“Does Human Papilloma Virus play a role in the histogenesis of the orthokeratinised jaw cyst?”

KALPESH LALLA

A research report submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in partial fulfilment of the requirements for the degree

of

Master of Science in Dentistry

Johannesburg, 2015

DECLARATION

I, Kalpesh Lalla, declare that this research report is my own work. It is being submitted for the degree of Master of Science in Dentistry at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

.....

.....day of ..... 2015.

ABSTRACT

Objectives: To analyse the clinico-pathological features of orthokeratinised jaw cysts (OJCs) and to determine whether human papillomavirus (HPV) DNA can be detected in OJCs.

Material and methods: The clinical and radiological information of 30 patients diagnosed with OJCs were reviewed and the respective histology samples were studied for light microscopic features characteristic of HPV infection. The 30 OJCs were further evaluated for the presence of HPV by using consensus HPV polymerase chain reaction (PCR).

Results: Patients with OJC ranged from 13 to 71-years (mean, 30.9 years; ± 12.9 years). There was a predilection for males (21/30). Most OJCs were found in the mandible (80%) and 44.8% were associated with an impacted tooth. Koilocyte-like characteristics were identified in 70% of cases, while 43.3% of cases showed a verruciform pattern of hyperkeratosis. All 30 OJCs were negative for HPV-DNA.

Conclusion: HPV infection does not appear to play a role in the OJC and is not responsible for the wart-like histological changes that may be encountered in OJCs.

ACKNOWLEDGEMENTS

I would like to express my gratitude to the Faculty Research Committee Individual Research Grant (Medical Endowment Grant) for financing this project. Grant number: 001 410 8469101 LALK000.

Mrs S Naidoo for her expertise in the preparation of the histological slides and her assistance in the undertaking of the PCR molecular analysis.

To my supervisor, Dr F Mahomed, for all your support, patience, guidance and constant encouragement, without which this research project would not have become a reality.

To my co-supervisor, Professor S Meer, for all your support, advice and assistance with my research project.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

1.0 INTRODUCTION

Cystic lesions occurring within the jaw bones and which are lined by orthokeratinised epithelium are known as orthokeratinised jaw cysts (OJCs).[1] Theories regarding the histogenesis of the OJC remain speculative. It is nevertheless currently endorsed that the OJC is not a histological variant of the odontogenic keratocyst (OKC),[2] as was described in earlier reports.[3-5] The rationale for this presumption includes the following findings; the OJC presents more frequently in a dentigerous relationship and shows a significantly lower recurrence rate than the OKC following conservative enucleation of the lesion.[1,6,7] Furthermore, aggressive features such as multiplicity, satellite cysts, proliferating epithelial islands and association with the nevoid basal cell carcinoma syndrome, although well documented for the OKC, have not been reported for the OJC.[8] Other theories suggest origin of the OJC from heterotopic ectodermal tissues or from post-traumatic implantation.[5] The high frequency of occurrence of these lesions in association with the crowns of impacted third molar teeth forms the basis of the premise that the follicular OJC most likely represents a dentigerous (follicular) cyst, in which the cyst lining has undergone histomorphological transformation (metaplasia) from a fairly non-descript, tenuous layer of flattened cuboidal cells to a robust orthokeratinised lining.[1,8] This orthokeratinised lining bears histological resemblance to the epidermoid cyst of the skin and other extragnathic sites.[9, 10]

Since the early nineties there has been a surge of interest in the association between human papillomavirus (HPV) and epidermoid cysts of the skin, in particular those of palmoplantar

location.[11] Although the origin of these cysts is still controversial, Egawa *et al.*[12] proposed the idea that certain palmoplantar epidermoid cysts might be a reflection of epidermoid metaplasia of eccrine duct epithelium and that HPV may play a role in this development. Cutaneous epidermoid cysts that occur in non-palmoplantar location are thought to arise from epidermal implantation due to trauma or a dilated follicular infundibulum.[13] Interestingly, even in these cases the presence of HPV has been shown by histopathological and molecular techniques.[13] Likewise HPV has also been identified in cholesteatomas, which are histologically defined as epidermoid cysts developing in the middle ear.[10]

Although papilloma viruses replicate exclusively in body surface tissues such as the skin or the mucosal surfaces of the anogenital region, mouth, or airways, it is thought that infection that is acquired at parturition or in utero may infect the invaginating primitive enamel organ.[14] After a period of latency, conversion from latent into productive infection is thought to occur.[14,15] It is considered unlikely that HPV acts alone, but rather that it may be a co-factor or initiator in the histogenesis of certain odontogenic epithelial lesions.[16] Odontogenic cysts and tumours that have been investigated for the presence of HPV DNA include the ameloblastoma,[14,16,17] and OKC.[18] In the study by Sand *et al.*[14] HPV DNA was detected by the polymerase chain reaction in 44.4% of ameloblastomas. Kahn[16] found HPV 16/18 in a peripheral ameloblastoma with the in situ hybridisation technique. Subsequently, HPV-18 was also demonstrated by in situ hybridisation in a verrucous hyperplastic area of a primary intraosseous ameloblastoma.[17] HPV-16 DNA was isolated by Cox *et al.*[18] from an OKC using Southern blot hybridisation.

HPV-induced histomorphological changes, as observed by light microscopy, have often been used as an adjunctive measure when assessing tissue specimens for underlying HPV infection.[19,20] The histological features that characterise flat viral warts (verruca plana), for example, includes hyperkeratosis, often absence of parakeratosis, slight or no papillomatosis, hypergranulosis and vacuolated keratinocytes with perinuclear clearing.[20] Despite the histological similarities between the OJC, cutaneous epidermoid cyst,[9, 19] cholesteatoma[10] and verruca plana,[20, 21] the OJC has not yet been studied for a possible association with HPV.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 Clinical, radiological and histological features of the orthokeratinised jaw cyst

OJCs are distinctly uncommon with less than 100 cases reported in the literature. This rare developmental cyst accounts for about 10% of cases that were previously coded as OKCs.[8] Clinically, orthokeratinised jaw cysts (OJCs) are usually non-expanding, asymptomatic, solitary lesions which occur predominantly in adults with an approximately 2:1 male to female ratio.[22] OJCs occur more frequently in the mandible than in the maxilla with a predilection for the posterior areas of the jaw.[22]

Radiographically, they present with no features that distinguish them from any other developmental or inflammatory odontogenic cyst.[23] The OJC frequently appears as a unilocular radiolucency associated with the crown of an unerupted mandibular third molar mimicking a dentigerous cyst. The size of the OJC may vary from 1cm to as large as 7cm in diameter.[22] The clinical behaviour of the OJC is similar to the dentigerous and radicular cyst showing little tendency for recurrence following conservative surgical enucleation.[8,22] It is, however, the histological features of the OJC that sets this particular cyst apart from the other jaw cysts. The OJC has an orthokeratinised stratified squamous epithelial often with a prominent stratum granulosum.[22,24] The basal layer comprises of cuboidal or flattened cells, which show little tendency to palisade or polarise.[25,26] Mature fibrous connective tissue constitutes the cyst wall that, unless secondarily infected, is devoid of inflammation.[25]

2.2 A historical perspective on the separation between the orthokeratinised jaw cyst and the odontogenic keratocyst

The OJC was first described as a dermoid cyst by Schultz[27] in 1927 and was later typed as a histological variant of the odontogenic keratocyst (OKC).[4] In 1981 Wright[5] began to describe the difference in clinical aggressiveness between the OJC and OKC. In subsequent studies the OJC was considered as a separate entity and no longer as a subtype of the OKC.[1,6-8] Orthokeratinised odontogenic cyst is a synonymous term for OJC that has been used in the literature.[6,8] The are several reasons for not considering the OJC as part of the spectrum of the OKC. From a clinical point of view, the OJC presents more frequently in a dentigerous relationship (60.8%) compared to the OKC (25-40%).[1,3,5,7,8,22] The OJC shows a significantly lower recurrence rate compared to the OKC following conservative enucleation of the lesion.[1,3,5,8]

Immunohistochemical studies have demonstrated that the epithelial linings of the OJC and the OKC are different in many respects. Ki-67-postive proliferating cells are dominant in the OKC and not the OJC lining.[28] The high suprabasal proliferative activity present in OKC is not evident in the OJC.[28] The presence of a high concentration of p53 expression in OKC is thought to be responsible for the maintenance of and differentiation of epithelial stem cells, suggesting that the lining of the OKC has a higher self-renewal potential when compared to the OJC.[29] Keratin profiling of the OJC and the OKC has also revealed these lesions to be distinct entities.[30] It was found in a study by Aragaki *et al.*[30] that the keratin profiling of the OJC lining resembled that of epidermis. By contrast the keratin profiling of the OKC resembled the dental lamina.[30]

Most authors believe that the OKC originates from the dental lamina or from surface mucosal epithelium.[22] Cohen and Shear[4] suggested that the origin of the OJC could also be related to the dental lamina which has the ability to keratinise. Other theories suggest origin of the OJC from heterotopic ectodermal tissues or from post-traumatic implantation.[5] The OJC that develops in association with the crowns of impacted teeth (follicular OJC) is further thought to represent a dentigerous (follicular) cyst in which the cyst lining has undergone squamous metaplasia.[31] The stimulus for transformation from a tenuous layer of flattened cuboidal cells to a robust orthokeratinised lining is still unknown.

Li *et al.*[24] suggested that the OJC origin cannot simply be attributed to metaplasia. These authors raised the possibility that those growth factors and their receptors that have been implicated in normal tooth development and in the pathogenesis of some odontogenic lesions may well be involved in the pathogenesis of the OJC.[24] Other authors have suggested that the underlying capsular fibrous tissue has an inductive influence on the lining of the cyst and that mesenchymal factors may influence the diversity of odontogenic lesions.[32]

Vuhahula *et al.*[1] suggested that the reduced enamel epithelium, a derivative of the odontogenic apparatus, has the ability to keratinise under the appropriate stimuli. Aldred *et al.*[33] later reported on the unusual histological findings of an odontogenic cyst associated with an impacted maxillary canine. In this case, transition from a thin layer of reduced enamel epithelium into a thicker, hyperplastic, orthokeratinised, verrucoid, cystic epithelium was observed prompting the authors to consider human papillomavirus (HPV) as a possible pathogenetic mechanism for this phenomenon.[33] Likewise there is vast research into HPV as a possible aetiologic trigger in a variety of extragnathic lesions that may show verrucoid

features. Such lesions include epidermoid cysts,[9] cholesteatoma,[10] cystic warts of the skin and flat warts.[19-21]

2.3 Human papillomavirus

The human papillomavirus (HPV) is part of a large family of viruses known as the papovaviridae.[34] HPVs are DNA viruses of the papovavirus subgroup A.[34] The virus is capable of becoming totally integrated with the DNA of the host cell.[34] The HPV genome encodes for 6 early DNA sequences namely (E1, E2, E4-E7).[34] These proteins are involved in viral gene regulation and cell transformation.[35] The late proteins (L1 and L2) are responsible for forming the shell of the virus as well as one region of the regulatory DNA sequences.[35] The characterisation of each HPV type is based on the genotypic variations in the DNA base-sequences of E6 and E7.[34] It was the discovery of these genotypic differences that allowed classification of the HPV into high, intermediate and low-risk oncogenic types.[34, 35]

HPV is one of the most prevalent infections in the world. These viruses have an exclusive tropism for epidermal and squamous mucosal epithelium.[35] Transmission by sexual and non-sexual person-to-person contact, contaminated objects, saliva and autoinoculation has been proposed.[34, 36] Of the more than 150 different types of HPV that have been identified, at least 25 types are associated with lesions of the head and neck (HPV-1, 2, 3, 4, 6, 7, 10, 11, 13, 16, 18, 31, 32, 33, 35, 40, 45, 52, 55, 57, 58, 59, 69, 72 and 73).[36,37] The association between HPV and the development of oral and oropharyngeal tumours (benign and malignant) is well established in the literature.[36,37] HPV has been demonstrated in diverse lesions such as

verruca vulgaris, condyloma acuminatum, papilloma, focal epithelial hyperplasia, proliferative verrucous leukoplakia, verruca plana and a subset of squamous cell carcinomas.[36,37]

HPV is capable of producing a variety of proliferative surface changes at the site of infection.[35] On light microscopy the presence of hyperkeratosis, koilocytosis and a verruco-papillary surface strongly suggest HPV related viral modification.[9,20] It is these alterations that reflect the ability of HPV to integrate viral DNA into that of the host cell, thus enabling itself to reproduce and act as an agent of clinico-pathological change.[35,38-39]

2.4 Laboratory methods for the detection of human papillomavirus

HPV can be found in normal oral mucosa, in varying degrees of concentration, depending on the method of detection used.[40-42] HPV is only able to replicate in the basal cells of stratified epithelium.[35] The virus infects epithelial tissues via micro-abrasions or trauma that exposes the cell basement membrane.[34, 39] The HPV virus has a slow infection process and takes anywhere from 12 to 24 hours to initiate transcription.[35] The lesions associated with HPV are believed to arise from the proliferation of infected basal keratinocytes. The release of viral particles occurs as a result of degeneration of desquamative cells.[35] HPV itself is a hardy virus and is able to survive for months at a time at low temperatures without a host cell.[34,35]

The detection of HPV can only be performed by molecular analysis of HPV DNA sequences as HPV cannot be cultured in vitro and serology assay has limited accuracy.[39,40] There are various molecular methods available for the analysis of HPV in clinical specimens.[40-42] These methods are largely based on the principles that were used for the discovery of the various

HPV subtypes.[34] Early testing methods relied on the use of nuclei acid probes labelled with radioactive phosphorus in a slot-blot hybridisation technique.[41] Besides being technically cumbersome, another key disadvantage of this technique is the lack of sensitivity and the inability to detect all HPV genotypes.[41,42] In the late eighties, nucleic acid in situ hybridisation (ISH) became available and has enjoyed a recent revival with the introduction of automated platforms that are manufactured by various companies.[40-42] HPV DNA ISH offers the advantages of high specificity, it provides visual detection of infected cells through the preservation of tissue context and allows for the distinction between episomal and integrated viral DNA.[40] To date, however, a major drawback of HPV DNA ISH is its reduced sensitivity, particularly when viral loads are low. Hence, while it is more sensitive than the Southern blot technique, it has a lower sensitivity than the polymerase chain reaction (PCR) technique.[42]

In essence, PCR is a technique that is able to amplify specific gene sequences of nuclei acids.[43] It then enables the user to apply the information in a variety of fields such as forensic science, prenatal diagnostics and histopathology.[43] PCR has become one of the most valuable and flexible biomedical tools in research and subsequent refinements in the technique have made PCR an accepted diagnostic tool in many pathology disciplines.[41,43]

There are several PCR screening assays available. The majority of these use consensus primer sets that bind to highly conserved regions, so-called consensus HPV PCR or qualitative end point PCR.[40] This method allows for the simultaneous identification of a large range of HPV types. The MY09/11 and GP5+/6+ primer sets fall into the consensus HPV PCR category and their efficacy has been compared by Remmerbach *et al.*[44] The authors concluded that

GP5+/6+ was more sensitive, especially in low viral load samples.[44] GP5+/6+ primer sets are also used more frequently with formalin-fixed paraffin-embedded (FFPE) tissue.[44] This is because the fixation process tends to cause fragmentation of the DNA into sequences that are often shorter than 200 base pairs.[45] The GP5+/6+ primer sets are well suited for this purpose as they target these short DNA sequences in FFPE samples thereby resulting in increased sensitivity.[44] The disadvantages of the PCR-based methods include the requirement for DNA extraction, the test yields no direct evidence of transcriptional activity, and there is a risk of contamination and detection of HPV from surrounding healthy tissue.[43]

Recently, p16 (INK4A) has emerged as a potential surrogate marker of HPV infection.[41] p16 is a tumour suppressor gene that prevents phosphorylation of the retinoblastoma (Rb) protein by inhibiting the cyclin D and cyclin dependent kinase (CDK) 4 complex at the G1-S interphase of the cell cycle.[46] In high-risk HPV related cancers p16 overexpression is frequently seen as a consequence of functional inactivation of the Rb gene by the E7 viral oncogene.[47] While p16 immunohistochemistry is a highly sensitive technique, it has been criticised as lacking specificity for the presence of HPV.[41,46] In other words, while p16 overexpression and high-risk HPV infections are highly correlated they are not always synonymous.[41,46] Given the advantages and disadvantages of the currently available HPV detection methods as explained above, consensus HPV PCR emerges as a highly sensitive, cost effective technique, that is feasible on FFPE material, targeting numerous HPV strains and adequate for screening for the presence or absence of HPV in most laboratories.[43,47]

2.5 Review of studies on HPV in odontogenic tumours and cysts

Studies demonstrating an association between HPV and odontogenic tumours and cysts have been noted in the literature and are summarised in Table 1.

Table 1. Summary of studies on human papillomavirus in odontogenic lesions

Reference	Lesion	*n*	HPV test	Result	HPV type/s
Kahn *et al.*[48]	Ameloblastoma	10	IHC	3/10 +	NT
Ayoub *et al.*[49]	Ameloblastoma	19	IHC	8/19 +	NT
van Heerden *et al.*[17]	Ameloblastoma	1	IHC, DNA ISH	1/1 +	18
Kahn *et al.*[16]	Peripheral ameloblastoma	1	DNA ISH	1/1 +	16/18
Sand *et al.*[14]	Ameloblastoma	18	C-PCR	8/18 +	6,11,18
Namin *et al.*[53]	Ameloblastoma	50	C-PCR	20/50 +	6
Migaldi *et al.*[54]	Ameloblastoma	18	C-PCR,N-PCR, DNA-ISH	0/18 +	-
Correnti *et al.*[55]	Ameloblastoma	18	IHC, N-PCR, ISH	6/18 +	6,11,16,33,42
Cox *et al.*[18]	Odontogenic keratocyst	1	SBH	1/1 +	16
Gonzalez-Moles *et al.*[56]	Odontogenic keratocyst	83	C-PCR	0/84 +	-
Rider *et al.*[57]	Radicular cyst	20	IHC	0/20 +	-
Aldred *et al.*[33]	Verrucous odontogenic cyst	1	IHC, C-PCR	0/1 +	-
Argyris *et al.*[58]	Verrucous odontogenic cyst	1	C-PCR	0/1 +	-

HPV=human papillomavirus, IHC=immunohistochemistry, SBH=Southern blot hybridisation, ISH=in situ hybridisation, C-PCR=consensus (qualitative) polymerase chain reaction, N-PCR=nested polymerase chain reaction, NT=not typed

The first documented study investigating for the presence of HPV in an odontogenic tumour was performed in 1989 by Kahn.[48] In this study ten cases of histologically confirmed ameloblastomas were subjected to HPV immunohistochemistry.[48] Of the 10 cases studied, three were positive for HPV. The HPV detected in these three cases were more prevalent in the columnar epithelial cell nuclei than in the stellate reticulum. No features of koilocytosis were, however, noted.[48]

In a more recent study, also using the immunohistochemical method of HPV detection, Ayoub *et al.*[49] analysed 19 cases of ameloblastoma for the presence of HPV. Fifteen of these cases were benign multicystic ameloblastomas and 4 were recurrent ameloblastomas with malignant features. The anti-HPV mouse monoclonal antibody used in this study is formulated for detecting major capsid proteins on most HPV subtypes.[49] The results demonstrated 6/15 (40%) of the benign ameloblastomas to be positive for HPV, with most of the reactions occurring in the cytoplasmic or membranous regions of the tumour cells.[49] The cells layers that were immunopositive were the basal ameloblastoma-like cells and the suprabasal stellate reticulum-like cells.[49] The two positive recurrent ameloblastomas with malignant features showed the HPV reactions to be mainly nuclear, suggesting viral integration and a role for the malignant transformation of these tumours. Ayoub *et al.,*[49] nevertheless, concluded that more sophisticated research techniques would have to be employed to accurately determine the exact aetiological role HPV plays in the development and progression of ameloblastoma. With regard to viral transmission, Ayoub *et al.*[49] postulated that the HPV infection may be acquired in utero or during parturition involving the invagination of the enamel organ.[49] HPV may subsequently influence various growth factors to induce pathologic proliferation of odontogenic epithelial residues that are normally present within the jaws. The alternative

possibility that these authors considered was that the virus could merely be a passenger and have no pathogenic potential in ameloblastomas.[49] Studies that have been conducted to ascertain the prevalence of HPV in normal oral mucosa have, however, yielded conflicting findings and no definitive conclusions can thus far be drawn thereby precluding a definitive role for HPV as a "by-stander" in these pathologic lesions.[50-52]

More sensitive techniques were used by other researchers to demonstrate the presence or absence of HPV in ameloblastomas. These techniques included HPV DNA ISH[16,17, 54, 55] and/or PCR.[14, 53,54,55] van Heerden *et al.*[17] were able to demonstrate the presence of HPV-18 DNA using DNA in situ hybridisation in an ameloblastoma. The HPV DNA was demonstrated in a papillomatous area within the ameloblastoma.[17] However, since HPV was only detected in the papillomatous area, the authors suggested that acquisition of HPV DNA within this lesion was most probably due to secondary infection from the overlying oral mucosa and that HPV could not be considered an aetiological factor in this instance.[17] In another study also using HPV DNA ISH, a peripheral ameloblastoma occurring in a 16-year old patient tested positively for HPV.[16] These authors maintained that a role for HPV in peripheral ameloblastomas and other benign and malignant lesions of the oral cavity remains a possibility.[16]

Sand *et al.*[14] examined 18 samples of central ameloblastoma from 12 patients for the presence of HPV DNA by PCR using the L1 consensus primer. The results of the study demonstrated that at primary surgery four of the samples tested positive for HPV-18 and HPV type 6/11, and three were positive for HPV type 6/11.[14] One HPV positive sample could not be typed.[14] The authors speculated that the presence of HPV at primary surgery may indicate a correlation

between ameloblastoma and HPV, while the presence of HPV at secondary surgery may be due to traumatic manipulation of the tissues which may decrease the resistance of the tissue to HPV infection.[14] The authors also suggested that the HPV detection could be attributed to presence of HPV in the overlying oral mucosa. This possibility could not be further investigated by Sands *et al.*[14] because normal oral mucosal samples were not included in their study. In the subsequent work by Terai *et al*.[50] and Sand *et al.*[51] the episomal form of HPV DNA was demonstrated in normal oral mucosa and the presence of HPV without any histological or clinical changes was considered to be latent infection.[50,51] With regard to viral transmission, Sand *et al.*[14] concurred with the hypothesis proposed by Kahn,[48] where it is speculated that the HPV in intrabony ameloblastomas may be acquired in utero or at parturition, involving the invaginating primitive enamel organ. The virus is then, at a later stage, thought to stimulate growth factors or inhibit natural control mechanisms which in turn may lead to the development of an ameloblastoma.[14]

Namin *et al.*[53] conducted a retrospective study for the presence of HPV by using the PCR technique on 50 cases of ameloblastomas and 50 impacted third molar follicles as the control group. The results of this study showed 20 samples in the study group positive for HPV while nine samples were HPV positive in the control group.[53] Statistical analysis proved this result to be significant. Eight cases were typed as HPV-6 while the remaining positive cases could not be subtyped. Namin *et al.*[53] also noted that further investigations would need to be carried out to determine the unidentifiable HPV types as this may be key to understanding the role that HPV has, if any, in the pathogenesis of the ameloblastoma.[53]

A study by Magaldi *et al.*[54] investigated 18 ameloblastomas for the presence of HPV using ISH, laser capture microdissection and nested PCR analysis. The authors used both the conventional as well as nested PCR approach.[54] The microdissection technique prevented contamination from the adjacent oral epithelium.[54] The results were negative on PCR and only a weak positive band was noted in the neoplastic tissue in one case and in one control sample. Magaldi *et al.*[54] concluded that that HPV should not be implicated in the development of ameloblastoma. It was also noted that previous studies failed to show any real viral integration.[14,48,49] Rather some authors eluded towards surgical contamination or a secondary event being the reason for HPV positivity in their studies.[14]

In a more recent study by Correnti *et al.*[55] 33% of intraosseous ameloblastomas were shown to be HPV positive. Methodology included immunohistochemistry, nested PCR, and DNA ISH.[55] Their results revealed six cases to be positive of which four were HPV-6 positive, one case was HPV-11 positive, and two cases were HPV-6 and HPV-42 and HPV-16 and HPV-33 positive respectively. Correnti *et al.*[55] were able to demonstrate koilocytic changes in the unicystic variants of ameloblastoma. These results were similar to those of Namin *et al.*[53] where most of the cases were positive for HPV-6. Correnti *et al.*[55] proposed that for HPV to replicate it requires differentiating epithelial cells to complete its life cycle. It was suggested by these authors that the source of this differentiating epithelium would have been provided for either by the lining of an odontogenic cyst or enamel organ residues.[55]

As far as odontogenic cysts are concerned, OKC,[18,56] radicular cysts[57] and two cases of verrucous odontogenic cysts were studied for the presence of HPV.[33, 58] Rider *et al.*[57] used a polyclonal rabbit anti-papilloma antibody to evaluate for the presence of HPV DNA in

radicular cysts. All 20 cases were negative for the presence of HPV by immunostaining.[57] The theory that was proposed by Rider *et al.*[57] was that in the case of radicular cysts the granulation tissue could be the source of growth factors, which in turn influence the proliferation of the epithelial rests of Malassez.

In the study by Cox *et al.*[18] HPV was detected in an OKC with signs suggestive of viral intergration. Eighty-three samples of OKC were analysed by Gonzalez-Moles *et al.*[56] for the presence of HPV DNA using PCR. Their results yielded no positive cases for HPV DNA. The OKCs used in their study included sporadic OKCs, recurrent OKCs and nevoid basal cell carcinoma syndrome associated OKCs. The PCR findings on two samples of verrucous odontogenic cysts that have thus far been investigated were negative for HPV DNA.[33,58]

2.6. HPV in extragnathic lesions that share overlapping histological features with OJC

The orthokeratinised lining of the OJC bears histomorphological resemblance to the epidermoid cyst,[9] cholesteatoma,[10] cystic warts of the skin and flat warts (verruca plana).[11,20,21] Whilst not confirmatory, HPV-induced histomorphological changes, as observed on light microscopy, are often used as an adjunctive measure when assessing tissue specimens for underlying HPV infection.[10, 19,20] In their study, Gross *et al.*[20] analysed the correlation between the HPV type and the histology of warts. It was found that the histology of HPV-induced warts is heterogenous.[19] The classical pattern of HPV-induced warts includes acanthosis, hypergranulosis, hyperkeratosis, papillomatosis, keratinocytes with perinuclear halos and pyknotic nuclei.[19] In addition to the classical pattern, HPV-induced warts with sickle-shaped nuclei that were pushed to the margin of vacuolated squamous cells were also

documented by Gross *et al.*[19] Whilst papillomatosis is a characteristic feature of some solid HPV-associated lesions such as squamous papillomas and verruca vulgaris, papillomatosis is notably slight or absent in HPV-associated epidermoid cysts (cystic warts).[11-13] Another variation in the morphological pattern is seen in verruca plana.[59] The histological features that characterise this HPV-associated lesion are hyperkeratosis, often absence of parakeratosis, slight or no papillomatosis, hypergranulosis and vacuolated keratinocytes with perinuclear clearing.[19,59]

Egawa *et al.*[60] were able to demonstrate HPV antigens in 37 of 119 palmoplantar epidermal cysts studied. HPV DNA was detected not only in cysts demonstrating all the HPV-induced light microscopic changes but also in those cysts demonstrating a single HPV-associated histological feature.[60] In another study, Jeon *et al.*[61] demonstrated, by the PCR technique, the presence of HPV in non-palmoplantar epidermal cysts. The epidermoid cysts were positive for HPV-60, which are typically found in palmoplantar epidermal cysts.[13]

Cholesteatomas which share a similar histological resemblance to OJCs have also been shown by various authors to have HPV present in their linings. Researchers postulate that the proliferative epithelium of some cholesteatomas may be associated with HPV infection.[10,62,63] Among the odontogenic cysts, those lined by orthokeratinised epithelium exhibit by far the most striking histological resemblance to HPV-induced histomorphological changes. However, other than two previous case reports on verrucous odontogenic cysts where HPV testing was undertaken,[33,58] the OJC has not yet been rigorously analysed for a possible association with HPV.

CHAPTER 3

3.0. AIM AND OBJECTIVES

3.1 Aim

To analyse the clinico-pathological features of orthokeratinised jaw cysts (OJCs) and to determine whether human papillomavirus (HPV) DNA can be detected in a series of OJCs.

3.2 Objectives

3.2.1. To determine the age and gender of patients diagnosed with OJC.

3.2.2. To determine the anatomical location of the OJC.

3.3.3. To determine the prevalence of the follicular type of OJC.

3.3.4. To compare the sites of occurrence and histological features between the follicular type of OJC and the extrafollicular OJC.

3.2.5. To histologically examine the OJC for light microscopic features suggestive of HPV infection.

3.2.6. To investigate for the presence of HPV DNA in the OJC by using consensus HPV PCR, also known as qualitative end point PCR.

CHAPTER 4

4.0 MATERIALS AND METHODS

4.1 Study sample

Formalin-fixed and paraffin embedded material of 30 biopsy specimens coded as OJCs were retrieved from the archives of the Department of Oral Pathology at the University of the Witwatersrand. For haematoxylin and eosin staining (H&E), 5-µm sections were cut from the tissue blocks and examined by light microscopy to confirm the diagnosis of OJC. OJCs comprised cystic lesions that were lined by orthokeratinised epithelium with a non-palisaded rows of basal cells. The age and gender of the patients, anatomical location of the cyst and the presence or absence of an associated impacted tooth was determined, wherever possible, from the surgical histopathology reports. The intraosseous location of the OJC was ascertained by evaluation of the radiographic findings as was described in the original histopathology laboratory report. Locations of the lesions were classified as anterior (incisor-canine region), premolar region, molar region or ramus.

4.2 Histological studies

The H&E stained OJC tissue sections were examined simultaneously by the student and supervisor with the use of a Nikon Eclipse 50*i* double-headed light microscope. The cyst linings were examined for the presence or absence of histomorphological features suggestive of HPV infection. The features included hyperkeratosis, hypergranulosis, vacuolated

keratinocytes with perinuclear clearing and verruciform hyperkeratosis. Verruciform hyperkeratosis was defined as squamous epithelium with an irregular surface exhibiting pointed projections with marked overlying hyperkeratosis (orthokeratosis or parakeratosis). Exclusion criteria included insufficient cyst lining for histological analysis, cysts showing a palisaded, hyperchromatic basal cell layer characteristic of OKC and those cysts where an intraosseous location could not be confirmed from the clinical information provided in the histopathology report.

4.3. Polymerase chain reaction

4.3.1 DNA extraction

Sections (10µm) were prepared from each formalin-fixed paraffin-embedded sample. These sections were deparaffinised using 1ml xylene and subsequently treated with 1ml ethanol. After centrifugation, DNA was extracted from the samples using the DNA Micro QIA amp kit (Qiagen, Whitehead Scientific) according to manufacturer's instructions.

4.3.2 Control of contamination

Tissue blocks were sectioned using new blades for each sample to prevent cross contamination. Work area and work tools were cleaned with 3% Virkon between each block handled. Work areas were decontaminated with ultraviolet light between subsequent procedures. The extraction procedure was assessed by PCR amplification of the internal ß-

globin control and DNA was quantified using the Nanodrop 1000 Spectrophotometer Thermo Scientific, Inqaba Biotec).

4.3.3 HPV PCR

HPV amplication with GP5+ /GP6+ primers (Table 2) was made in a reaction mix containing 5µl of template DNA, 200µM dNTP's (Roche), 0,2 µM of each primer(Whitehead Scientific), 1.0 U Taq DNA polymerase (Roche), 10x Reaction Buffer (with $MgCl_2$, 15mM) in a total volume of 50µl. The thermal conditions of amplification were as follows: initial denaturation to 95ºC for 4 minutes; subsequent 40 cycles consistent of 95 ºC for 1 minute, 55ºC for 1 minute and 72ºC for 1 minute and a final extension at 72 ºC for 5 minutes. PCR was carried out in the 9700 Gene Amp PCR System (Life Technologies). The ß-globin housekeeping gene (PC04/GH20) served as a control for efficacy of extraction and amplification of DNA from paraffin embedded tissue material.

Table 2. DNA sequences of HPV primers (GP5+/GP6+) and ß-globin housekeeping gene (PC04/ GH20)

Primer	DNA sequence
GP5+	5'- TTT GTT ACT GTG GTA GAT ACT AC–3'
GP6+	5'- GAA AAA TAA ACT GTA AAT CAT ATT C –3'
PCO4	5'-CAA CTT CAT CCA CGT TCA CC-3'
GH20	5'-GAA GAG CCA AGG ACA GGT AC-3'

4.3.4 Gel Electrophoresis

Amplified PCR products were examined by agarose gel electrophoresis. Samples were electrophoresed at 100 volts using a 3 % agarose gel (Celtic Diagnostics), stained with ethidium bromide (Merck). The gel was visualised under ultraviolet light. Positive samples appeared as a visible band with a molecular size of 150 base pairs (bp).

Controls used: The PCR procedure was controlled with the use of both positive and negative controls. The positive control included paraffin embedded samples that had previously tested positive with HPV PCR. The negative control used included a *no-template* control in which nuclease- free water was substituted as a template.

4.3.5 Real-time amplification of the ß-globin gene

This assay was performed in a Corbett Research RotoGene 6000 (Whitehead Scientific) RT-PCR machine using the Bioline SensiMix™SYBR No-Rox kit(Celtic Diagnostics). A final volume of 20 µl reaction mix was made using 0.2 mM of each primer, 10 µl 2x SensiMix™SYBR No-Rox Master Mix (with $MgCl_2$, 50mM) and 2µl of template DNA. The thermal cycling profile of this assay consisted initial denaturation step at 95ºC for 10 minutes, followed by 50 cycles consistent of 95ºC for 10 seconds (denaturation), 55ºC for 10 seconds(annealing) and 72ºC for 15 seconds (elongation). After amplification, melt curve analysis was carried out at 95ºC with a ramp rate 1ºC/5seconds. The average melting temperature ($T_{m)}$ of the β-globin amplicon is 85.5 +/-1.0°C. Gel electrophoresis was performed on specimens with doubtful T_m values to confirm the presence of the 268bp PCR fragment.

4.4 Data collection and statistical analysis

Descriptive statistics was used to describe the clinico-pathological features of the data in this study. This was in the form of basic statistical analyses and table summaries describing what the data showed. Fisher's exact test was used to determine the association between the frequency of the occurrence of the extrafollicular OJC and follicular OJC in the upper and lower jaws. The Chi-Square test was used to determine the association between the presence or absence of an impacted and whether that had an impact on the histological features of the cyst lining. *P*-values of <0.05 were considered statistically significant.

The histological features observed on light microscopy were also captured in the form of a table. The histological features that were analysed included: hyperorthokeratosis (HK), hypergranulosis (HG), vacuolated keratinocytes with perinuclear clearing (VK) and verruciform hyperkeratosis (VH).

The PCR findings were captured by photographing of the gel electrophoresis.

4.5 Ethical considerations

Ethics approval for this study was granted by the Human Research Ethics Committee of the University of the Witwatersrand for the use of stored tissue and archived patient histopathology reports. The ethics clearance reference number is M120956 (Appendix 1).

CHAPTER 5

5.0. RESULTS

5.1 Clinico-pathological findings

The clinico-pathological findings are summarised in Table 3.

Table 3. Clinical data and histopathological findings in patients with orthokeratinised jaw cyst

Case No.	Age (yr)	Gender	Jaw	OJC type	Histological findings			
					HK	HG	VK	VH
1	20	Male	Maxilla – M	Extrafollicular	+	+	+	-
2	25	Female	Mandible	Follicular (38)	+	-	+	+
3	13	Male	Mandible	Follicular (38)	+	+	+	+
4	25	Female	Mandible	Follicular (35)	+	-	+	-
5	34	Male	Maxilla	Extrafollicular	+	-	+	-
6	32	Male	Mandible – M	Extrafollicular	+	+	+	+
7	28	Male	Mandible	Follicular (38)	+	+	+	-
8	20	Male	Mandible	Follicular (48)	+	-	+	+
9	27	Male	Mandible – A	Extrafollicular	+	+	+	+
10	42	Female	Mandible – M	Extrafollicular	+	+	+	+
11	33	Female	Mandible	Extrafollicular	+	-	+	-
12	22	Male	Mandible	INA	+	+	+	-
13	34	Male	Mandible – R	Extrafollicular	+	-	+	-
14	INA	Male	Mandible – M	Extrafollicular	+	+	+	-
15	22	Male	Mandible	Follicular (48)	+	-	+	-
16	26	Female	Mandible – M	Extrafollicular	+	+	-	+
17	29	Male	Maxilla – A	Extrafollicular	+	+	+	+
18	21	Male	Mandible	Follicular (38)	+	-	-	-
19	33	Female	Maxilla	Extrafollicular	-	-	+	+
20	47	Male	Mandible – M	Extrafollicular	+	+	+	-
21	22	Male	Mandible – M	Extrafollicular	+	+	-	-
22	66	Male	Mandible	Follicular (48)	+	+	+	+
23	22	Female	Maxilla – M	Extrafollicular	+	+	-	-
24	31	Male	Mandible	Follicular (38)	+	+	-	+
25	21	Male	Mandible	Follicular (48)	+	-	-	+
26	32	Male	Mandible – M	Extrafollicular	+	+	+	-
27	71	Female	Mandible – A	Extrafollicular	+	+	-	-
28	33	Male	Mandible	Follicular (48)	+	+	+	-
29	43	Female	Maxilla	Follicular (13)	+	+	-	-
30	23	Male	Mandible	Follicular (48)	+	-	-	+

INA, information not available; M, molar region; A, anterior region; R, ramus; HK, hyperorthokeratosis; HG, hypergranulosis; VK, vacuolated keratinocytes; VH, verruciform hyperkeratosis; tooth numbers are denoted in parentheses

5.1.1 Clinical findings

The OJC showed a male predominance with 21 cases occurring in males and 9 in females yielding a 2.3:1 male to female ratio. The mean age at presentation was 30.9 ±12.9 years (age range = 13-71 years; median = 27.5years). There was a strong predilection for the mandible (24/30 cases; 80%). The mandibular molar region was the most commonly affected site (18/26; 69.2%). The maxilla was affected in 6 cases (20%). In 13 patients (44.8%) the OJC occurred in association with the crown of an impacted tooth (follicular OJC), most frequently an impacted mandibular third molar (11/13; 84.6%). There was no significant association between the frequency of occurrence of the follicular OJC versus extrafollicular OJC in the upper or lower jaw. A *P*-value of 0,18 was obtained with the Fisher's exact test (Table 4).

Table 4. 2x2 Contingency table for follicular OJC and extrafollicular OJC in mandible versus maxilla

	Follicular OJC	**Extrafollicular OJC**
Mandible	12	11
Maxilla	1	5

5.1.2 Light microscopic findings

On light microscopy, all the cysts were lined by stratified squamous epithelium, showing keratinisation of the epidermoid type in 29/30 (96.7%) cases (Fig 1). None of the cases showed a palisaded basal cell layer, a characteristic histological feature of the OKC. Cyst lining showing foci of verruciform hyperkeratosis was present in slightly less than half of all cases (13/30; 43.3%) (Fig 2; Fig 3), while a prominent stratum granulosum was noted in about

two-thirds of the cases (63.3%) (Fig 4). Vacuolated keratinocytes with perinuclear clearing were identified in 21/30 cases (70%) (Fig 5A, B), of which 2 cases showed the presence of keratinoctyes with marginal sickle-shaped nuclei (Fig 6A, B). Sebaceous cells were identified in the cyst lining in 1 case (Fig 7) while epithelial plaques were noted in 1 case (Fig 8).

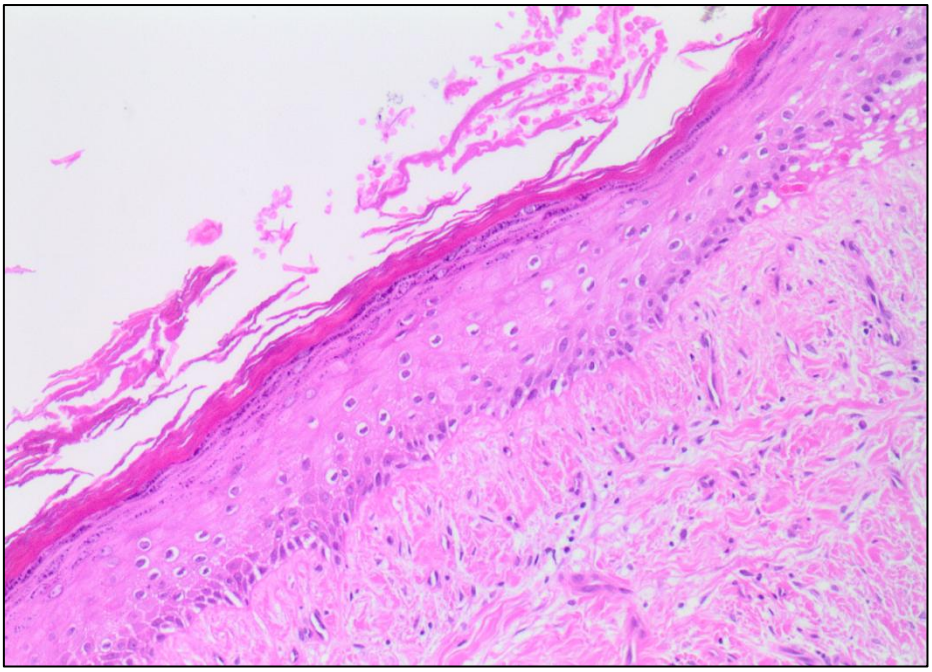


Figure 1. Orthokeratinised jaw cyst lined by stratified squamous epithelium with a discernible granular cell layer and laminations of keratin present at the surface. The basal cells show little tendency to palisade or polarise (Haematoxylin and eosin; original magnification X10).

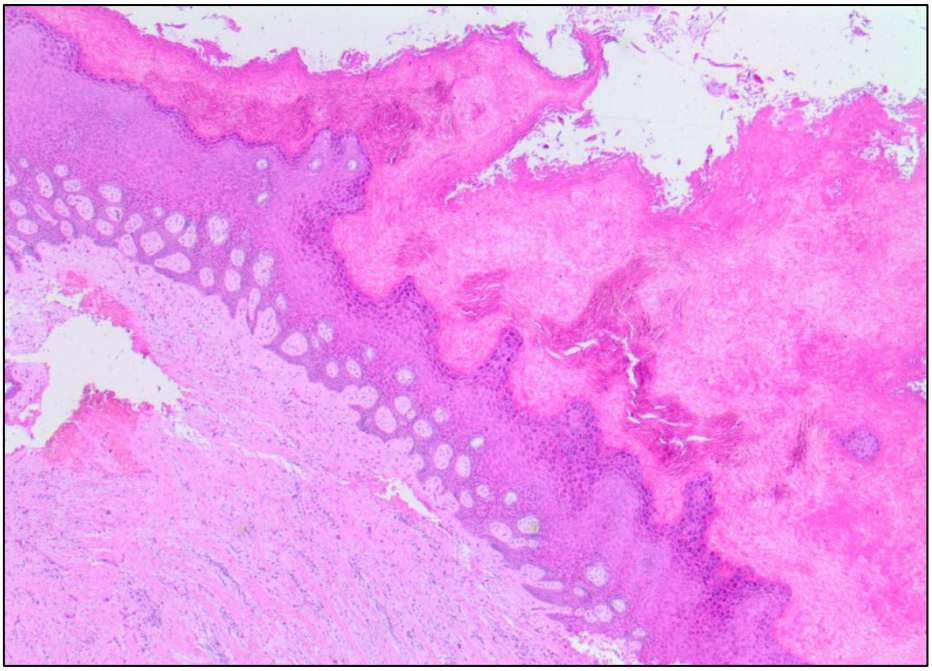


Figure 2. Orthokeratinised jaw cyst showing acanthosis, hypergranulosis and a verrucous-like pattern of hyperkeratosis (Haematoxylin and eosin; original magnification X4).

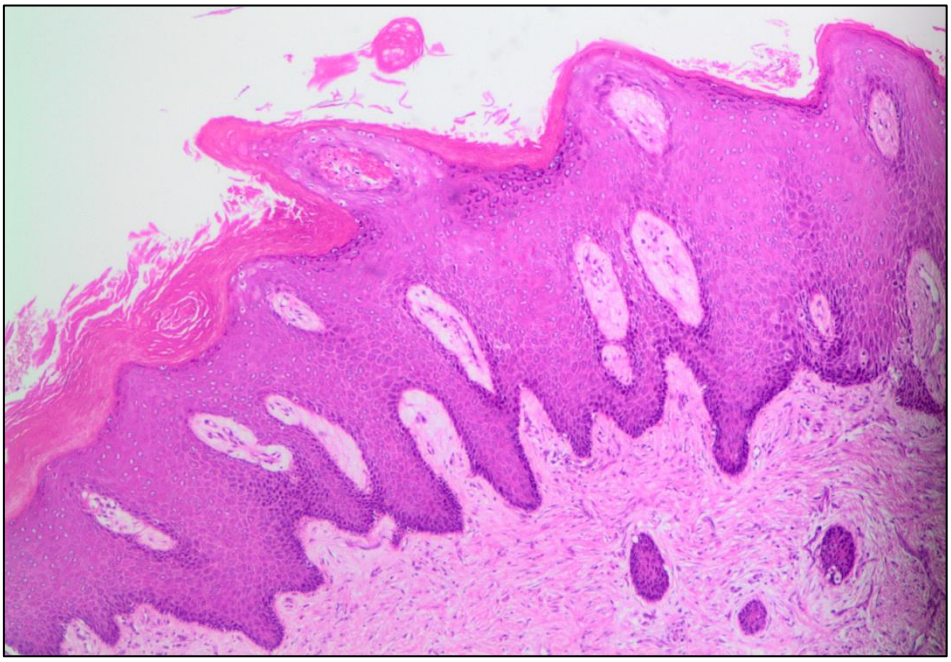


Figure 3. Orthokeratinised jaw cyst showing hyperkeratotic, surface projections reminiscent of wart-viral changes (Haematoxylin and eosin; original magnification X10).

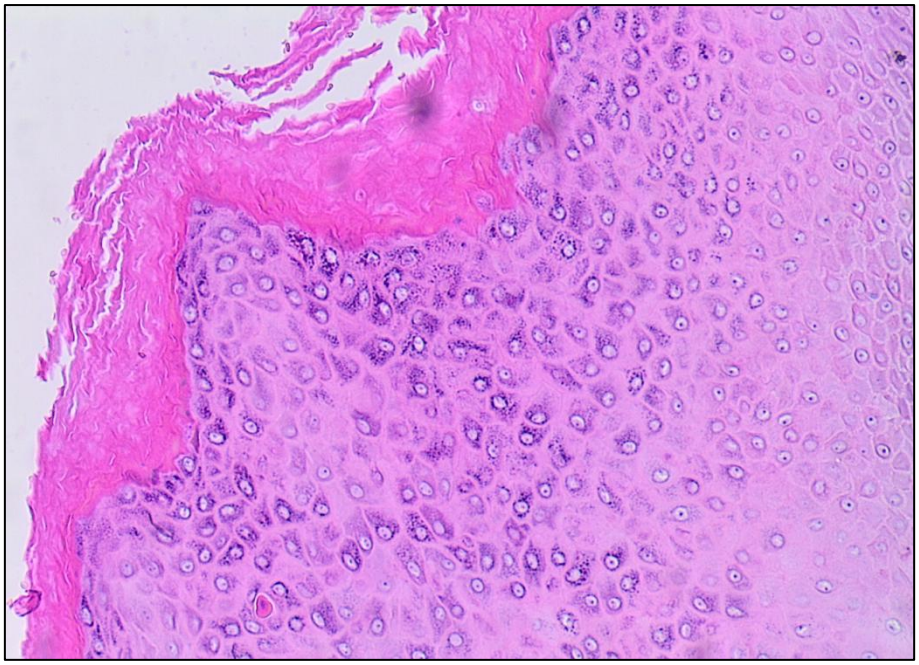


Figure 4. High-power magnification illustrating hypergranulosis with marked overlying hyperkeratosis and surface irregularities within an orthokeratinised jaw cyst lining (Haematoxylin and eosin; original magnification X20).

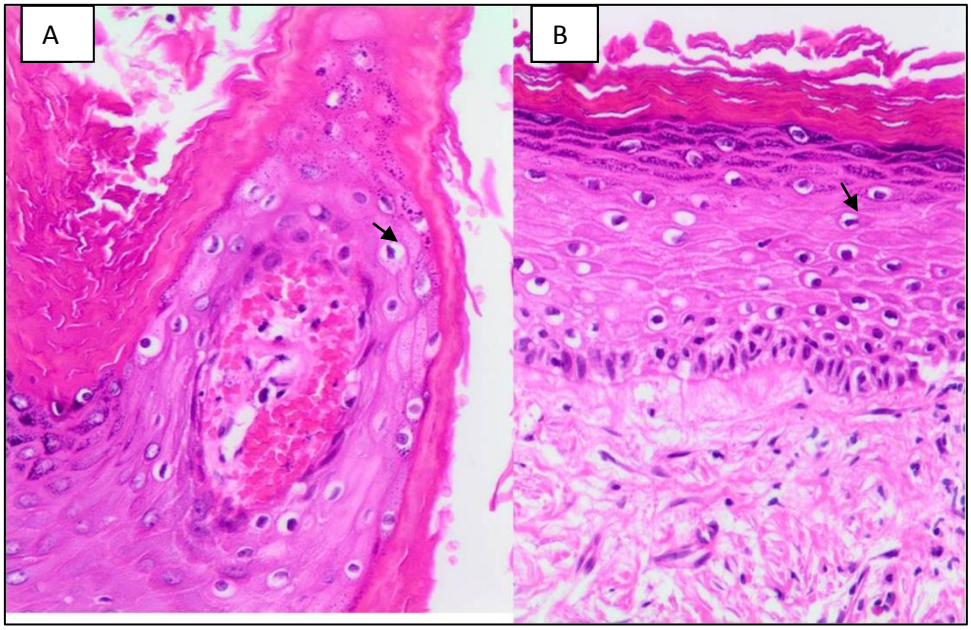


Figure 5. A. Orthokeratinised jaw cyst lining with cytoplasmic vacuolisation seen in the stratum spinosum and persisting up to the corneal layer. B. Pyknotic nuclei (arrows) are more or less prominent in some areas of the cyst lining (Haematoxylin and eosin; A, B original magnification X40).

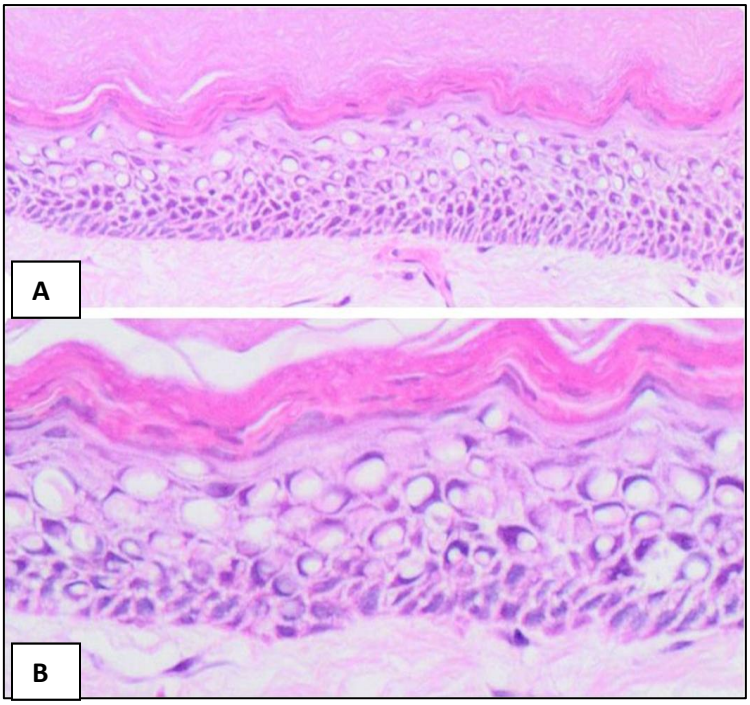


Figure 6. Orthokeratinised jaw cyst lining associated with vacuolised squamous cells with pyknotic, marginal, sickle-shaped nuclei. (Haematoxylin and eosin; A, original magnification X20; B, original magnification X40)

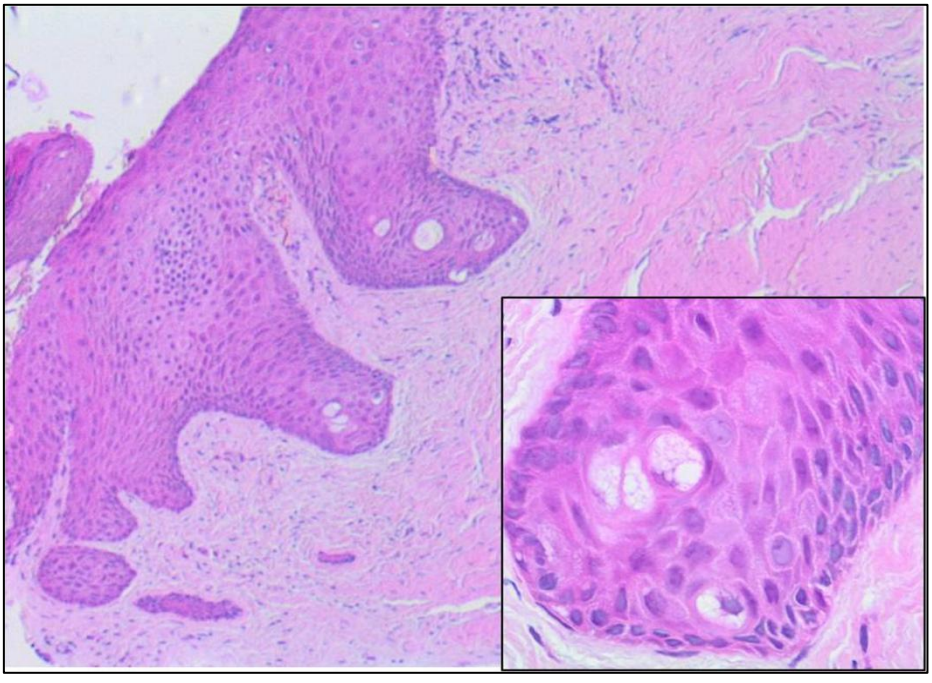


Figure 7. Cluster of sebaceous cells were focally identified within the lower third of an orthokeratinised jaw cyst lining (Haematoxylin and eosin; original magnification X20).

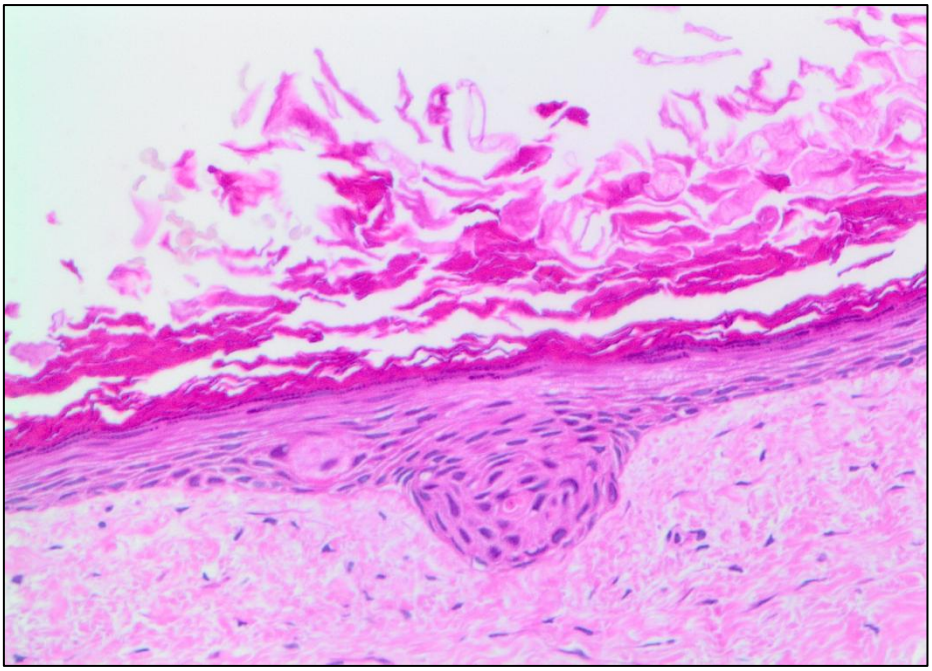


Figure 8. Orthokeratinised jaw cyst lining showing a nodular thickening of squamous cells arranged in a swirling pattern and constituting a so-called "localised epithelial plaque" (Haematoxylin and eosin; original magnification X40).

No significant association was found between the presence (follicular type of OJC) or absence of an impacted tooth (extrafollicular type of OJC) and the histological features of the cyst lining (Table 5). The Chi-Square test yielded a *P*-value of 0.67.

Table 5. 4x2 Contingency table for histological features of the cyst lining and OJC type

	Hyperorthokeratosis	**Hypergranulosis**	**Vacuolated keratinocytes**	**Verruciform hyperkeratosis**
Extrafollicular	15	12	12	6
Follicular	14	6	9	7

5.2 Molecular findings

The PCR products obtained from the positive controls were strongly amplified with the GP5+ and GP6+ primers, whereas all OJCs were consistently negative.

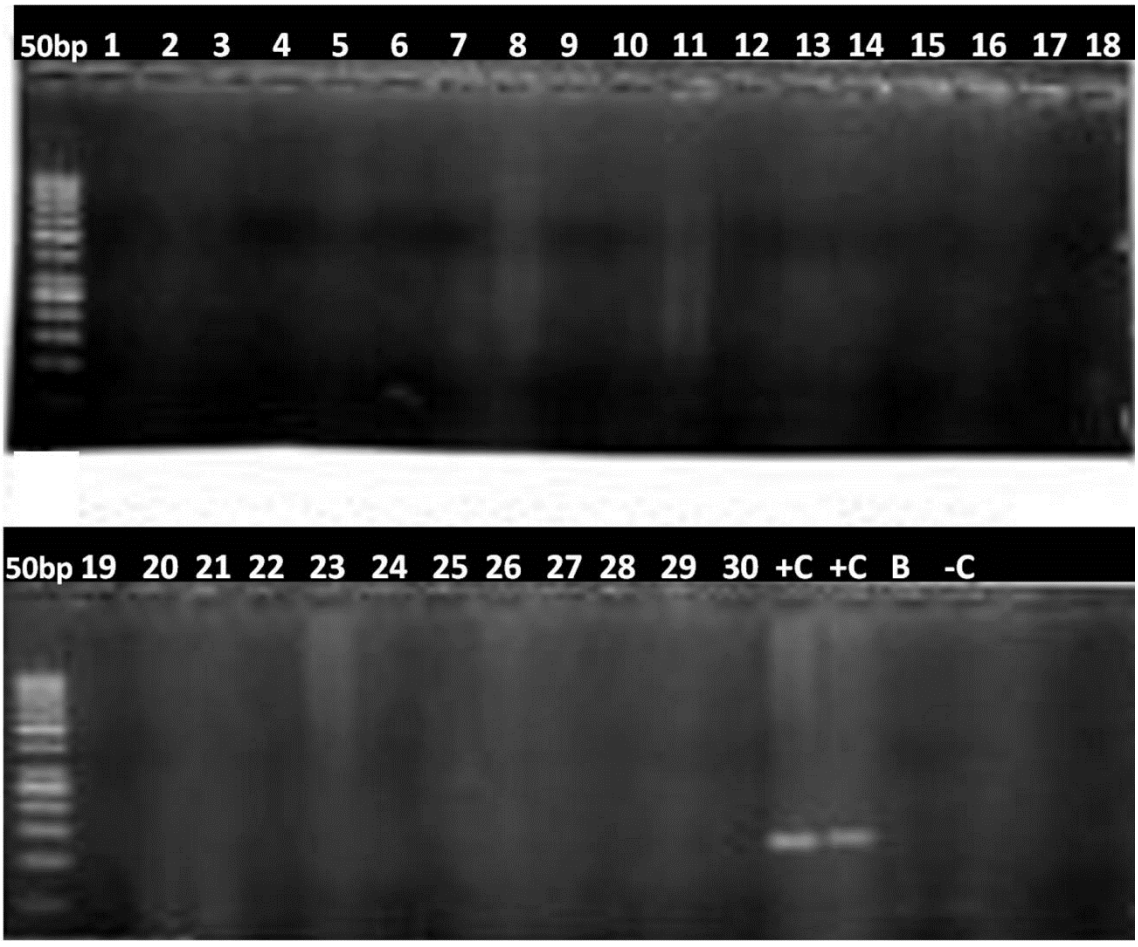


Figure 9. The polymerase chain reaction amplified products were separated on an agarose gel and photographed after ethidium-bromide staining. The molecular marker constitutes 50 base pairs; lanes 1 to 30, orthokeratinised jaw cyst samples; +C, positive controls indicating the expected size of the HPV amplified product; B, blank; -C, negative control.

CHAPTER 6

6.0. DISCUSSION

It is generally estimated that the OJC represents about 10% of cases that were previously classified as orthokeratinised variants of the OKC.[8] This relatively uncommon cyst has been shown to exhibit significant clinico-pathological differences from the OKC thereby warranting its designation as a distinct and separate entity.[1,2,6-8] The present study analysed the clinico-pathological features in 30 cases of OJC in a South African population. The average age at diagnosis of patients with OJC was 30.9 years. Previous studies on OJC have shown a predilection for patients in their third and fourth decades.[1,8] The male to female ratio in the current study was 2.3:1, a very near distribution to the 2.26:1 male to female ratio reported in data pooled from all OJC cases reported in the English language literature.[1,3,5,7,8,24] Further, in keeping with previous studies,[8,22] the mandible was found to be more commonly affected than the maxilla (80% versus 20%). When comparing the frequencies of occurrence of follicular versus extrafollicular OJC in the upper and lower jaw no significant differences were found (P=0.18).

Thirteen cysts (44.8%) were found to be associated with an impacted tooth. This frequency is relatively lower than the 60.8% average described in the literature for the follicular type of OJC.[8] It is possible that this discrepancy may be the result of a relatively small sample size in our study. Of interest, nevertheless, is the observation that 11/13 follicular OJCs occurred in association with an impacted mandibular third molar while the case in the maxilla occurred in relation to an impacted canine. Since these teeth are among those most frequently associated

with the development of dentigerous cysts, this finding may support the hypothesis that the follicular OJC most likely represents a dentigerous (follicular) cyst in which the cyst lining has undergone squamous metaplasia with transformation to a robust orthokeratinised lining that bears histological resemblance to epidermoid cysts of other sites.[9,10] The stimulus for metaplasia in the context of the OJC, however, remains unknown.

On light microscopy, all the cysts (follicular and extrafollicular OJC) were lined by hyperkeratinised stratified squamous epithelium, which was of the epidermoid type (anucleated cells in the stratum corneum) in 29/30 (96.7%) cases. There was no significant difference in the histological features of the cyst linings between follicular and extrafollicular OJCs (P=0.67). None of the cases showed a palisaded basal cell layer, which is a characteristic histological feature of the OKC.[64] Sebaceous cells were noted in an extrafollicular OJC and epithelial plaques were identified in a follicular OJC associated with an impacted mandibular premolar tooth. While sebaceous cells are common to cutaneous tissue and may also be seen in normal oral mucosa, to the best of our knowledge, epithelial plaques have not yet been described in non-odontogenic epithelia. These epithelial plaques are furthermore a histological feature associated with odontogenic cysts derived from reduced enamel epithelium.[64]

The OJC shows some histological features that are reminiscent of HPV-induced histomorphological changes seen in cutaneous and mucosal squamous epithelia. HPV has been demonstrated in intraosseous and peripheral odontogenic lesions with the ameloblastoma being most frequently investigated for the presence of HPV. Of the eight studies that have thus far been undertaken on HPV in ameloblastoma,[14,16,17,48,49,53-55] HPV was detected in seven

studies with various methodologies and frequencies reported across these studies.[14,16,17,48,49,53,55] By consensus PCR, HPV was detected in 40% to 44.4% of ameloblastomas,[14,53] and by nested PCR and ISH in 33.3% of ameloblastomas.[55] Correnti *et al.*[55] proposed that for HPV to replicate it requires differentiating epithelial cells to complete its life cycle. It was suggested by these authors that the source of this differentiating epithelium would have been provided for either by the lining of an odontogenic cyst or enamel organ residues.[55] In the odontogenic cyst category, OKC and radicular cysts were investigated for the presence of HPV. Of the 84 OKCs tested thus far,[18,56] HPV viral integration was demonstrated in one case using Southern blot hybridisation.[18] All twenty cases of radicular cysts were HPV negative in the study by Rider *et al.*[57] These findings correlate well with the lack of HPV associated histomorphological changes in these lesions. By contrast, the OJC shares overlapping histomorphological features with lesions that have shown an HPV association. These lesions include cystic papillomas,[11,19] flat warts,[20,38] a subset of cholesteatomas,[10,62,63] and epidermoid cysts.[9-13,60,61] The OJC was investigated for the presence of HPV DNA for the first time in this study. Hypergranulosis was noted in 63.3% of cases while cyst linings showing a verruciform pattern of hyperkeratosis were present in 43.3% of cases. Two cases also showed the presence of vacuolated keratinoctyes with marginal sickle-shaped nuclei. The latter feature was described by Gross *et al.*[20] as an HPV-2 cytopathic effect in cutaneous verruca vulgaris.

All 30 cases in this study were subjected to HPV-DNA PCR using consensus HPV GP5+ and GP6+ primers. The PCR products obtained from the positive controls in the current study were strongly amplified with the GP5+ and GP6+ primers, however, all 30 OJC samples were negative for HPV DNA. Previous studies have shown that GP5+/6+ primers are highly

sensitive, especially in low viral load samples.[43] GP5+/6+ primer sets are also used more frequently with FFPE tissue since the fixation process tends to cause fragmentation of the DNA sequences.[43,44] The GP5+/6+ primer sets are well suited for this purpose as they target these short DNA sequences in FFPE samples thereby resulting in increased sensitivity.[44] The GP5+/GP6+ primers are further capable of amplifying 20 HPV genotypes (HPV-6, -11, -13, -16, -18, -30, -31, 32,-33,-35, -39, -40, -43, -45, -51, -54, -55, -56, -59 and -66),[65] which includes five of the six HPV genotypes that have thus far been identified in odontogenic epithelial tumours.[14,16-18,53,55] The absence of HPV in the 30 cases studied makes it unlikely that HPV plays a role in the histogenesis of the OJC, despite the wart-like morphology that may be noted in some cases. Similarly, HPV has been suggested as a possible causative agent in benign verrucous acanthomas and non-genital seborrheic keratosis based on their morphological overlap with verruca, but despite extensive research HPV involvement has not yet been detected in these lesions.[66,67] Future studies using larger samples of cases and primers covering a larger spectrum of HPV types are required to clarify whether the same applies to the OJC.

While HPV may play no role in the development of the OJC, it is tempting to speculate whether those cysts that exhibited a verruciform pattern of hyperkeratosis in this study fall under the category of cysts termed as "verrucous odontogenic cysts". There are three reported examples in the literature of these keratinising odontogenic cysts with a verrucous pattern of the cyst lining.[33,58,68] Two of these cases were extrafollicular and involved the third molar region of the mandible,[58,68] while one occurred in association with an unerupted maxillary canine.[33] In the latter case the cyst lining comprised a thin layer of epithelium resembling reduced enamel epithelium, whilst in other areas the cyst lining showed verrucous projections

into the lumen with areas of hypergranulosis and some cells resembling koilocytes raising the possibility of a viral aetiology.[33] The possibility of HPV involvement was, however, not supported in their case by immunohistochemical and PCR amplification for HPV DNA.[33] Aldred *et al.*[33] have suggested that this verrucous change in odontogenic cysts may represent an unusual secondary change in a pre-existing cyst.

In the verrucous odontogenic cyst reported by Argyris *et al.*[58] the cyst was lined by hyperorthokeratinising and partially verrucoid stratified squamous epithelium, a prominent granular cell layer, multiple sharp and blunt rounded epithelial projections. Argyris *et al.*[58] also recorded epithelial dysplasia focally. The authors further noted numerous keratinocytes within the superficial epithelial layers showing round, pyknotic nuclei surrounded by a clear halo or vacuolated cytoplasm resembling koilocytes phenotypically.[58] The presence of transcriptionally active HPV infection was investigated by HPV DNA PCR and the tissue was also subject to p16 immunohistochemistry.[58] Areas of nuclear and cytoplasmic immunopositivity were observed in the cyst epithelium.[58] The PCR assay, however, failed to identify HPV-DNA in both incisional and excisional biopsy thereby ruling out HPV infection.[58] There is no data on the HPV status in the case reported by Ueeck *et al.*[68] because technical limitations impeded further investigation.

Two of the reported verrucous odontogenic cysts were treated by enucleation with no signs of recurrence,[33,58] while the third case required segmental mandibulectomy following recurrence of the lesion 7-months after enucleation.[68] We are unable to make comparisons regarding the biologic behaviour of these lesions, since the lack of clinical follow-up and incomplete data on the therapeutic approach are limitations of this study. Notwithstanding these limitations, the

current study findings suggest that HPV infection is not a pathogenetic factor for the OJC. The results further support the findings by Aldred *et al.*[33] and Argyris *et al.*[58] which suggested that HPV is probably not responsible for the verrucous histological changes that may be encountered in some cysts of the jaws.

CHAPTER 7

7.0. CONCLUSIONS

- The average age at diagnosis of patients with OJC was 30.9 years.
- OJC had a male predominance with a male to female ratio of 2.3:1.
- The mandible was more commonly affected than the maxilla, with mandibular involvement seen in 80% of cases.
- 44.8% of OJCs were found to be associated with an impacted tooth.
- There was no significant difference in the histological features of the cyst linings between follicular and extrafollicular OJCs.
- Vacuolated keratinocytes with perinuclear clearing were identified in 66.7% of cases, while 63.3% of cases showed a verruciform pattern of hyperkeratosis suggesting a possible human papilloma virus (HPV) aetiology.
- PCR amplification using consensus GP5+/6+ primer sets for HPV DNA was negative in all 30 OJC analysed in this study.
- The study findings suggest that OJC is not associated with HPV DNA even though some of these lesions may histologically mimic the architecture of verruca.
- HPV infection does not play a role in OJC.

CHAPTER 8

8.0 REFERENCES

1. Vuhahula E, Nikai H, Ijuhin N, *et al.* Jaw cysts with orthokeratinization: analysis of 12 cases. J Oral Pathol Med 1993; 22: 35-40.

2. Barnes L, Everson JW, Reichart P, *et al*. World Health Organization classification of tumours: pathology and genetics of head and neck tumours. Lyon: International Agency for Research on Cancer, 2005.

3. Crowley TE, Kaugars GE, Gunsolley JC. Odontogenic keratocysts: a clinical and histologic comparison of the parakeratin and orthokeratin variants. J Oral Maxillofac Surg 1992; 50: 22-6.

4. Cohen MA, Shear M. Histological comparison of parakeratinised and orthokeratinised primordial cysts (keratocysts). J Den Assoc S Afr. 1980; 35: 161-5.

5. Wright JM. The odontogenic keratocyst: orthokeratinized variant. Oral Surg 1981; 51: 609-18.

6. da Silva MJ, de Sousa SOM, Correa L, *et al*. Immunohistochemical study of the orthokeratinized odontogenic cyst: A comparison with the odontogenic keratocyst. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2002; 94: 732-7.

7. Siar CH, Ng KH. Orthokeratinized odontogenic keratocysts in Malaysians. Br J Oral Maxillofac Surg 1988; 26: 215-20.

8. Dong Q, Pan S, Sun LS, *et al*. Orthokeratinized odontogenic cyst: a clinicopathologic study of 61 cases. Arch Pathol Lab Med 2010; 134: 271-5.

9. Tomasini C, Aloi F, Pippione M. Papillomavirus-infected epidermoid cysts. J Cutan Pathol 1994; 21: 94.

10. Ferekidis E, Nikolopoulos TP, Yiotakis J, *et al.* Correlation of clinical and surgical findings to histological features (koilocytosis, papillary hyperplasia) suggesting papillomavirus involvement in the pathogenesis of cholesteatoma. Med Sci Monit 2006; 12: CR368-71.

11. Egawa K, Inaba Y, Ono T, *et al.* 'Cystic papilloma' in humans? Demonstration of human papillomavirus in plantar epidermoid cysts. Arch Dermatol 1990; 126: 1599-603.

12. Egawa K, Egawa N, Honda Y. Human papillomavirus-associated plantar epidermoid cyst related to epidermoid metaplasia of the eccrine duct epithelium: a combined histological, immunohistochemical, DNA-DNA in situ hybridization and three-dimensional reconstruction analysis. Br J Dermatol 2005; 152: 961-7.

13. Lee S, Lee W, Chung S, *et al*. Detection of human papillomavirus 60 in epidermal cysts of nonpalmoplantar location. Am J Dermatopathol 2003; 25: 243-7.

14. Sand L, Jalouli J, Larsson PA, *et al*. Presence of human papilloma viruses in intraosseous ameloblastoma. J Oral Maxillofac Surg 2000; 58: 1129-34; discussion 1135-6.

15. Gravitt PE. The known unknowns of HPV natural history. J Clin Invest 2011; 121: 4593-9.

16. Kahn MA. Demonstration of human papillomavirus DNA in a peripheral ameloblastoma by in situ hybridization. Hum Pathol 1992; 23: 188-91.

17. van Heerden WF, van Rensburg EJ, Raubenheimer EJ, *et al*. Detection of human papillomavirus DNA in an ameloblastoma using the in situ hybridization technique. J Oral Pathol Med 1993; 22: 109-12.

18. Cox M, Eveson J, Scully C. Human papillomavirus type 16 DNA in an odontogenic keratocyst. J Oral Pathol Med 1991; 20: 143-5.

19. Meyer LM, Tyring SK, Little WP. Verrucous cyst. Arch Dermatol 1991; 127: 1810-2.

20. Gross G, Pfister H, Hagedorn M, *et al*. Correlation between human papillomavirus (HPV) type and histology of warts. J Invest Dermatol 1982; 78: 160-4.

21. Egawa K, Inaba Y, Honda Y, *et al*. 'Cystic papilloma': human papillomavirus in a palmar epidermoid cyst. Arch Dermatol 1992; 128: 1658-9.

22. Damm DD, Bouquot JE, Neville BW, *et al*. Oral and Maxillofacial Pathology. 2nd edition. Philadelphia, PA; Saunders. 2002: 590-7.

23. MacDonald-Jankowski DS, Li TK. Orthokeratinized odontogenic cyst in a Hong Kong community: the clinical and radiological features. Dentomaxillofac Radiol 2010; 39: 240-5.

24. Li TJ, Kitano M, Chen XM, *et al*. Orthokeratinized odontogenic cyst: a clinicopathological and immunocytochemical study of 15 cases. Histopathology 1998; 32: 242-51.

25. Deyhimi P, Hashemzade Z. Comparative study of TGF-alpha and P53 markers' expression in odontogenic keratocyst and orthokeratinaized odontogenic cyst. Dent Res J (Isfahan) 2012; 9: S39-44.

26. Thosaporn W, Iamaroon A, Pongsiriwet S, *et al*. A comparative study of epithelial cell proliferation between the odontogenic keratocyst, orthokeratinized odontogenic cyst, dentigerous cyst, and ameloblastoma. Oral Dis 2004; 10: 22–6.

27. Schultz L. Cysts of the maxillae and mandible. J Am Dent Assoc 1927; 14: 1395-402.

28. Gani F, Mahomed F, Meer S. Evaluation of Ki-67 and cyclin D1 expression in odontogenic keratocysts and orthokeratinised jaw cysts. Sadj; 67: 370-3.

29. Baghaei F, Eslami M, Sadri D. Evaluation of Ki-67 antigen and protein P53 expression in orthokeratinized and parakeratinized odontogenic keratocyst. J Dentistry (Iran). 2004; 1: 53-8.

30. Aragaki T, Michi Y, Katsube K, *et al.* Comprehensive keratin profiling reveals different histopathogenesis of keratocystic odontogenic tumor and orthokeratinized odontogenic cyst. Hum Pathol 2010; 41: 1718-25.

31. Pavelic B, Levanat S, Crnic I. *et al.* PTCH gene altered in dentigerous cysts. J Oral Pathol Med 2001; 30: 569-76.

32. Gadbail AR, Hande A, Chaudhary M, *et al.* Tumor angiogenesis in keratocystic odontogenic tumor assessed by using CD-105 antigen. J Oral Pathol Med 2011; 40: 263-9.

33. Aldred MJ, Talacko AA, Allan PG, *et al*. Odontogenic cyst with verrucous proliferation. J Oral Pathol Med 2002; 31: 500-3.

34. zur Hausen H, de Villiers EM. Human papillomaviruses. Annu Rev Microbiol 1994; 48: 427-47.

35. Doorbar J, Quint W, Banks L, *et al.* The biology and life-cycle of human papillomaviruses. Vaccine; 30 Suppl 5: F55-70.

36. Kumaraswamy KL, Vidhya M. Human papilloma virus and oral infections: an update. J Cancer Res Ther 2011; 7: 120-7.

37. Castro TP, Bussoloti Filho I. Prevalence of human papillomavirus (HPV) in oral cavity and oropharynx. Braz J Otorhinolaryngol 2006; 72: 272-82.

38. Mitsuishi T, Ohsawa I, Kato T, *et al*. Molecular cloning and characterisation of a novel type of human papillomavirus 160 isolated from a flat wart of an immunocompetent patient. PLoS One 2013; 8: e79592.

39. Schiller JT, Day PM, Kines RC. Current understanding of the mechanism of HPV infection. Gynecol Oncol 2010; 118: S12-7.

40. Molijn A, Kleter B, Quint W, *et al*. Molecular diagnosis of human papillomavirus (HPV) infections. J Clin Virol 2005; 32 Suppl 1: S43-51.

41. Mirghani H, Amen F, Moreau F, *et al.* Human papilloma virus testing in oropharyngeal squamous cell carcinoma: what the clinician should know. Oral Oncol 2014; 50: 1-9.

42. Hubbard RA. Human papillomavirus testing methods. Arch Pathol Lab Med 2003; 127: 940-5.

43. Baumforth KRN, Nelson PN, Digby JE, *et al*. The polymerase chain reaction. J Clin Pathol: Mol Pathol 1999; 52: 1-10.

44. Remmerbach TW, Brinckmann UG, Hemprich A, *et al*. PCR detection of human papillomavirus of the mucosa: comparison between MY09/11 and GP5+/6+ primer sets. J Clin Virol 2004; 30: 302-8.

45. Specht K, Richter T, Muller U, *et al*. Quantitative gene expression analysis in microdissected archival formalin-fixed and paraffin-embedded tumor tissue. Am J Pathol 2001; 158: 419-29.

46. Singhi AD, Westra WH. Comparison of human papillomavirus in situ hybridization and p16 immunohistochemistry in the detection of human papillomavirus-associated head and neck cancer based on a prospective clinical experience. Cancer 2010; 116: 2166-73.

47. zur Hausen H. Papillomaviruses and cancer: from basic studies to clinical application. Nat Rev Cancer 2002; 2: 342-50.

48. Kahn MA. Ameloblastoma in young persons: a clinicopathologic analysis and etiologic investigation. Oral Surg Oral Med Oral Pathol 1989; 67: 706-15.

49. Ayoub MS, Elmalahy MH, Elshafei MM. Expression of human papilloma virus and Epstein Barr virus in benign and malignant ameloblastoma. International journal of academic research 2011; 3: 393-7.

50. Terai M, Hashimoto K, Yoda K, *et al*. High prevalence of human papillomaviruses in the normal oral cavity of adults. Oral Microbiol Immunol 1999; 14: 201-5.

51. Sand L, Jalouli J, Larsson PA, *et al*. Human papilloma viruses in oral lesions. Anticancer Res 2000; 20: 1183-8.

52. Boy S, Van Rensburg EJ, Engelbrecht S, *et al*. HPV detection in primary intra-oral squamous cell carcinomas--commensal, aetiological agent or contamination? J Oral Pathol Med 2006; 35: 86-90.

53. Namin AK, Azad TM, Eslami B, *et al*. A study of the relationship between ameloblastoma and human papilloma virus. J Oral Maxillofac Surg 2003; 61: 467-70.

54. Migaldi M, Pecorari M, Rossi G, *et al*. Does HPV play a role in the etiopathogenesis of ameloblastoma? An immunohistochemical, in situ hybridization and polymerase chain reaction study of 18 cases using laser capture microdissection. Mod Pathol 2005; 18: 283-9.

55. Correnti M, Rossi M, Avila M, *et al*. Human papillomavirus in ameloblastoma. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2010; 110: e20-4.

56. González-moles MA, Mosqueda-Taylor A, Delgado-Rodríguez M, *et al.* Analysis of p53 protein by PAb240, Ki-67 expression and human papillomavirus DNA detection in different types of odontogenic keratocysts. Anticancer Research 2006; 26: 175-82.

57. Rider CA, Rupkalvis R, Miller AS, *et al*. Search for evidence of three viral agents in radicular (periapical) cysts with immunohistochemistry. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1995; 80: 87-91.

58. Argyris PP, Nelson AC, Koutlas IG. Keratinizing odontogenic cyst with verrucous pattern featuring negative human papillomavirus status by polymerase chain reaction. Oral Surg Oral Med Oral Pathol Oral Radiol 2014; [Epub ahead of print].

59. Chen SL, Tsao YP, Lee JW, *et al*. Characterization and analysis of human papillomaviruses of skin warts. Arch Dermatol Res 1993; 285: 460-5.

60. Egawa K, Honda Y, Inaba Y, *et al*. Detection of human papillomaviruses and eccrine ducts in palmoplantar epidermoid cysts. Br J Dermatol 1995; 132: 533-42.

61. Jeon J, Lee HS, Ahn HH, *et al*. Human papillomavirus 60-positive epidermal cyst and wart at a nonpalmoplantar location. Br J Dermatol 2005; 152: 1361-2.

62. Bergmann K, Hoppe F, He Y, *et al.* Human-papillomavirus DNA in cholesteatomas. Int J Cancer 1994; 59: 463-6.

63. Chao WY, Chang SJ, Jin YT. Detection of human papillomavirus in cholesteatomas. Eur Arch Otorhinolaryngol 2000; 257: 120-3.

64. Shear M, Speight PM. Cysts of the oral and maxillofacial regions. 4th edition. Oxford, UK; Blackwell Munksgaard. 2007: 45-58.

65. de Roda Husman AM, Walboomers JM, van den Brule AJ, *et al.* The use of general primers GP5 and GP6 elongated at their 3' ends with adjacent highly conserved sequences improves human papillomavirus detection by PCR. J Gen Virol 1995; 76: 1057-62.

66. Zhu WY, Leonardi C, Kinsey W, *et al.* Irritated seborrheic keratoses and benign verrucous acanthomas do not contain papillomavirus DNA. J Cutan Pathol 1991; 18: 449-52.

67. Lee ES, Whang MR, Kang WH. Absence of human papillomavirus DNA in nongenital seborrheic keratosis. J Korean Med Sci 2001; 16: 619-22.

68. Ueeck BA, Woo BM, Stewart JC, *et al.* Keratinizing odontogenic cyst with verrucous proliferation. J Oral Maxillofac Surg 2007; 65: 585-8.

CHAPTER 9

9.0 APPENDICES

9.1 Ethics clearance form

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Dr Kalpesh Lalla

CLEARANCE CERTIFICATE	M120956
PROJECT	Does Human Papilloma Virus Play A Role in the Histogenesis
INVESTIGATORS	Dr Kalpesh Lalla.
DEPARTMENT	School of Oral Health Sciences/Oral Pathology
DATE CONSIDERED	28/09/2012
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

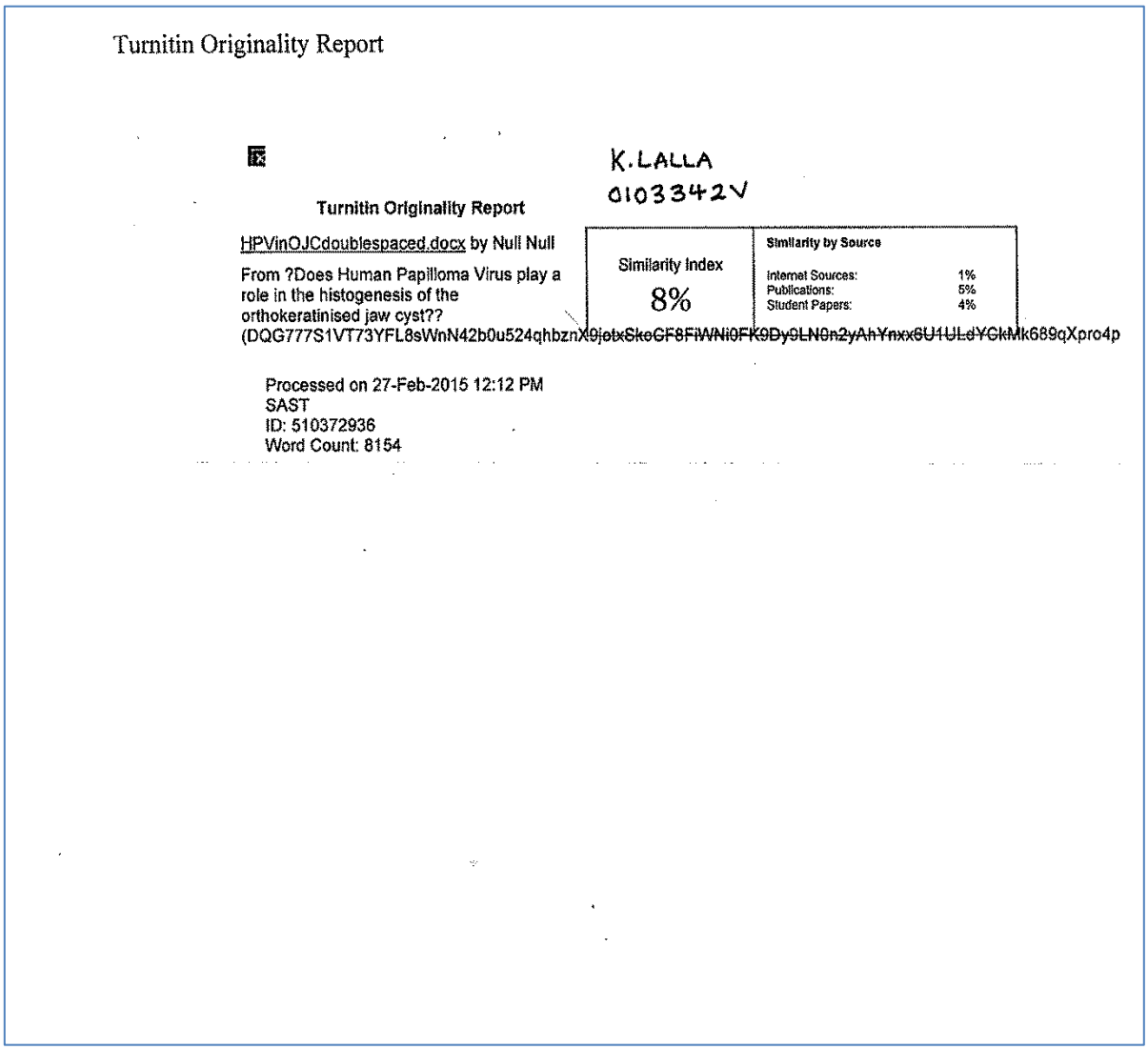
DATE 28/09/2012 CHAIRPERSON .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor : Dr Farzana Mohamed

DECLARATION OF INVESTIGATOR(S)
To be completed in duplicate and ONE COPY returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. I agree to a completion of a yearly progress report.

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...

9.2 Turnitin



Turnitin Originality Report

K.LALLA
0103342V

Turnitin Originality Report

HPVinOJCdoublespaced.docx by Null Null

From ?Does Human Papilloma Virus play a role in the histogenesis of the orthokeratinised jaw cyst??
(DQG777S1VT73YFL8sWnN42b0u524qhbznX9jotxSkoCF8FiWNi0FK9Dy9LN0n2yAhYnxx6U1ULdYCkMk689qXpro4p

Similarity Index	Similarity by Source	
8%	Internet Sources:	1%
	Publications:	5%
	Student Papers:	4%

Processed on 27-Feb-2015 12:12 PM
SAST
ID: 510372936
Word Count: 8154