hemorrhagic, the viral load values in the fluid should remain comparable to that of viral load in the blood of the same patients. Therefore the theory that a ranula might, like the BLEC, be a HIV-1 viral reservoir is strongly supported.

Based on the observed prevalence of ranula in HIV-positive patients (66.7%), combined with the possibility that ranula may be a viral reservoir, the current authors believe that ranula should, like the BLEC, be considered as a HIV-SGD.

Notwithstanding the limitation due to the small sample in this study, there is, however, no statistically significant correlation between the level of viral load in the blood and that of viral load in the cystic fluid for the 14 tested patients. The clinical presentation of the lesion; simple or plunging ranula, does not seem to statistically affect the viral content. A larger size sample study might provide a more conclusive outcome in relation to study of this rare pathological entity.

CONCLUSION

It was demonstrated that all 14 ranula fluid samples studied, contained HIV-1 RNA at various concentrations, ranging from <25 to 1600000 copies/ml. In 6 of the 14

specimens, the level of HIV-1 RNA copies/ml was higher than in the corresponding blood samples. Therefore the ranula might represent an HIV-1 viral reservoir.

REFERENCES

1. Shanti RM, Aziz SR. HIV-associated salivary gland disease. *Oral Maxillofac Surg Clin N Am* 2009;21:339-43.
2. Schiødt M. HIV-associated salivary gland disease: A review. *Oral Surg Oral Med Oral Pathol* 1992;73:164-7.
3. Leao JC, Ribeiro CMB, Carvalho AAT, Frezzini C, Porter S. Oral complications of HIV disease. *Clinics* 2009;64:459-70.
4. Shiboski CH, Patton LL, Webster-Cyriaque JY, Greenspan D, Traboulsi RS, Ghannoum M, et al. The oral HIV/AIDS research alliance: updated case definitions of oral disease endpoints. *J Oral Pathol Med* 2009;38:481-8.
5. Robert L, Peel and Raja R. Seethala. Pathology of salivary gland disease. In: Myers EN, Ferris LR, editors. *Salivary gland disorders*. Berlin Heidelberg: Springer;2007.p.33-104.
6. Chidzonga MM, Mabomva L. Ranula: Experience with 83 cases in Zimbabwe. *J Oral Maxillofac Surg* 2007;65:79-82.
7. Syebele K. Regression of both oral mucocele and parotid swellings, following antiretroviral therapy. *Int J Pediatr Otorhinolaryngol* 2010;74:89-92.
8. Butt FM, Chindia ML, Kenyanya T, Gathece LW, Rana F. An audit of ranulae occurring with the human immunodeficiency virus infection. *J Oral Maxillofac Pathol*. 2010;14:33-5.

9. Syebele K, Bütow K-W. Oral mucoceles and ranulas may be part of initial manifestations of HIV infection. *AIDS Res Hum Retrov.* 2010;26:1079-85.
10. Uccini S, D'Offizi G, Angelici A, Prozzo A, Riva E, Antonelli G, et al. Cystic lymphoepithelial lesions of parotid gland in HIV-1 infection. *AIDS Patient Care ST.* 2000;14:143-7.
11. Uccini S, Riva E, Antonelli G, D'Offizi G, Prozzo A, Angelici A, et al. The benign cystic lymphoepithelial lesion of the parotid gland is a reservoir in HIV type 1-infected patients. *AIDS Res Hum Retrov.* 1999;15:1339-44.
12. Stewart S, Levy R, Karpel J, Stoopack J. Lymphoepithelial (branchial) cyst of the parotid gland. *J Oral Surg.* 1974;32:100.
13. Fujibayashi T, Itoh H. Lymphoepithelial (so-called branchial) cyst within the parotid gland. Report of a case and review of the literature. *Int J Oral Surg.* 1981;10:183-92.
14. Basu D, Williams FM, Ahn CW, Reveille JD. Changing spectrum of the diffuse infiltrative lymphocytosis syndrome. *Arthrit Rheum-Arthr.* 2006;55:466-72.
15. McArthur CP, Subtil-DeOliveira A, Palmer D, Fiorella RM, Gustafson S, Tira D, et al. Characteristics of salivary diffuse infiltrative lymphocytosis syndrome in West Africa. *Arch Pathol Lab Med.* 2000;124:1773-9.
16. Ellis GL. Lymphoid lesions of salivary glands: malignant and benign. *Med Oral Pathol Oral Cir Bucal.* 2007;12:479-85.
17. Liuzzi G, Chirianni A, Clementi M, Bagnarelli P, Valenza A, Cataldo PT, et al. Analysis of HIV-1 load in blood, semen and saliva: evidence for different viral compartments in a cross-sectional and longitudinal study. *AIDS.* 1997;11:1058-9.

18. Shugars DC, Slade GD, Patton LL, Fiscus SA. Oral and systemic factors associated with increased levels of human immunodeficiency virus type 1 RNA in saliva. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod.* 2000;89:432-40.
19. Campo J, Perea MA, Romero JD, Cano J, Hernando V, Bascones A. Oral transmission of HIV, reality or fiction? An update. *Oral diseases* 2006;12:219-28.
20. Rothenberg RB, Scarlett M, Carlos del Rio, Reznik D, O'Daniels C. Oral transmission of HIV. *AIDS.* 1998;12:2095-2105.
21. Cohen MS, Shugars DC, Fiscus SA. Limits on oral transmission of HIV-1. *Lancet.* 2000;356:272.

Reprint request:

Dr Kabunda Syebele

Maxillo-Facial and Oral Surgery

P O Box 1266

Pretoria 0001 - South Africa

E-mail: kabunda.syebele@up.ac.za