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VIRUSES AND CANCER

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ABSTRACT
Viruses are ubiquitous and are also the pathogenic agents that are most commonly associated with neoplastic transformation of cells of several organs in human beings – thereby causing cancer of epithelial cells (carcinomas) or cancer of mesenchymal cells (leukemias, lymphomas and sarcomas) depending on the type and location of the infected host cell. This review highlights the six major groups of viruses that have established aetiological association with cancer in human populations. The epidemiology and the processes through which these pathogens cause malignant transformation of the infected host cells are discussed – with particular emphasis on the evolving and changing natures of the diseases as they parallel changes in human behaviours. Also discussed is a brief overview of the current understanding of molecular pathology as they emerge with the advent of new technological capabilities for studying these processes at subcellular (genomic) levels.

INTRODUCTION

Viruses have notoriety for causing acute epidemic infections that may result in pandemics and global crisis within a short period of time. But also of an ever increasing need for attention due to the public health risk they constitute, are endemic diseases of viral origin and the associated malignancies. Viruses cause chronic infections that are endemic in many communities and such chronic infections have been shown to have aetiological associations with certain malignant disease processes that are also of endemic proportions in the affected communities. Considering the ubiquity of viruses and the high prevalence of viral infections world-wide, viruses are probably second only to tobacco abuse as the most common risk factor for developing cancer. In some cases, the development of cancer is directly related to the activities of the virus. In other cases, the virus remains dormant in host cells (carrier state) until factors such as immunosuppression and/or infection with other oncogenic viruses activates the oncogenic potentials of the virus in the carrier. This review highlights the interplay between environmental factors, host factors and viral factors that influence the oncogenic transformation of virus-infected host cells.

1. EPSTEIN BARR VIRUS

1:1 Epstein Barr Virus (EBV) Infection and The Immune System.

Epstein Barr Virus (EBV) is a gamma herpesvirus that was first discovered in 1964 in SubSahara Africa by Epstein, Achong and Barr when they observed and reported the presence of herpes-virus-like particles in electron micrographs of cultured Burkitt lymphoma (Burkitt's Lymphoma) cells (1). Shortly thereafter, higher titers of antibody to EBV antigens were reported in Burkitt's Lymphoma patients (2), infectious mononucleosis and undifferentiated nasopharyngeal cancer (3,4). Since then, EBV has been shown to be an ubiquitous virus and the putative agent for an array of neoplastic lymphoproliferative disorders that include Hodgkin's disease, non-Hodgkin's B-cell lymphomas, immunosuppression-associated lymphomas, some T-cell lymphomas; and a subset of gastric adenocarcinoma. All these tumours have been shown to contain EBV genome and EBV-coded latent genes which play active roles in the malignant transformation of infected cells.

EBV is a linear double-stranded DNA gamma-herpesvirus with global presence. It infects about 95% of adult populations in every part of the world and has a life-long persistence in infected individuals. The primary infection may be asymptomatic and occur early in life or it may occur later during pubertal years or adulthood as infectious mononucleosis (5,6). Although the virus can infect epithelial cells, T-lymphocytes and B-lymphocytes, the latter are the preferred target cells (7). EBV is orally transmitted and the oropharynx is the primary site of infection and also the site for virus replication; hence, the spread of the virus through the saliva of infected persons (8,9). During the primary infection of B-cells, there is an initial short-lived but critical

burst of lytic viral replication which enhances spread of the virus to other cells and culminates in transformation of the host cells and establishment of non-productive latent infection (7). In the latency state, instead of replicating in the infected cells, the virus produces a variety of latent genes and proteins that mediate adaption of the virus to the host cells physiology to ensure survival of the virus, persistence of infection and avoidance of host immune surveillance mechanisms.

Inhibition or removal of the immune T-cells in *in-vitro* systems results in spontaneous transformation of resting EBV-infected B-cells into indefinitely proliferating lymphoblastoid cell lines (LCLs) that express high levels of B-cell activation markers (CD23, CD30, CD39 and CD70) (10); and contain multiple copies of the viral episome; nine latent proteins comprised of six EBV nuclear antigens (EBNAs) and three latent membrane proteins (LMPs). The nuclear antigens consist of EBNAs 1, 2, 3A, 3B, 3C and LP; and the LMPs include LMP1, 2A and 2B (11). EBERs 1 and 2 are small non-polyadenylated (non-coding) EBV RNAs that are also abundantly expressed in latently infected cells. Although they are not essential for transformation of EBV-infected cells into immortalized LCLs (6); they are useful as markers of latent EBV infections (5). Also identified in latently infected cells and also in EBV-associated tumours, are the BARTs (Bamh1 A rightward transcripts). These are believed to play some role in enhancing virus persistency through yet to be defined mechanism(s) (12). It appears viral persistence in host cells is enhanced and ensured through a host of mechanisms including limited expression of viral antigens during latency (> 80 antigens expressed during lytic replication compared with 9 in latency), reduced copy member of the expressed viral antigens, production of virus encoded RNAs (EBERs) which may protect EBV-infected cells from apoptosis, and use of putative latency program (latency 0) in which no detectable latency gene is expressed in the infected cells – thus rendering such infected cells invisible to the host's immune surveillance machinery (13).

Under normal circumstances, latent EBV infection is kept in check and precluded from actively spreading to infect and immortalize other host cells by a combination of T and B cell responses to the detectable EBV latent antigens. Abrogation or suppression of these host immune responses may tilt the balance in favor of active viral replication and so facilitate the development of EBV associated malignancies. Such situations may be iatrogenic immunosuppression as in transplant patients or due to co-infection by other pathogens such as Human Immunodedeficiency Virus (HIV) and *Plasmodium falciparum*, or local immune suppression resulting from changes in the tissue microenvironment (13).

1:2 EBV and Burkitt's Lymphomas

The discovery of EBV resulted from a striking observation that Burkitt's lymphoma was endemic in the SubSahara African region that was also holoendemic for *Plasmodium falciparum* infection (1). In that region, Burkitt's Lymphoma is 50 times more common compared with other regions with sporadic cases of the tumour. Also, in endemic Burkitt's Lymphoma, EBV positivity in tumour cells is 100% compared to only 15-20% in sporadic cases (11,14). Burkitt's lymphoma is also common in HIV-infected patients – about 40% of AIDS-related Burkitt's lymphoma has been associated with EBV (15).

A characteristic chromosomal abnormality consistently present in all cases of Burkitt's Lymphoma is translocation of the region of *c-myc* proto-oncogene from the long arm of chromosome 8 (8q24) to the region of the immunoglobulin heavy chain gene on chromosome 14; or less commonly to the regions of the immunoglobulin light chain genes on chromosomes 2 or 22. This results in dysregulation of the *c-myc* oncogene with consequent activation of pathways that favor malignant proliferation of the infected cells. (16,17). EBNA1 which is the only EBV protein consistently detected in Burkitt's Lymphoma interact with the dysregulated *c-myc* to alter B-cell growth and so transforms the cell into a state of continuous proliferation (5).

Malaria infection causes polyclonal activation of B-lymphocytes and general immunosuppression including downregulation of EBV specific cellular T lymphocyte (CTL) response. These result in up to five-fold increase in the number of circulating EBV-infected B-cells during acute malaria infection. (18). In children with Burkitt's Lymphoma, CTL response against EBNA1 is significantly decreased while CTL responses against other EBV antigens are unaffected (19)

1:3 EBV and Hodgkin's Lymphoma

Hodgkin's lymphoma is the most common EBV-associated lymphoma in the western world with

18-50% EBV positivity rate in Hodgkin's lymphomas (20-22). In developing countries, the frequency of EBV associated Hodgkin's lymphoma is (much) higher and may be as high as 100% with EBV RNA and/or antigens being detected in virtually all cases in some published series, depending on the sensitivity of the assay employed (23,24).

EBV having a possible casual role in the pathogenesis of Hodgkin's lymphoma was deduced from the observation of two to three fold increased risk for developing Hodgkin's lymphoma by patients with history of infectious mononucleosis (25). The first concrete evidence that showed EBV as a causative agent for Hodgkin's lymphomas was the detection of higher EBV antibodies titers in Hodgkin's lymphoma patients compared with other lymphoma patients (26), and observations that elevation of EBV antibodies titers in Hodgkin's lymphoma patients occurred several years before the development of the tumour (27).

The presence of EBV DNA in Hodgkin's lymphoma tissue samples was first demonstrated in 1987 with the use of in-situ hybridization probe. Since then, several studies have confirmed the presence of EBV DNA, RNAs and antigens in tissue samples of Hodgkin's lymphoma – using improved in-situ hybridization and immunohistochemical methods (5). Type 1 EBV is the usually detected "variant" but type 2 virus has also been reported in Hodgkin's lymphoma that develops in clinical settings of immunodeficiency. Transcripts analysis and hybridization studies have also shown clonality for EBV in Hodgkin's lymphoma tissues samples – thus confirming those tumours resulted from clonal expansion of single EBV–infected cells (28). EBV is usually associated with the mixed cellularity histopathologic form of Hodgkin's lymphoma and is more common in males, and also in pediatric patients and older patients (>55 years of age) (29,30).

Immunohistochemical studies and transcriptional analysis of Hodgkin's lymphoma tissue samples have shown EBV infected Hodgkin's lymphoma cells (Reed-Sternberg's cells) contain high levels of the EBV latent antigen LMP1 in the absence of EBNA2 (31-33). In the Hodgkin's lymphoma cells, LMP1 causes nuclear activation of NF-Kappa B, decrease the cell's susceptibility to apoptosis, and upregulate the production of immunosuppressive cytokines like IL-10, IL-13 and TAF-B (6,34-36). Thus EBVs LMP1 latent antigen appears to stimulate and enhance the clonal expansion of Reed-Sternberg's cells by dysregulating the cell-cycle/apoptosis mechanisms and also by activating the cells to produce substances that have immunosuppressive effects on the microenvironment – thereby allowing the cells to escape the host's immune mechanisms (13).

1:4 EBV and Immunosuppression – Related Lymphoproliferative Diseases

EBV is also implicated as the causative agent in a number of lymphoproliferative disorders that occur in the clinical settings of immunosuppression. These include: post-transplant lymphoproliferative disorders (PTLDs), inherited immunodeficiency associated lymphoproliferative syndromes and HIV infection – related lymphomas. In these disease conditions, immunosuppressions results in unabated chronic antigenic stimulation (in the transplanted organ and also in the host patient's own tissue), enhance the activation and transformation of cells with latent EBV infection and allows the virus to escape from EBV-specific immune control mechanisms. These events combined in due course culminate in aggressive and uncontrolled proliferation of transformed EBV-infected cells.

EBV-associated HIV-related nonHodgkin's lymphomas include primary CNS lymphoma, Human herpes virus 8 (HHV8)-positive primary effusion lymphoma and it's solid variant, and plasmablastic lymphoma. Other EBV-associated lymphomas that may also occur in HIV-infected patients are diffuse large cell lymphomas with immunoblastic features and Burkitt's lymphoma (37). About 40% of AIDS associated Burkitt lymphomas are positive for EBV (15). In PTLDs and HIV-associated diffuse large cell lymphomas, majority of the cases are EBV-positive and expresses all the latent antigens (latency III) (6).

Primary effusion lymphoma (PEL) is a tumour that occurs frequently in HIV-infected patients and is very rare in immunocompetent individuals. These tumours appear to develop as a result of co-infection by EBV and Kaposi's sarcoma herpes virus - HHV8 (also a gamma herpes virus) (20). Both PEL and EBV positive HIV-related Burkitt's lymphoma express EBNA1 as the only EBV protein and both are also characterized by upregulation of *c-myc* oncogene

to stimulate cell proliferation and increase resistance to apoptosis (38-40).

1:5 Nasopharyngeal Carcinoma

Undifferentiated nasopharyngeal carcinoma (UNPC; WHO type III) has a consistent global association with EBV. This tumour has a rather high incidence in Southeast Asia particularly in Southeast China and neighboring Hong Kong with a peak incidence of 20-30 cases per 100,000.- and is the eighth leading cause of death in China (41-43). A relationship between EBV and UNPC was first suggested based on presence of high titers of EBV antibodies in UNPC patients. That was followed with in-situ demonstration of EBV DNA and EBNA proteins in the tumour cells (4). Also, EBV genomes in UNPC tumour cells have been shown to be monoclonal, suggesting the tumour results from clonal proliferation of cells with latent EBV-infection (44).

Irrespective of geographic location, UNPC is invariably positive for EBV with a 100% positivity rate (45-47). EBNA1 and EBERs are detected in 100% of cases and LMP1 in up to 65% cases depending on the sensitivity of the assay (48,49). Elevation of IgA titers against EBV antigens may occur several years before development of UNPC. The antibody titer also correlates with tumour burden, remission and recurrence (50,51)). Therefore, EBV-specific serology (titers of IgA antibodies to EBV capsid antigen and early antigens) is now employed as invaluable tool in the management of UNPC for diagnosis and monitoring of response to therapy (52,53).

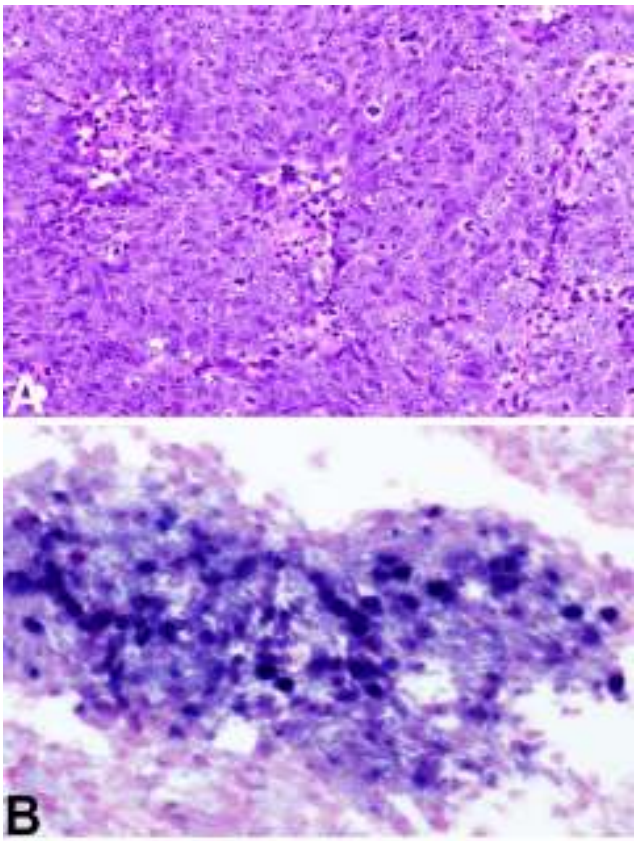

Figure 1

A. Hematoxylin and Eosin-stained tissue section micrograph of EBV-associated nasopharyngeal undifferentiated carcinoma.
B. In-situ hybridization showing presence of EBER in the tumour cells.

In addition to EBV infection, other non-viral factors contribute to the development of UNPC in affected individuals. These include genetic predisposition, consumption of salted fish and meats containing the preservative nitrosamine and a prolonged exposure to domestic wood cooking (for at least 10 years) (43,50). The report of high incidence rates of this tumour in individuals of chinese descent (especially Cantonese males) that are residents in other parts of the world that are not endemic for EBV infection, suggests genetic predisposition as a key underlying factor for the development of EBV-associated nasopharyngeal carcinoma (5).

1:6 EBV and T-cell lymphomas

Although B-cells are the preferred cells for EBV infection, the virus can also infect CD4+ and CD8+ T-cells and Natural Killer (NK) cells (54). A number of EBV-associated T-cell/Natural Killer cells lymphoproliferative disorder have been reported; the most common of these is sinus-nasal T-cell nonHodgkins lymphomas (SNTL). SNTL have been reported in Asia, Europe, North and South America and do not appear to have distinctive geographical or racial distribution (55). These tumours characteristically express NK markers, may lack T-cell antigens and do not express T-cell receptor gene re-arrangements. In *in-vitro* experimental settings, T-cells are refractory to EBV infection; therefore the exact mechanism(s) of how EBV infects T-cells and participate in development of T/NK cells lymphoma is still an enigma (15).

Since most EBV-associated T-cell lymphomas develop in the context of chronic active EBV infection (of B-cells), there are suggestions that EBV-associated T-cell oncogenesis may be a result of proliferating cytotoxic T-cells trying to kill the EBV infected cells. This assertion is supported by the fact that most EBV-associated T-cell lymphomas have cytotoxic phenotype (positive expression of granzyme B and T-cell intracytoplasmic antigen-1 [TIA1]). Type II latency (expression of EBNA1 and LMP 1, 2A and 2B) is characteristic but interestingly, EBV is detected only in a fraction (5-50%) of tumour cells - implying EBV infection may have occurred after development of the tumour (56). EBV-infected B cells with type III latency are frequently detected in T-cell lymphomas and may contribute to the neoplastic T-cells through secretion of tumour-enhancing cytokines or direct stimulatory interactions with the T-cells (6).

1:7 EBV and Gastric Carcinoma

EBV-associated gastric carcinoma is defined by the presence of EBV in the tumour cells – irrespective of the histologic appearance of the tumour. In 1990, Burke et al using PCR-based detection method, reported the presence of EBV genome in a group of gastric carcinoma characterized by a lymphoepithelioma-like histologic appearance (57). Subsequent studies with in-situ hybridization in the 1990s reported the abundant presence of monoclonal EBV (positive EBER signals) in a subset of gastric adenocarcinoma including those that lack the lymphoepithelioma-like histomorphology. The studies also showed the abundant presence of EBER in virtually all tumour cells; and none in surrounding benign epithelial cells (58-61). Also suggesting possible etiological role for EBV in these tumours is the presence of elevated titers of EBV prior to the diagnosis of EBV-associated gastric carcinoma (62). According to pooled (meta-analysis) data from America, Asia and Europe, about 10% of gastric carcinomas are associated with EBV (63).

In-situ hybridization has also shown the virus (EBER signals) to be present in the tumour cells at all stages – from pre-invasive intramucosal to invasive stage. Genomic terminal repeat studies have confirmed the monoclonality of the EBV in the tumour cells and also showed EBV infection in the tumour cells is latent (with no active viral replication); and that the virus exist in the tumour cells in unintegrated (episomal) forms (59,61,64). These data taken together show EBV infection and its role in neoplastic transformation of gastric epithelial cells occur early in the premalignant stage.

Unlike EBV-associated undifferentiated nasopharyngeal carcinoma which is characterized by intense stromal lymphocytic infiltrate, the overwhelming majority (about 80%) of EBV-associated gastric carcinoma has notably less but varying degrees of lymphocytic infiltrate – i.e. akin to what obtains with regular gastric carcinomas. However, irrespective of their histomorphologic appearance, all EBV-associated gastric carcinomas share the following clinicopathologic features: They occur predominantly in males, relatively younger patients and unlike *Helicobacter pylori*-associated carcinomas, tend to be localized to proximal regions (cardia and body) of the stomach. Compared with regular gastric carcinomas, EBV-

associated tumours also tend to have relatively better prognosis with lower rate of lymph node metastasis (65,66).

Exactly how EBV infects gastric mucosa epithelial cells and establishes latency therein is still a matter of speculation. However, in-situ hybridization studies have shown EBV infected epithelial cells in the fundic glands to be the likely nidus of the viral infection that eventually results in malignant transformation of the epithelial cells. In the infected cells, EBV through its oncoproteins (LMP1 and LMP2A), causes global non-random aberrant (CρG island) methylation of the promoter regions of several oncogenes such as p14, p16, p73, CDH1 and PTEN (57,60,67). Carcinoma with Lymphoepithelima-like histologic appearance has been reported in other organs including liver, oral cavity and breast, but EBV has not been conclusively associated with any of these tumours.

2. CHRONIC VIRAL HEPATITIS AND HEPATOCELLULAR CARCINOMA

2:1 Viral Hepatitis and Hepatocellular Carcinoma

Hepatocellular carcinoma is the fifth most common cancer and the third leading cause of cancer related deaths worldwide (68). The incidence of hepatocellular carcinoma is rising in the western world. In the United States of America, both the incidence of hepatocellular carcinoma and rate of cancer-related death due to hepatocellular carcinoma is on the rise, even though the overall rate of cancer deaths is declining. This paradoxical situation has been attributed to an increase in HBV related hepatocellular carcinoma among immigrants from endemic countries and also partly to an increase in the incidence of hepatitis C infection in the American populace (69) in which up to two thirds of hepatocellular carcinoma are related to chronic HCV infection (70)

Infection with hepatitis B virus (HBV) and hepatitis C virus (HCV) is the number one risk factor for hepatocellular carcinoma worldwide, with these viral infections accounting for over 80% of all hepatocellular carcinoma cases globally (71). The remaining 20% of hepatocellular carcinoma is attributed to other risk factors such as alcohol consumption, aflatoxin B1, and other chronic liver disease such as non-alcoholic fatty liver disease, and inherited metabolic diseases (hemochromatosis and alpha one antitrypsin deficiency) (69).

2:2:1 Hepatitis B Virus and Hepatocellular Carcinoma

The association of hepatocellular carcinoma with chronic hepatitis B infection was first noted in 1970 and confirmed by subsequent studies in the following two decades leading to the established and now undisputed fact that more than 80% of patients with hepatocellular carcinoma reside in high incidence areas (East Asia and Sub-Saharan Africa) where more than 80 % of patients with hepatocellular carcinoma were seropositive hepatitis B surface antigen (HBsAg), compared with 10-15% positive rate in control populations. Also, serologic evidence of active or previous HBV infection evidenced by positive hepatitis B core antigen (serum positivity) has been reported in more than 90% cases of hepatocellular carcinoma (69). A definite aetiological association of HBV with hepatocellular carcinoma was reported in 1981 by Beasley et al (72) following their large prospective cohort study of 22,707 male municipal workers in Taiwan between 1975 and 1980. 3,454 (15.2%) were seropositive for HBsAg with 40 of them dying from hepatocellular carcinoma compared with only one hepatocellular carcinoma -related death among the 19,253 HBsAg seronegative participants. The incidence of hepatocellular carcinoma among the HBsAg carriers was 1,158 per 100,000 compared with 5/100,000 in their seronegative counterparts; and a calculated relative risk of 223 for hepatocellular carcinoma developing in seropositive individuals. A more recent and larger prospective cohort study involving 90,000 participants and including 11,000 HBsAg positive patients followed over a period 8 years, showed that hepatitis B virus infected patients have 18.8 and 33.2 risk for hepatocellular carcinoma in men and women respectively (73). These studies and others have proven unequivocally, the association between hepatitis B virus infection and hepatocellular carcinoma.

Another direct evidence of causative linkage between hepatitis B infection and hepatocellular carcinoma is the relationship between level of serum HBV DNA (marker of HBV replication) and the risk of hepatocellular carcinoma. Several studies have shown that there is a direct relationship between serum levels of HBV DNA and risk of hepatocellular carcinoma; with

elevated baseline serum level of HBV DNA greater than 10,000 copies per mL (approximately 2,000 IU/mL) being a strong predictor of subsequent development of hepatocellular carcinoma. This association has also been shown to be independent of serum Hepatitis B e antigen (HBeAg) status, serum aminotransferases levels, or the presence of cirrhosis (74,75). Serum positivity for HBeAg was previously considered to be a risk factor for development of hepatocellular carcinoma. However, subsequent studies have not shown it to be an independent predictor of development of hepatocellular carcinoma. It is now thought it effects may be dependent on the underlying levels of HBV DNA in the serum as well as the genotype of the HBV involved (74,76). There is a definite association between certain types of HBV genotypes and higher risk and rate of hepatocellular carcinoma. Of the eight known HBV genotypes (A-H), based on genomic sequence divergence, HBV genotype C infected patients are generally believed to have increased risk of developing hepatocellular carcinoma compared with patients infected with other genotypes (75,77). HBV genotype C has also been associated with poorer surgical outcomes and higher hepatocellular carcinoma recurrence rate following surgical resection of the primary tumour. This is probably due to the higher pathogenicity of this genotype – as the patients tend to have more active disease in the residual liver (78). However, a few studies have also shown that in some populations, genotype B or even genotype F may be associated with higher risk for developing hepatocellular carcinoma (79)

2:2:2 Hepatits B Virus and Viral Hepatocarcinogenesis

Hepatitis B virus is a double stranded DNA virus that belongs to the Hepadnavirus family. The virion has a diameter of 42 nanometer (nm) and is composed of an outer envelope formed by hepatitis B surface antigen surrounding an inner nucleocapsid, the hepatitis core antigen, which contains the viral genome and polymerase (68). The process through which HBV causes oncogenic transformation of infected hepatocytes is yet to be fully elucidated. It involves a complex multistep process involving the activation of cellular oncogenes, the inactivation of tumour suppression genes, and dysregulation of multiple signal transduction pathways that are critical to control of cell proliferation and viability, apoptosis, and cellular DNA repair mechanisms.

Although, HBV can replicate on its own without integrating into the HBV DNA, its integration into the host genome does allow for persistence of the viral genome within the host cells (80). It appears HBV causes hepatocellular carcinoma principally through the integration of its DNA into the host genome. Integration of HBV into the genome as evidenced by the presence of HBV DNA in tumour cells has been found in about 80% of HBV related hepatocellular carcinoma. HBV DNA has also been found to be integrated into non hepatocellular carcinoma tissue, but the DNA in individual hepatocellular carcinoma has been shown to be monoclonal in nature, thus supporting the observation that HBV DNA integration is a critical aetiologic factor in those tumours (81). HBV DNA integration into the host genome has been found to occur at both critical and non-critical locations and appears to occur in a random manner with marked variation in the length and the components of the integrant HBV DNA (82). The integration of HBV DNA into the host cell genome results in several mutagenic events that culminate in chromosomal instability and malignant transformation of affected cell (80). Integration of HBV induces a host of genetic alterations within the host genome including chromosomal deletions, translocations and upregulation of genetic mechanisms responsible for maintaining cellular homeostasis and general genomic stability (68).

A number of HBV genes and their products (proteins) have been implicated in HBV-related hepatocarcinogenesis. These include *HBx* gene, *pre-s2/s* gene, and the HBV spliced protein. Of these, *HBx* gene has been shown to be the most important and also the most commonly integrated gene – being present in over 90% of HBV-related cirrhosis and dysplasia; and in 70% of HBV related hepatocellular carcinoma (80).
Integration of the *HBx* gene into host DNA results in transcriptional activation of several cellular genes associated with growth control (83), and promotion of host genetic instability through a variety of mechanisms that include compromise of host cell nucleotide excision repair (DNA repair) mechanisms, inactivation of p53 (a tumour suppressor gene) and disruption of p53-associated DNA repair pathways. *HBx* also transactivates several cytoplasmic signaling pathways in ways that further compromise cellular proliferation and differentiation - with the balance tilted towards enhanced proliferation and decreased apoptosis (68). *HBx* has also been shown to be a factor in promoting TGF-beta mediated fibrosis, thus promoting development

of cirrhosis in HBV infected patients. Animal models studies also provide evidence supporting the role of *HBx* in hepatocarcinogenesis. It has been shown for instance, that woodchucks animal models in which *HBx* gene is present and integrated into the host genome develop hepatocellular carcinoma within 3-4 years following infection; whereas, duck hepatitis B virus infection in which the virus lacks the *HBx* gene never results in the development of cancer in infected ducks (84).

2:2:3 Hepatitis B Viral Hepatocarcinogenesis and Cirrhosis

Although hepatitis B virus may cause hepatocellular carcinoma directly without cirrhosis, it appears that the high propensity of the virus to cause hepatocellular carcinoma is due to the virus's ability to induce cirrhosis in the liver. Between 70-80% of HBV related hepatocellular carcinoma occur in cirrhotic liver - with the risk of cirrhosis being greater in older patients (69,80,85). Cirrhosis irrespective of its aetiology, is known to be associated with increased risk for hepatocellular carcinoma. 80-90% of all hepatocellular carcinomas develop in cirrhotic liver (86). Liver cirrhosis develops in 20-30% of patients after 20-30 years of chronic infection and hepatocellular carcinoma develops at an annual of 1-7% in HCV infected cirrhotic patients and 3-8% in HBV infected cirrhotic patients (68,87,88). This may explain in part, the higher incidence of HBV associated hepatocellular carcinoma in HBV endemic regions (sub-Saharan Africa and East Asia). In those regions, HBV is acquired at birth or early in life (through maternal-infant transmission), with consequent high rate of lifelong chronic infection (that is often unabated) and eventual evolution of the disease into cirrhosis and hepatocellular carcinoma; compared with western populations where hepatitis B infection is usually acquired during adolescence or adulthood (commonly through sexual contact).

Since HBV infection is the major aetiologic risk factor for hepatocellular carcinoma globally, the most effective way of preventing and controlling hepatocellular carcinoma secondary to HBV infection is to prevent HBV infection and so reduce the seroprevalence of HBV in the general population. Use of HBV vaccination (large scale national programs of vaccination against of hepatitis B) in many countries has proven to be an effective way (cost effective and efficient way) of achieving this objective. The resultant decrease in hepatitis B infection and HBV related cirrhosis has resulted in marked reduction in the incidence of childhood HBV related hepatocellular carcinoma and it is expected that this would also later transform to reduction in the incidence and prevalence of HBV related hepatocellular carcinoma as the vaccinated children populations grow into adulthood (69). Also, since high level of serum of HBV DNA has been shown to be associated with increased risk of hepatocellular carcinoma, aggressive and effective use of antiviral therapy of hepatitis B infection is another practicable way of preventing HBV related hepatocellular carcinoma (89).

3. HEPATITIS C VIRUS HEPATOCELLULAR CARCINOMA

HCV is an RNA virus and belongs to the Flaviviridae family. It has an open reading frame that encodes a single polyprotein which is processed into both structural (core and envelope) and non-structural proteins. Patients infected with HCV have 11.5 to 17 fold increased risk for developing hepatocellular carcinoma (90,91).

In the host cells, HCV being an RNA virus is unable to integrate itself into the host genome. Therefore, the viral RNA genome is replicated and packaged into new viral particles in the cytoplasm of host cells through the combined machinery of viral and host proteases. The associated translation of viral protein results in host responses that facilitate oncogenic (malignant) transformation of the host cells. HCV core protein has been shown to be the most important factor in HCV-induced hepatocarcinogenesis (68). The envelope proteins (E1 and E2) appear to have synergistic effects in accelerating tumour development. When treated with Diethylnitrosamine, transgenic mice expressing core protein, E1 and E2 envelope proteins develop tumours faster than mice expressing only core protein (92). This recent finding confirms earlier reports that in transgenic mice, the presence of the complete HCV polyprotein enhances hepatocellular carcinoma development in a more efficient manner compared with the presence of structural proteins alone (93). In the absence of structural proteins, HCV non-structural proteins do not cause any known liver pathology (94).

The exact molecular mechanism through which HCV induces hepatocarcinogenesis is yet to be fully understood. HCV proteins induce

hepatocarcinogenesis through a combination of complex and sometimes overlapping molecular events that ultimately result in suppression of apoptosis, and enhancement of proliferation of the infected hepatocytes. This is done mainly through modulation of p53 and p53 dependent pathways, such as those mediated by p21, p73 and pRb; and results in derangement of cell cycle control mechanisms (68). HCV core protein through Smad3 activation reverses TGF-Beta cell growth-suppressing effect to tumour-promoting responses in the infected cells (95). In addition, by upregulating TGF-Beta, HCV core protein induces fibrogenesis, causing liver cirrhosis and therefore increasing the risk of hepatocellular carcinoma (96).

HCV infection is also associated with steatosis and that has been shown to occur in as high as 70% of patients with chronic HCV infection (97). Steatosis worsens fibrosis and accelerates the development of cirrhosis and is also an independent high risk factor for the development of hepatocellular carcinoma in HCV infected patients (98). HCV induced steatohepatitis and fibrogenesis result from increased oxidative stress and lipid peroxidation. These result in generation of radical oxidative species (ROS) and consequent oxidative DNA damage, increased chromosomal instability, chromosomal aberrations and eventually, oncogenic transformation of the host cell.

Patients with chronic hepatitis infection often have elevated serum iron and increased hepatic iron deposition. Free iron in hepatocytes cytoplasm results in increased oxidative stress and so enhances lipid peroxidation and also facilitates fibrogenesis and development of cirrhosis (99-101).

In summary, chronic HCV infection is associated with three distinct pathologic events that are associated with increased risk of hepatocellular carcinoma, viz: steatosis, hepatocytic accumulation of iron and cirrhosis.

4. HUMAN HERPES VIRUS 8 (KAPOSI'S SARCOMA-ASSOCIATED HERPES VIRUS)

Kaposi's sarcoma (KS), a multicentric tumour of endothelial cells was first described in 1872 by Moritz Kaposi as "idiopathic multiple pigmented sarcoma of skin" that affects elderly Jewish men of Ashkenazi origins and was later named Kaposi's sarcoma (KS) by Koebner in 1895. During the 1940s through 1980, endemic KS was described in parts of Central and East Africa (Uganda, Malawi and Kenya) where it most commonly affects children and presents with disseminated lymphadenopathy, hence the designation lymphadenopathic KS (102,103). In 1969, the first case of KS occurring after an organ transplant was reported (104) and thereafter, it has been established in the literature that iatrogenic KS occurs in immunosuppressed individuals such as organ transplant patients 105). In 1981, report of KS epidemic affecting young homosexual men in the USA was the first indicator of a soon to-be-discovered HIV1 infection pandemic (106-108). As of today, KS is the most common neoplasm in untreated HIV infected patients; and remains the most common AIDS-associated cancer in both developed and developing countries (109). In the western world, it continues to be most prevalent among HIV-infected homosexual men; whereas, in Africa, due to sexual practices that may include multiple sexual partners in heterosexuals and bisexual individuals, HIV-related KS epidemic is common among both male and female genders (110).

The incidence of KS in the general population is 1 in 100,000, 1 in 20 in HIV-infected individuals and was as high as 1 in 3 in HIV-infected homosexual men before the introduction of highly active antiretroviral therapy (HAART) (111,112). Due to lack of adequate or appropriate treatment of HIV infection, about 90% of all KS cases now occur in Sub-Saharan Africa and only about 12% of patients are alive five years after diagnosis (110,113,114). In the developed countries, the advent and success of HAART led to a dramatic drop in the incidence and mortality of HIV-related KS (115,116)

Since the 1950s, an infectious agent has been suspected to cause KS. Herpes viral particles in tissue culture of KS lesional cells were demonstrated by electron microscopy in 1972 (117). However, it was much later (in 1990) following the emergence of HIV/AIDS pandemic that an infectious agent was proposed as the etiologic factor for KS (112). In 1994, a definite association was established between a new human gamma herpes virus and KS through isolation and characterization of DNA fragments obtained from KS tissue biopsy specimens. In that study, the new herpes virus DNA sequences were isolated from more than 90% of KS tissues and 15% of non-KS tissues of AIDS patients and in none of the tissue samples from non-AIDS patients (118). The new virus was named Kaposi's Sarcoma-associated Herpes Virus

(KSHV - now also known as Human Herpes Virus 8 {HHV8}). HHV8 latency associated nuclear antigen (LANA) is expressed in all cells infected by the virus and irrespective of the underlying clinical condition, over 95% of KS have been found to be infected with this virus (119). The immunohistochemical detection of the viral latency protein (LANA) has become a useful tool in the histopathologic diagnosis of the tumour using light microscopy (120,121). The strong association of KS epidemic with the HIV infection pandemic eventually resulted in the identification of KS as an AIDS-defining illness in HIV-infected patients (122).

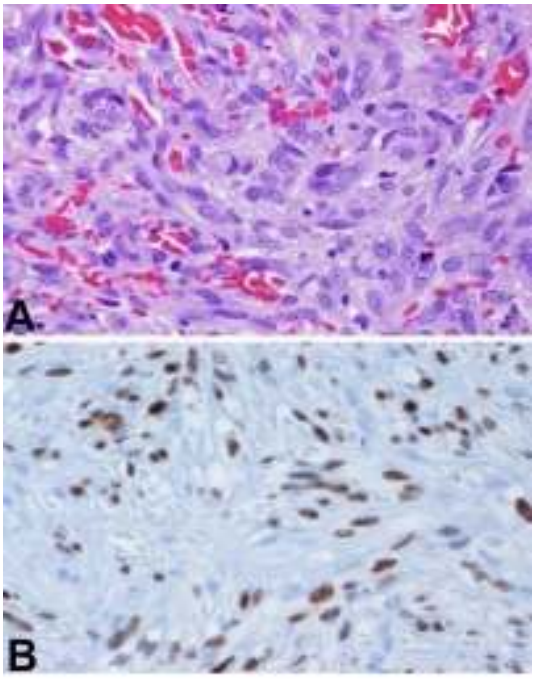

Figure 2

A. Kaposi's sarcoma with spindle cell proliferation of endothelial cells.
B. Positive immunohistochemical staining for HHV8 in the nuclei of the tumour cells.

Iatrogenic KS (also known as post-transplant KS) is immunosuppression-associated KS that occurs in transplant patients who receive solid organs such as kidney, pancreas and heart; and it is most common in renal transplant patients (123). In this group of patients, long-term immunosuppression which is essential for preventing allograft rejection creates a microenvironment that is conducive for KSHV-induced malignant transformation. Fortunately, this form of KS usually resolves with withdrawal or reduction of the immunosuppressive therapy.

In addition to KS, two highly aggressive and rapidly fatal non-Hodgkin's B-cell lymphomas have also been associated with KSHV. These are (1) primary effusion lymphoma (PEL) – a tumour that is also very common in HIV-infected patients with AIDS (124) and (2) plasmablastic lymphoma arising in patients with multicentric Castleman's disease, especially in those who are also HIV positive (125,126).

Oncogenetic transformation of endothelial cells and infected B lymphocytes by KSHV occurs through a complex multistep process that may result in genomic chromosomal instability, alternation of cell cycle and programmed cell death regulatory genes, increase in cell proliferation and long-term survival, and up-regulation of cells' invasive properties (103). KSHV like other herpesviruses has a double stranded DNA genome and possesses a long unique region in which 15 unique open reading frames (ORFs) are located. These are designated sequentially K1 through K15 based on their relative locations in the KSHV genome and are involved in signal transduction, cell cycle regulation, inhibitions of apoptosis and immune modulation. The virus also possesses some microRNAs (a total of 12 discovered so far); they

play critical roles in KSHV pathogenesis (103). KSHV has a two-phased life cycle that is typical for herpesviruses. The lytic phase characterized by active replication of linear viral genomes and production of immediate early (α), early (β) and late (γ) transcripts and an episomal latent phase during which the virus exist in the host cell as circularized extrachromosomal viral genome – a state in which no infectious or functional viral particles are produced. This state of latency is established and maintained principally by the latency nuclear antigen (LANA) (127-129). LANA and other viral proteins interact with p53 and other cell cycle regulatory proteins and as a result derange a host of cellular pathways involved in cell proliferation, programmed cell death and tumourigenesis (103,130).

5. HUMAN PAPILLOMA VIRUS

5:1 HPV and Human Cancers - Historical Account

A probable relationship between cervical cancer and sexually transmitted agents was first noted in 1842 by Rigoni-Stern who reported a high frequency of cervical cancer in married women, widows, and prostitutes; but rare in virgins and nuns (131). With the advent of better bacteriology and virology techniques in the latter part of the 20th century, in the late 1960's and early 1970's, a number of investigators reported Herpes simplex virus type 2 as the likely causative agent for sexual disease - related cervical cancer. But large scale prospective studies exonerated Herpes simplex type 2 virus as the likely candidate (132).

In the late 1970s and early 1980s, Vonka et al (133,134) conducted a large prospective study in attempts to establish a relationship between Herpes simplex type 2 virus and its causative role in cervical neoplasia. In a study conducted over 8 years (1975 to 1983), 10,683 women were enrolled and 10,389 had complete set of data. In the subset of women with moderate to severe squamous dysplasia, squamous cell carcinoma in situ and invasive squamous cell carcinoma either at enrollment or during the follow-up period, the prevalence of Herpes simplex type 2 virus antibody was the same as in control women

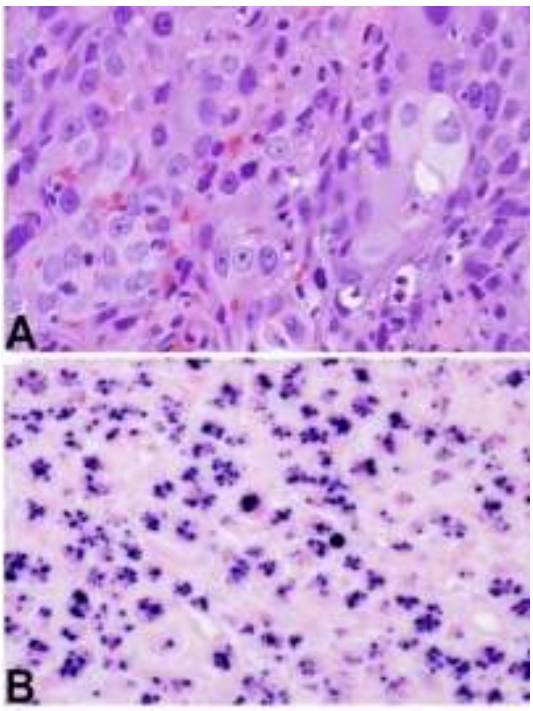

Figure 3

A. Hematoxylin and Eosin-stained tissue section micrograph of HPV-associated squamous cell carcinoma of the oropharyngeal.

B. In-situ hybridization showing presence of HPV in the nuclei of the tumour cells with diffuse and dot like staining patterns.

appropriately matched by age, age at first intercourse, number of sexual partners, smoking habits and clinical integrity of the cervical ectopic epithelium (ectocervix) and the transformation zone. That landmark report was followed by intense investigation and search for the culprit pathogenic agent and eventually led to the identification and confirmation of Human Papilloma Virus (HPV) as the causative agent. In the 1970's, there were anecdotal reports of association between HPV induced genital warts (Condyloma acuminata) and cervical cancer. In 1982, the presence of HPV DNA in invasive squamous cell carcinoma was reported (135) and soon confirmed by additional reports in the following year. That was followed by a burst of additional confirmatory epidemiological, histopathologic and molecular data in the years that follow (136,137). Today, there are more than 200 known types and 20% of them known to cause human infections.

5:2 HPV and Anogenital/Uterine Cervical Cancers

Cervical cancer is the second most common cancer in women worldwide with about 500,000 new cases and 274,000 cervical cancer deaths occurring each year (138). A disproportionately high percentage of these new cancer cases and deaths (about 80%) occur in developing countries (138). There is an abundance of epidemiological and experimental data that show HPVs have causative roles in genital as well as extragenital mucocutaneous squamous cell carcinomas. HPVs are the most sexually transmitted viruses, the most prevalent viruses implicated in causing cancer in human populations and second only to tobacco usage as most important risk factors for developing cancer in human beings (139).

HPV has now been shown to be the causative agent in almost 100% of cervical cancer, and is also responsible for about 50% of vulva squamous cell carcinoma (140), 30 to 50% of penile carcinomas (141-143), 60 to 90% of vaginal carcinomas (140,144) and also with a similar percentage for anal and perineal cancers (145). A large study of cervical cancer material collected from patients in 22 countries all over the world revealed HPV genome in 99.7% of cases (144).

A recent Medline review of 1,824 cases from 37 studies using PCR or hybrid capture for HPV detection in anal squamous dysplasia and invasive carcinoma shows the prevalence of HPV 16 and 18 to be 90% (n=832) in squamous dysplasia and about 70% in invasive carcinoma (n=992); similar to the prevalence observed in invasive cervical cancer (146). A similar review of HPV distribution in vulvar and vaginal cancers show an HPV prevalence of 40% in invasive vulvar carcinoma (n=1379) and about 80% in vulvar squamous dysplasia; and a much higher rate in the vaginal cancers and premalignant (dysplastic) vagina epithelium – 65% and greater than 90% respectively (147).

Meta–analysis of pooled data on HPV type distribution and prevalence between 1990 and 2010 and including 243 studies with a total of 30,848 cases of invasive cervical cancer showed a steady increase from 85.9% in the 1990s to 92.9% in studies published between 2006 and 2010. The prevalence of HPV 16 and/or 18 was 82% in Western/Central Asia and 68% in Eastern Asia (148). In recent study of uterine cervix squamous dysplasia (premalignant lesions that predate HPV induced invasive carcinoma), HPV was detected in 98.4% of women (n=643) with more than half of them (52.5%) infected with more than one HPV serotype; and HPV 16 was the most commonly detected type. Other HPV types detected include HPV 31, 33, 52, 18 and 51 (149). A multisite study of HPV types specific prevalence in women with cervical cancer and premalignant intraepithelial changes in the cervix in England and involving 6,234 women showed the prevalence of HPV 16 and/or 18 to be 76% in squamous cell carcinomas, 82% in adeno/adenosquamous carcinoma and 63% and 91% respectively in severe cervical squamous intraepithelial dysplasia and cervical glandular intraepithelial neoplasia (150).

5:3 HPV and Cancer of the Head and Neck Region

The first evidence of HPV involvement as causative agents of oropharyngeal cancers was first reported in 1983 by Syrjanen et al (156) when they showed the presence of positive immunoperoxidase staining for HPV in oral epithelial hyperplasia and squamous cell papillomas. That was followed by a report of specific HPV types in cancer of the tongue and oropharyngeal region (157). Subsequent studies have since confirmed the association of HPV with cancer of the head and neck regions especially that of the oropharyngeal region with rate of positivity varying between 25 to 60% (132,158). It is now conservatively estimated between 25 to 30% of oropharyngeal cancers are caused by HPV and that the virus does have some aetiological role in these tumours (132). There is increasing incidence of oropharyngeal, tongue base and tonsillar cancer in the young men and women in the United States and evidence abounds that HPV infection is the underlying aetiological factor (159).

In a multicenter case control study involving 9 countries, it was shown that HPV is involved in significant proportion of oropharyngeal cancers and in a smaller group of squamous carcinoma of the oral cavity. Also in that study, it was found that 70% of the tumours contain HPV DNA with HPV 16 as the most frequently observed; a scenario that is similar to that observed in HPV-related genital cancers (160). Studies using very reliable techniques (quantitative PCR and in-situ hybridization) have shown that HPV occur in only a small proportion of oral cavity squamous cell carcinoma and in up to 60% of oropharyngeal tumours (161,162). Kreimer, AR et al (163) reviewed published studies that employ PCR based method to detect and genotype HPV in order to describe the prevalence and HPV type distribution by anatomic cancer sites and geographic location, as possible source of variability. With a total of 5,046 cases of squamous cell carcinoma from 60 eligible studies from 26 countries, the overall HPV prevalence in head and neck cancer was reported as 25.9% and HPV prevalence was significantly higher in oropharyngeal squamous cell carcinoma (35.6% compared to 23.5% in oral squamous cell carcinoma and 24.0% in laryngeal squamous cell carcinoma). HPV 16 accounted for the majority of HPV positive oropharyngeal carcinoma (86.7%) compared with 68.2% and 69.2% in HPV positive oral and HPV associated laryngeal squamous cell carcinoma. HPV 18 which is the second most common detected high risk type was found in only very small number of cases. HPV 18 detection rate was 8%, 3.9% in oral and laryngeal cancers and only 1% oropharyngeal cancers. HPV prevalence in oral squamous carcinoma was found to be similar in Europe and North America (16 and 16.1% respectively) but was significantly greater in Asia (33%). However, with oropharyngeal carcinoma, HPV prevalence was significantly higher in North America (47%) compared with Europe (28.2%). In the cases from Asia, the HPV prevalence rate for oropharyngeal carcinoma was rather high (46.3%) but the number of cases studied was very small (n=54). Thus in all, HPV prevalence in oral squamous cell carcinoma from Asia was considerably higher than other geographic locations and HPV prevalence was higher in oropharyngeal squamous cell carcinoma from North America and Asia compared with Europe (162). However, in a multicontinent study conducted by IARC, HPV prevalence did not differ significantly among Europe, North and South America, Asia and Africa (162,163).

Squamous cell carcinoma of the head and neck region is traditionally believed and has been shown to be related to environmental factors such as tobacco use and alcohol abuse. However, it has also been shown that a small but significant proportion (15-20%) of this cancer occur in patients without these traditional risk factors (164). This is further confirmed by the fact that the incidence of oropharyngeal cancer is increasing and tends to occur more in young male patients while the incidence of tobacco use and over all incidence of squamous carcinoma of the head and neck region are decreasing (158,165).

Similar to what occurs with genital HPV related malignancies, HPV positive head and neck squamous carcinoma is related to sexual history. The risk factors include increasing number of oral and vaginal sexual partners, history of genital warts, and younger age at first intercourse (161,166,167). Also, Gillison et al (167) reported increasing association of HPV 16 positive head and neck cancer with increased use of marijuana. While the means by which HPV is transmitted to the upper airways is unclear, it does appear to be related to sexual activity and sexual behaviors – since oral HPV infection is rare in newborn babies of infected mothers and in children prior to sexual activity (168,169). Additional sexual behaviours strongly associated

with high risk HPV positive tumour include history of oral sex and oral-anal contact (170,171). There is also an increase in individuals with a history of HPV associated anogenital cancers and in husband of women with HPV related in-situ squamous cell carcinoma and invasive cancer (164). Patient with anogenital cancers have a 4.3 fold increase risk of tonsillar cancers and there are remarkable histopathological and molecular biological similarities between HPV tumours at these two sites (172).

HPV-associated head and neck cancer have characteristically distinct features that distinguish them from the non HPV-related head and neck cancers. HPV-related squamous cell carcinoma of head and neck tends to be more common in the oropharyngeal region, and affects men and women equally; unlike tobacco associated cancers that are more frequent in men. HPV-associated cancers patients also tend to be non smokers, non drinkers, and tend to be younger (164). HPV positive head and neck cancer also have distinct histologic appearance. They tend to be more poorly differentiated, non Keratinizing and have basaloid appearance. However, other patterns such as papillary squamous cell carcinoma have also been described (158).

5:4 HPV and Carcinogenesis

Human papilloma virus (HPV) is a double stranded DNA virus with all putative open reading frames (ORFs) on one DNA strand, and the presumably non coding strand containing short ORFs which are conserved regardless of localization and composition (164). The putative genes consist of early genes (E1-E8) which are expressed shortly after infection and prior to onset of DNA replication, and late genes (L1 & L2) which code for structural proteins of viral capsids and are activated late during the final stages of the virus circle.

HPVs are epitheliotropic viruses that cause mucocutaneous infections. Currently, more than 200 types are known with about 40 of them having been described to infect human mucosa and the individual types have varying predilections for different epithelial surfaces and different oncogenic potentials (173). Based on their oncogenic potential (ability to cause cancer), they are divided into low risk and high risk types (174). With infection of epithelial cells through abrasions or micro trauma, the virus integrates into the epithelial cells and cause abnormal growth of the cells. The high risk HPV types by virtue of their E7 proteins integrate themselves into the genome of the host epithelial cells, immortalize the cells infected and cause dysregulation of the host cells' cell cycle and apoptosis regulatory proteins and pathways. The high risk HPV types include types 16, 18, 31 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68 and 73 (175). HPV types 16 and 18 are responsible for approximately 70% of all cervical cancers and are also the culprits in the squamous carcinoma of the ano-genital region including vulva, anal, and penile cancers (164). They have also been shown to be the main types involved in HPV related head and neck squamous cell carcinomas (176).

HPV infects the basal cells and the virus early genes E6 and E7 (both of which encode oncoproteins) integrate into the host cell genome. In the actively dividing basal epithelial cells, virus maturation parallels that of the infected host cells as they mature and differentiate into more mature cells. Hence, the early viral genes are found in basal cells and late genes are in the more superficial keratinizing epithelial layer. The E6 protein of the high risk HPV binds to and induces degradation of the tumour suppressor protein p53 (through an ubiquitin- mediated process); resulting in dysregulation of the innate cellular tumour supressor process. The E7 protein of high risk HPV types on the other hand, causes destruction of phosphorylated retinoblastoma proteins mediated transcriptional repression of genes that are involved in regulation of the cell cycle. The combined subcellular (molecular) activities of E6 and E7 proteins of high risk HPV types, results in loss of control of the cell cycle and apoptosis control mechanisms. Thus the infected cells become immortalized and acquire oncogenic potentials that enable them to go into uncontrolled cellular proliferation and tumour formation (177-183). Although integration of the E6 and E7 proteins into the host genome is believed to be central to HPV-mediated carcinogenesis with the integration of HPV DNA into the host genome as the key event, recent studies have shown that transcription of HPV-16E6-E7 mRNA is not necessarily dependent on DNA integration - as the viral DNA which is predominately in episomal (unintegrated) form takes part in the carcinogenesis process (184,185). E6 through binding of p53 and inducing its degradation, impairs host cell apoptosis and causes instability of the host genome (increased predisposition of the genome to mutations and changes that supports cellular proliferation). E6 binding also

inactivates telomerase (enzyme responsible for maintaining telomeric DNA stability). E7 binding to phosphorylated retinoblastoma proteins and related proteins, results in upregulation of transcriptional factors that activates or turns on genes that regulate cell proliferation. The down regulation of phosphorylated retinoblastoma protein by E7 also result in the upregulation of p16 which is now used as a surrogate marker for HPV infection in squamous cell dysplasia – the histopathologic manifestation of the premalignant transformation of HPV infected cells (186-188)

Fortunately, majority of HPV infection of epithelial cells is self-liming - cleared by the host's humoral and cell mediated immune responses through poorly understood mechanisms (189). However, infection caused by high risk HPV types results in subversion and evasion of the host's virus clearing immune mechanism and persistent infection that may culminate in malignant transformation of infected host cells. It appears that there are other factors in play to initiate the process of carcinogenesis and for the progression through low grade dysplasia to high grade dysplasia and ultimately invasive squamous cell carcinoma. This is supported by report that high risk HPV serotypes are three fold more frequently encountered in patients with malignant lesions than in those with benign or precancerous lesions (190). The precursor lesions of infection by high risk HPV types persist longer and also progress more quickly in individuals infected with these high risk oncogenic viruses compared with those infected by the low risk non oncogenic HPV types (191,192). Hence, the need for typing the HPV detected in individuals that undergo HPV screening. In so doing, individuals at risk would be followed with the appropriate aggressive management scheme; while those infected with less aggressive types are spared unnecessary aggressive management.

Although HPV DNA integration into the host genome results in genomic instability, it is noteworthy that HPV positive cancers tend to have significantly lower levels of chromosomal mutations and loss compared to the HPV negative tumours at the same sites (191,194). Presence and high levels of active HPV infection as demonstrated by elevated levels of p16 in tumour cells, have been shown to be associated with higher survivor rates of 79% five year survival compared with 20% five year survival in individuals with HPV negative tumours (195). This improved survival in HPV related squamous cell carcinoma compared with HPV negative tumours has since been confirmed by other studies (196,197). HPV associated head and neck squamous cell carcinoma are more responsive to treatment - being more radiosensitive and have better survivor rates compared to their non HPV-related counterparts. Also, HPV related tumours have been shown to have higher response rate to induction chemotherapy and chemoradiation (198). The higher response to chemotherapy and radiation by HPV associated tumour has been attributed to improved apoptotic response facilitated by the presence of unmutated p53 and absence of other chromosomal abnormalities in HPV related cancers; in contrast to p53 mutation and other chromosomal abnormalities that tend to occur in HPV negative tumours. Also in HPV negative tumours, the inducing agents such as tobacco and alcohol are known to cause p53 mutation and field cancerization – processes that result in subcellular genetic events that make the tumour cells in those cases to be less radiosensitive and also predispose those individuals to development of a second primary cancer (176). Similar to what obtains with HPV associated head and neck tumours, HPV positive vulva and penile carcinoma also seem to have better prognosis and more favorable therapeutic response in comparison with HPV negative tumours (163).

6. HUMAN T- CELL LYMPHOTROPHIC VIRUS 1

6:1 Human T-cell Lymphotrophic Virus Subtypes

Human T-cell lymphotrophic virus (HLTV) 1 was the first retrovirus to be associated with human disease. It was first isolated and identified in 1980 by Poresz, et al from the T-cells of a patient with cutaneous lymphoma (199). Soon thereafter, Human T-cell lymphotrophic virus type 2 (HLTV2) was isolated from a patient with hairy-cell leukemia (200). Both HLTV1 and HLTV2 infect helper T-cells and have similar genomic structure with 66% nucleotide sequence homology in their genome; hence the serologic cross-reactivity between the two. Recently (in 2005), two additional related viruses – HLTV-3 and HTLV-4 were reported in Central Africa (201). However, only HLTV-1 has been shown to have etiological association with a number of diseases (202). There are six subtypes of HLTV-1 (subtypes A-F); but they all appear to have similar pathogenicity (203).

6:2 HLTV-1 Infections.

It is estimated that there are about 20 million HLTV-1 infected individuals in the world with the highest prevalence rates in southwestern Japan, sub-Sahara Africa, Caribbean Islands, South America, Papa New Guinea, and Iran (204). The most common mode of transmission is mother-to-child vertical transmission - mostly through breast feeding (205,206) and that has been shown to be directly proportional to the duration of breast feeding and the provirus load in the milk (207-210). Other modes of transmission include sexual contact (211), blood transfusion (212) and sharing of contaminated needles (206). Fortunately, the overwhelming majority (about 90%) of individuals infected by HLTV-1 are asymptomatic throughout their lifetime (213). HLTV-1 was originally associated with Adult T-cell leukemia/lymphoma (ATLL), but it has also been associated with the following non neoplastic diseases: uveitis, arthropathy, polymyositis, thyroiditis, Sjögren's syndrome, inflammatory dermatopathic disease conditions, myelopathy and tropical spastic paraparesis (213,214). Some of the inflammatory dermatologic conditions associated with HLTV-1 occur as part of the disease entity called infective dermatitis associated with HLTV1 – a severe and recurrent eczema that is characterized by occurrence in childhood, chronic rhinorrhea and chronic relapsing dermatitis. There is associated infection of the skin and nasal vestibules by non-virulent *Staphylococcus aureus* or *Beta-hemolytic streptococcus;* therefore the dermatitis responds promptly to antibiotic therapy but also recurs promptly on withdrawal of the antibiotic treatment. (215-217). HLTV-1 infected patients are also known to be more predisposed to developing other skin conditions including xerosis, acquired ichthyosis, seborrheic dermatitis, non infectious erythematous dermatitis and infectious skin diseases (dermatophytosis, scabies, verruca vulgaris and bacterial infections) (218-221). The presence of chronic (recurrent and sometimes refractory) skin conditions in HLTV-1 infected patients is thought to result from immune dysregulation and is associated with protracted high levels of cytokines which support the development of lymphoproliferative disorder. Therefore, the presence HLTV-1 associated skin conditions is an indicator for increased risk for developing HLTV-1 associated Leukemia/Lymphoma (222-226).

6:3 HLTV1 and ATLL (Pathogenesis)

Following infection of a T-cell, the proviral DNA (generated from the viral RNA), integrates into the host genome and spreads only as the infected cells divide. Two viral accessory genes *tax* and HTLV-I b-ZIP factor (HBZ) enhance infectivity by inducing cell proliferation, modulate programmed cell death and induce a series of processes that support cellular transformation and leukemogenesis (227,228). HLTV-1 infection of T-lymphocytes results in T-cell activation and a hyperactive immune response that is characterized by exaggerated type 1 (Th_1) immune response and spontaneous lymphoproliferation with increase in $CD4^+$ counts, $CD8^+$ counts and $CD4^+/CD8^+$ ratio (215,216,229). B-cell activity is also markedly increased resulting in elevation of Ig A, Ig D, Ig E, and Ig G levels (215,216). In the infected cells, the HLTV-1 protein *tax* also transactivates genes responsible for production of proinflammatory Th_1 and Th_2 cytokine such as tumour necrosis factor-alpha (TNF α), interferon-gamma (1FN-γ), interleukin 1, interleukin 5 and interleukin 6 (214,217,230,231).

In HLTV-1 carriers, elevated pro-viral load has been shown to strongly predispose such infected patients to the development of ATLL (232-234) and patients with HLTV-1 associated inflammatory skin conditions tend to have high pro-viral load. In addition, associated bacterial infections results in synergistic stimulation of T-lymphocytes by the bacterial antigens; further worsening exaggeration of the Th_1 immune response and associated T-cell proliferation. Eventually, malignant transformation ensues, with the emergence of clones of T-cells that undergo uncontrolled proliferation (214).

ATLL is an aggressive malignant lymphoproliferative disorder with heterogeneous clinical presentation. There are four subtypes, viz; smoldering, chronic, lymphoma and acute types. The lymphoma and acute forms are more aggressive and have worse prognosis with less than 1 year survival after diagnosis (202,213). The incidence of ATLL in HLTV-1-infected is estimated to be less than 5% (235) and occurs almost exclusively in individuals who acquire the infection with vertical transmission through breast feeding (202,236). ATLL usually occur two to three decades after the onset of HLTV-1 infection. As a result, ATLL is generally reported in adults, and is more common in males (235,237).

ATLL has variable histopathologic appearances with features similar to those observed in other non HLTV-1 related lymphoproliferative disorders. Therefore, the diagnosis of ATLL is based on criteria that include positive HLTV-1 serology; the presence of hypercalcemia, detection of HTLV-1 pro-viral DNA is tumour cells (in lymphoma type) and the presence of abnormal T-cells in the peripheral blood (including flower cells) in the leukemic subtypes (202). The flower cells have characteristic polylobulated nuclei and are pathognomonic of ATLL. They are more commonly seen in acute ATLL - and can be readily identified with Giemsa or Wright staining of a blood smear – an attribute that allows for a quick and reliable diagnosis of acute ATLL – even in resources-limited settings (238).

REFERENCES

1. Epstein MA, Achong BG, Barr YM. Virus particles in cultured lymphoblasts from Burkitt's lymphoma. Lancet. 1964;1:702-3.
2. Henle G, Henle W. Immunofluorescence in cells derived from Burkitt's lymphoma. J Bacteriol. 1966;91:1248-56.
3. Henle G, Henle W, Diehl V. Relation of Burkitt's tumor-associated herpes-type virus to infectious mononucleosis. Proc Natl Acad Sci U S A. 1968;59:94-101.
4. zur Hausen H, Schulte-Holthausen H, Klein G, Henle W, Henle G, Clifford P, Santesson L. EBV DNA in biopsies of Burkitt tumours and anaplastic carcinomas of the nasopharynx. Nature. 197012;228:1056-8.
5. Baumforth KR, Young LS, Flavell KJ, Constandinou C, Murray PG. The Epstein-Barr virus and its association with human cancers. Mol Pathol. 1999;52:307-22.
6. Young LS, Murray PG. Epstein-Barr virus and oncogenesis: from latent genes to tumours. Oncogene. 2003;22:5108-21.
7. Halder S, Murakami M, Verma SC, Kumar P, Yi F, Robertson ES. Early events associated with infection of Epstein-Barr virus infection of primary B-cells. PLoS One. 2009 ;4:e7214.
8. Sixbey JW, Nedrud JG, Raab-Traub N, Hanes RA, Pagano JS. Epstein-Barr virus replication in oropharyngeal epithelial cells. N Engl J Med. 1984;310:1225-30.
9. Gerber P, Lucas S, Nonoyama M, Perlin E, Goldstein LI. Oral excretion of Epstein-Barr virus by healthy subjects and patients with infectious mononucleosis. Lancet. 1972;2:988-9.
10. Rowe M, Rowe DT, Gregory CD, Young LS, Farrell PJ, Rupani H, Rickinson AB. Differences in B cell growth phenotype reflect novel patterns of Epstein-Barr virus latent gene expression in Burkitt's lymphoma cells. EMBO J. 1987;6:2743-51.
11. Kieff E and Richardson AB. *Fields Virology*. 4th Eds. Knipe DM and HowleyPM (Eds). Lippincott Williams and Wilkins Publishers: Philadelphia, 2001; pp. 2511-2574
12. Smith P. Epstein-Barr virus complementary strand transcripts (CSTs/BARTs) and cancer. Semin Cancer Biol. 2001;11:469-76.
13. Münz C, Moormann A. Immune escape by Epstein-Barr virus associated malignancies. Semin Cancer Biol. 2008;18:381-7.
14. Brady G, MacArthur GJ, Farrell PJ. Epstein-Barr virus and Burkitt lymphoma. J Clin Pathol. 2007;60:1397-402.
15. Young LS, Rickinson AB. Epstein-Barr virus: 40 years on. Nat Rev Cancer. 2004;4:757-68.
16. de Leval L, Hasserjian RP. Diffuse large B-cell lymphomas and burkitt lymphoma. Hematol Oncol Clin North Am. 2009;23:791-827.
17. Bellan C, Stefano L, Giulia de F, Rogena EA, Lorenzo L. Burkitt lymphoma versus diffuse large B-cell lymphoma: a practical approach. Hematol Oncol. 2010;28:53-6.
18. Lam KM, Syed N, Whittle H, Crawford DH. Circulating Epstein-Barr virus-carrying B cells in acute malaria. Lancet. 1991;337:876-8.
19. Moormann AM, Heller KN, Chelimo K, Embury P, Ploutz-Snyder R, Otieno JA, Oduor M, Münz C, Rochford R. Children with endemic Burkitt lymphoma are deficient in EBNA1-specific IFN-gamma T cell responses. Int J Cancer. 2009;124:1721-6.
20. Küppers R. B cells under influence: transformation of B cells by Epstein-Barr virus. Nat Rev Immunol. 2003;3:801-12.
21. Herbst H, Niedobitek G, Kneba M, Hummel M, Finn T, Anagnostopoulos I, Bergholz M, Krieger G, Stein H. High incidence of Epstein-Barr virus genomes in Hodgkin's disease. Am J Pathol. 1990;137:13-8.
22. Wu TC, Mann RB, Charache P, Hayward SD, Staal S, Lambe BC, Ambinder RF. Detection of EBV gene expression in Reed-Sternberg cells of Hodgkin's disease. Int J Cancer. 1990;46:801-4.
23. Chang KL, Albújar PF, Chen YY, Johnson RM, Weiss LM. High prevalence of Epstein-Barr virus in the Reed-Sternberg cells of Hodgkin's disease occurring in Peru. Blood. 1993;81:496-501.
24. Weinreb M, Day PJ, Niggli F, Green EK, Nyong'o AO, Othieno-Abinya NA, Riyat MS, Raafat F, Mann JR. The consistent association between Epstein-Barr virus and Hodgkin's disease in children in Kenya. Blood. 1996;87:3828-36.

25. Gutensohn N, Cole P. Epidemiology of Hodgkin's disease. Semin Oncol. 1980;7:92-102.

26. Levine PH, Ablashi DV, Berard CW, Carbone PP, Waggoner DE, Malan L. Elevated antibody titers to Epstein-Barr virus in Hodgkin's disease. Cancer. 1971;27:416-21.

27. Mueller N, Evans A, Harris NL, Comstock GW, Jellum E, Magnus K, Orentreich N, Polk BF, Vogelman J. Hodgkin's disease and Epstein-Barr virus. Altered antibody pattern before diagnosis. N Engl J Med. 1989;320:689-95.

28. Anagnostopoulos I, Herbst H, Niedobitek G, Stein H. Demonstration of monoclonal EBV genomes in Hodgkin's disease and Ki-1-positive anaplastic large cell lymphoma by combined Southern blot and in situ hybridization. Blood. 1989;74:810-6.

29. Jarrett RF, Gallagher A, Jones DB, Alexander FE, Krajewski AS, Kelsey A, Adams J, Angus B, Gledhill S, Wright DH, et al. Detection of Epstein-Barr virus genomes in Hodgkin's disease: relation to age. J Clin Pathol. 1991;44:844-8.

30. Armstrong AA, Alexander FE, Cartwright R, Angus B, Krajewski AS, Wright DH, Brown I, Lee F, Kane E, Jarrett RF. Epstein-Barr virus and Hodgkin's disease: further evidence for the three disease hypothesis. Leukemia. 1998;12:1272-6.

31. Pallesen G, Hamilton-Dutoit SJ, Rowe M, Young LS. Expression of Epstein-Barr virus latent gene products in tumour cells of Hodgkin's disease. Lancet. 1991;337:320-2.

32. Murray PG, Young LS, Rowe M, Crocker J. Immunohistochemical demonstration of the Epstein-Barr virus-encoded latent membrane protein in paraffin sections of Hodgkin's disease. J Pathol. 1992;166:1-5.

33. Deacon EM, Pallesen G, Niedobitek G, Crocker J, Brooks L, Rickinson AB, Young LS. Epstein-Barr virus and Hodgkin's disease: transcriptional analysis of virus latency in the malignant cells. J Exp Med. 1993;177:339-49.

34. Herbst H, Foss HD, Samol J, Araujo I, Klotzbach H, Krause H, Agathanggelou A, Niedobitek G, Stein H. Frequent expression of interleukin-10 by Epstein-Barr virus-harboring tumor cells of Hodgkin's disease. Blood. 1996;87:2918-29.

35. Kapp U, Yeh WC, Patterson B, Elia AJ, Kägi D, Ho A, Hessel A, Tipsword M, Williams A, Mirtsos C, Itie A, Moyle M, Mak TW. Interleukin 13 is secreted by and stimulates the growth of Hodgkin and Reed-Sternberg cells. J Exp Med. 1999;189:1939-46.

36. Hsu SM, Lin J, Xie SS, Hsu PL, Rich S. Abundant expression of transforming growth factor-beta 1 and -beta 2 by Hodgkin's Reed-Sternberg cells and by reactive T lymphocytes in Hodgkin's disease. Hum Pathol. 1993;24:249-55.

37. Carbone A, Gloghini A, Dotti G. EBV-associated lymphoproliferative disorders: classification and treatment. Oncologist. 2008;13:577-85.

38. Kelly GL, Milner AE, Baldwin GS, Bell AI, Rickinson AB. Three restricted forms of Epstein-Barr virus latency counteracting apoptosis in c-myc-expressing Burkitt lymphoma cells. Proc Natl Acad Sci U S A. 2006;103:14935-40.

39. Hammerschmidt W, Sugden B. Epstein-Barr virus sustains Burkitt's lymphomas and Hodgkin's disease. Trends Mol Med. 2004;10:331-6.

40. Bubman D, Guasparri I, Cesarman E. Deregulation of c-Myc in primary effusion lymphoma by Kaposi's sarcoma herpesvirus latency-associated nuclear antigen. Oncogene. 2007;26:4979-86.

41. Wei WI, Sham JS. Nasopharyngeal carcinoma. Lancet. 2005;365:2041-54.

42. Heussinger N, Büttner M, Ott G, Brachtel E, Pilch BZ, Kremmer E, Niedobitek G. Expression of the Epstein-Barr virus (EBV)-encoded latent membrane protein 2A (LMP2A) in EBV-associated nasopharyngeal carcinoma. J Pathol. 2004;203:696-9.

43. Guo X, Johnson RC, Deng H, Liao J, Guan L, Nelson GW, Tang M, Zheng Y, de The G, O'Brien SJ, Winkler CA, Zeng Y. Evaluation of nonviral risk factors for nasopharyngeal carcinoma in a high-risk population of Southern China. Int J Cancer. 2009;124:2942-7.

44. Raab-Traub N, Flynn K. The structure of the termini of the Epstein-Barr virus as a marker of clonal cellular proliferation. Cell. 1986;47:883-9.

45. Klein G. The relationship of the virus to nasopharyngeal carcinoma. In: Epstein MA, Achong BG, eds. *The Epstein-Barr virus*. Berlin: Springer-Verlag, 1979:339–50.

46. Weiss LM, Movahed LA, Butler AE, Swanson SA, Frierson HF Jr, Cooper PH, Colby TV, Mills SE. Analysis of lymphoepithelioma and lymphoepithelioma-like carcinomas for Epstein-Barr viral genomes by in situ hybridization. Am J Surg Pathol. 1989;13:625-31.

47. Niedobitek G, Hansmann ML, Herbst H, Young LS, Dienemann D, Hartmann CA, Finn T, Pitteroff S, Welt A, Anagnostopoulos I, et al. Epstein-Barr virus and carcinomas: undifferentiated carcinomas but not squamous cell carcinomas of the nasopharynx are regularly associated with the virus. J Pathol. 1991;165:17-24.

48. Young LS, Dawson CW, Clark D, Rupani H, Busson P, Tursz T, Johnson A, Rickinson AB. Epstein-Barr virus gene expression in nasopharyngeal carcinoma. J Gen Virol. 1988;69 (Pt 5):1051-65.

49. Niedobitek G, Young LS, Sam CK, Brooks L, Prasad U, Rickinson AB. Expression of Epstein-Barr virus genes and of lymphocyte activation molecules in undifferentiated nasopharyngeal carcinomas. Am J Pathol. 1992;140:879-87.

50. Zheng X, Yan L, Nilsson B, Eklund G, Drettner B. Epstein-Barr virus infection, salted fish and nasopharyngeal carcinoma. A case-control study in southern China. Acta Oncol. 1994;33:867-72.

51. Mazeron MC. [Value of anti-Epstein-Barr antibody detection in the diagnosis and management of undifferentiated carcinoma of the nasopharynx]. Bull Cancer Radiother. 1996;83:3-7.

52. Hu B, Hong G, Li Z, Xu J, Zhu Z, Li L. Expression of VCA (viral capsid antigen) and EBNA1 (Epstein-Barr-virus-encoded nuclear antigen 1) genes of Epstein-Barr virus in Pichia pastoris and application of the products in a screening test for patients with nasopharyngeal carcinoma. Biotechnol Appl Biochem. 2007;47(Pt 1):59-69.

53. Li S, Deng Y, Li X, Chen QP, Liao XC, Qin X. Diagnostic value of Epstein-Barr virus capsid antigen-IgA in nasopharyngeal carcinoma: a meta-analysis. Chin Med J (Engl). 2010;123:1201-5.

54. Mitarnun W, Suwiwat S, Pradutkanchana J, Saechan V, Ishida T, Takao S, Mori A. Epstein-Barr virus-associated peripheral T-cell and NK-cell proliferative disease/lymphoma: clinicopathologic, serologic, and molecular analysis. Am J Hematol. 2002;70:31-8.

55. Rezk SA, Weiss LM. Epstein-Barr virus-associated lymphoproliferative disorders. Hum Pathol. 2007;38:1293-304.

56. Niedobitek G and Young LS. Non-Hodgkin's Lymphomas. 2nd edn. Magrath I (ed). Edward Arnold:London, 1997:pp. 309–329.

57. Akiba S, Koriyama C, Herrera-Goepfert R, Eizuru Y. Epstein-Barr virus associated gastric carcinoma: epidemiological and clinicopathological features. Cancer Sci. 2008;99:195-201.

58. Shibata D, Weiss LM. Epstein-Barr virus-associated gastric adenocarcinoma. Am J Pathol. 1992;140:769-74.

59. Levine PH, Stemmermann G, Lennette ET, Hildesheim A, Shibata D, Nomura A. Elevated antibody titers to Epstein-Barr virus prior to the diagnosis of Epstein-Barr-virus-associated gastric adenocarcinoma. Int J Cancer. 1995;60:642-4.

60. Tokunaga M, Land CE, Uemura Y, Tokudome T, Tanaka S, Sato E. Epstein-Barr virus in gastric carcinoma. Am J Pathol. 1993;143:1250-4.

61. Fukayama M, Hayashi Y, Iwasaki Y, Chong J, Ooba T, Takizawa T, Koike M, Mizutani S, Miyaki M, Hirai K. Epstein-Barr virus-associated gastric carcinoma and Epstein-Barr virus infection of the stomach. Lab Invest. 1994;71:73-81.

62. Imai S, Koizumi S, Sugiura M, Tokunaga M, Uemura Y, Yamamoto N, Tanaka S, Sato E, Osato T. Gastric carcinoma: monoclonal epithelial malignant cells expressing Epstein-Barr virus latent infection protein. Proc Natl Acad Sci U S A. 1994;91:9131-5.

63. Fukayama M. Epstein-Barr virus and gastric carcinoma. Pathol Int. 2010;60:337-50.

64. Uozaki H, Fukayama M. Epstein-Barr virus and gastric carcinoma—viral carcinogenesis through epigenetic mechanisms. Int J Clin Exp Pathol. 2008;1:198-216.

65. Murphy G, Pfeiffer R, Camargo MC, Rabkin CS. Meta-analysis shows that prevalence of Epstein-Barr virus-positive gastric cancer differs based on sex and anatomic location. Gastroenterology. 2009;137:824-33.

66. Lee JH, Kim SH, Han SH, An JS, Lee ES, Kim YS. Clinicopathological and molecular characteristics of Epstein-Barr virus-associated gastric carcinoma: a meta-analysis. J Gastroenterol Hepatol. 2009;24:354-65.

67. Chang MS, Uozaki H, Chong JM, Ushiku T, Sakuma K, Ishikawa S, Hino R, Barua RR, Iwasaki Y, Arai K, Fujii H, Nagai H, Fukayama M. CpG island methylation status in gastric carcinoma with and without infection of Epstein-Barr virus. Clin Cancer Res. 2006;12:2995-3002.

68. Tsai WL, Chung RT. Viral hepatocarcinogenesis. Oncogene. 2010;29:2309-24.

69. Di Bisceglie AM. Hepatitis B and hepatocellular carcinoma. Hepatology. 2009;49(5 Suppl):S56-60.

70. Yang JD, Roberts LR. Epidemiology and management of hepatocellular carcinoma. Infect Dis Clin North Am. 2010;24:899-919.

71. Perz JF, Armstrong GL, Farrington LA, Hutin YJ, Bell BP. The contributions of hepatitis B virus and hepatitis C virus infections to cirrhosis and primary liver cancer worldwide. J Hepatol. 2006;45:529-38.

72. Beasley RP, Hwang LY, Lin CC, Chien CS. Hepatocellular carcinoma and hepatitis B virus. A prospective study of 22 707 men in Taiwan. Lancet. 1981;2(8256):1129-33.

73. Evans AA, Chen G, Ross EA, Shen FM, Lin WY, London WT. Eight-year follow-up of the 90,000-person Haimen City cohort: I. Hepatocellular carcinoma mortality, risk factors, and gender differences. Cancer Epidemiol Biomarkers Prev. 2002;11:369-76.

74. Chen CJ, Yang HI, Su J, Jen CL, You SL, Lu SN, Huang GT, Iloeje UH; REVEAL-HBV Study

Group. Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level. JAMA. 2006;295:65-73.

75. Chan HL, Hui AY, Wong ML, Tse AM, Hung LC, Wong VW, Sung JJ. Genotype C hepatitis B virus infection is associated with an increased risk of hepatocellular carcinoma. Gut. 2004;53:1494-8.

76. Yang HI, Lu SN, Liaw YF, You SL, Sun CA, Wang LY, Hsiao CK, Chen PJ, Chen DS, Chen CJ; Taiwan Community-Based Cancer Screening Project Group. Hepatitis B e antigen and the risk of hepatocellular carcinoma. N Engl J Med. 2002;347:168-74.

77. Ding X, Mizokami M, Yao G, Xu B, Orito E, Ueda R, Nakanishi M. Hepatitis B virus genotype distribution among chronic hepatitis B virus carriers in Shanghai, China. Intervirology. 2001;44:43-7.

78. Liang TJ, Mok KT, Liu SI, Huang SF, Chou NH, Tsai CC, Chen IS, Yeh MH, Chen YC, Wang BW. Hepatitis B genotype C correlated with poor surgical outcomes for hepatocellular carcinoma. J Am Coll Surg. 2010;211:580-6.

79. Livingston SE, Simonetti JP, McMahon BJ, Bulkow LR, Hurlburt KJ, Homan CE, Snowball MM, Cagle HH, Williams JL, Chulanov VP. Hepatitis B virus genotypes in Alaska Native people with hepatocellular carcinoma: preponderance of genotype F. J Infect Dis. 2007;195:5-11.

80. Fung J, Lai CL, Yuen MF. Hepatitis B and C virus-related carcinogenesis. Clin Microbiol Infect. 2009;15:964-70.

81. Bonilla Guerrero R, Roberts LR. The role of hepatitis B virus integrations in the pathogenesis of human hepatocellular carcinoma. J Hepatol. 2005;42:760-77.

82. 16: Kew MC, Miller RH, Chen HS, Tennant BC, Purcell RH. Mutant woodchuck hepatitis virus genomes from virions resemble rearranged hepadnaviral integrants in hepatocellular carcinoma. Proc Natl Acad Sci U S A. 1993;90:10211-5.

83. Muroyama R, Kato N, Yoshida H, Otsuka M, Moriyama M, Wang Y, Shao RX, Dharel N, Tanaka Y, Ohta M, Tateishi R, Shiina S, Tatsukawa M, Fukai K, Imazeki F, Yokosuka O, Shiratori Y, Omata M. Nucleotide change of codon 38 in the X gene of hepatitis B virus genotype C is associated with an increased risk of hepatocellular carcinoma. J Hepatol. 2006;45:805-12.

84. Tennant BC, Toshkov IA, Peek SF, Jacob JR, Menne S, Hornbuckle WE, Schinazi RD, Korba BE, Cote PJ, Gerin JL. Hepatocellular carcinoma in the woodchuck model of hepatitis B virus infection. Gastroenterology. 2004 Nov;127(5 Suppl 1):S283-93.

85. Kew MC, Hodkinson J, Paterson AC, Song E. Hepatitis-B virus infection in black children with hepatocellular carcinoma. J Med Virol. 1982;9:201-7.

86. Caldwell S, Park SH. The epidemiology of hepatocellular cancer: from the perspectives of public health problem to tumor biology. J Gastroenterol. 2009;44 Suppl 19:96-101.

87. El-Serag HB, Rudolph KL. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. Gastroenterology. 2007;132:2557-76.

88. Fattovich G. Natural history and prognosis of hepatitis B. Semin Liver Dis. 2003;23:47-58.

89. Bruix J, Sherman M; Practice Guidelines Committee, American Association for the Study of Liver Diseases. Management of hepatocellular carcinoma. Hepatology. 2005;42:1208-36.

90. Donato F, Boffetta P, Puoti M. A meta-analysis of epidemiological studies on the combined effect of hepatitis B and C virus infections in causing hepatocellular carcinoma. Int J Cancer. 1998;75:347-54.

91. Donato F, Tagger A, Gelatti U, Parrinello G, Boffetta P, Albertini A, Decarli A, Trevisi P, Ribero ML, Martelli C, Porru S, Nardi G. Alcohol and hepatocellular carcinoma: the effect of lifetime intake and hepatitis virus infections in men and women. Am J Epidemiol. 2002 ;155:323-31.

92. Kamegaya Y, Hiasa Y, Zukerberg L, Fowler N, Blackard JT, Lin W, Choe WH, Schmidt EV, Chung RT. Hepatitis C virus acts as a tumor accelerator by blocking apoptosis in a mouse model of hepatocarcinogenesis. Hepatology. 2005;41:660-7.

93. Lerat H, Honda M, Beard MR, Loesch K, Sun J, Yang Y, Okuda M, Gosert R, Xiao SY, Weinman SA, Lemon SM. Steatosis and liver cancer in transgenic mice expressing the structural and nonstructural proteins of hepatitis C virus. Gastroenterology. 2002;122:352-65.

94. Majumder M, Steele R, Ghosh AK, Zhou XY, Thornburg L, Ray R, Phillips NJ, Ray RB. Expression of hepatitis C virus non-structural 5A protein in the liver of transgenic mice. FEBS Lett. 2003;555:528-32.

95. Battaglia S, Benzoubir N, Nobilet S, Charneau P, Samuel D, Zignego AL, Atfi A, Bréchot C, Bourgeade MF. Liver cancer-derived hepatitis C virus core proteins shift TGF-beta responses from tumor suppression to epithelial-mesenchymal transition. PLoS One. 2009;4(2):e4355.

96. Matsuzaki K, Murata M, Yoshida K, Sekimoto G, Uemura Y, Sakaida N, Kaibori M, Kamiyama Y, Nishizawa M, Fujisawa J, Okazaki K, Seki T. Chronic inflammation associated with hepatitis C

virus infection perturbs hepatic transforming growth factor beta signaling, promoting cirrhosis and hepatocellular carcinoma. Hepatology. 2007;46:48-57.

97. Lonardo A, Adinolfi LE, Loria P, Carulli N, Ruggiero G, Day CP. Steatosis and hepatitis C virus: mechanisms and significance for hepatic and extrahepatic disease. Gastroenterology. 2004 Feb;126:586-97.

98. Pekow JR, Bhan AK, Zheng H, Chung RT. Hepatic steatosis is associated with increased frequency of hepatocellular carcinoma in patients with hepatitis C-related cirrhosis. Cancer. 2007;109:2490-6.

99. Kato J, Miyanishi K, Kobune M, Nakamura T, Takada K, Takimoto R, Kawano Y, Takahashi S, Takahashi M, Sato Y, Takayama T, Niitsu Y. Long-term phlebotomy with low-iron diet therapy lowers risk of development of hepatocellular carcinoma from chronic hepatitis C. J Gastroenterol. 2007;42:830-6.

100. Fujita N, Takei Y. Iron, hepatitis C virus, and hepatocellular carcinoma: iron reduction preaches the gospel for chronic hepatitis C. J Gastroenterol. 2007;42:923-6.

101. Missiha SB, Ostrowski M, Heathcote EJ. Disease progression in chronic hepatitis C: modifiable and nonmodifiable factors. Gastroenterology. 2008;134:1699-714.

102. Mesri EA, Cesarman E, Boshoff C. Kaposi's sarcoma and its associated herpes virus. Nat Rev Cancer. 2010;10:707-19.

103. Wen KW, Damania B. Kaposi sarcoma-associated herpesvirus (KSHV): molecular biology and oncogenesis. Cancer Lett. 2010;289:140-50.

104. Siegel JH, Janis R, Alper JC, Schutte H, Robbins L, Blaufox MD. Disseminated visceral Kaposi's sarcoma. Appearance after human renal homograft operation. JAMA. 1969;207:1493-6.

105. Lebbé C, Legendre C, Francès C. Kaposi sarcoma in transplantation. Transplant Rev (Orlando). 2008;22:252-61.

106. Centers for Disease Control. Kaposi's sarcoma and Pneumocystis pneumonia among homosexual men--New York City and California. MMWR Morb Mortal Wkly Rep. 1981;30:305-8.

107. Gottlieb GJ, Ragaz A, Vogel JV, Friedman-Kien A, Rywlin AM, Weiner EA, Ackerman AB. A preliminary communication on extensively disseminated Kaposi's sarcoma in young homosexual men. Am J Dermatopathol. 1981;3:111-4.

108. Friedman-Kien AE. Disseminated Kaposi's sarcoma syndrome in young homosexual men. J Am Acad Dermatol. 1981 Oct;5(4):468-71.

109. Casper C, Wald A. The use of antiviral drugs in the prevention and treatment of Kaposi sarcoma, multicentric Castleman disease and primary effusion lymphoma. Curr Top Microbiol Immunol. 2007;312:289-307.

110. Wabinga HR, Parkin DM, Wabwire-Mangen F, Mugerwa JW. Cancer in Kampala, Uganda, in 1989-91: changes in incidence in the era of AIDS. Int J Cancer. 1993;54(1):26-36.

111. Gallo RC. The enigmas of Kaposi's sarcoma. Science. 1998;282(5395):1837-9.

112. Beral V, Peterman TA, Berkelman RL, Jaffe HW. Kaposi's sarcoma among persons with AIDS: a sexually transmitted infection? Lancet. 1990;335(8682):123-8.

113. Parkin DM, Sitas F, Chirenje M, Stein L, Abratt R, Wabinga H. Part I: Cancer in Indigenous Africans--burden, distribution, and trends. Lancet Oncol. 2008;9:683-92.

114. Parkin DM. The global health burden of infection-associated cancers in the year 2002. Int J Cancer. 2006;118:3030-44.

115. Eltom MA, Jemal A, Mbulaiteye SM, Devesa SS, Biggar RJ. Trends in Kaposi's sarcoma and non-Hodgkin's lymphoma incidence in the United States from 1973 through 1998. J Natl Cancer Inst. 2002;94:1204-10.

116. Tam HK, Zhang ZF, Jacobson LP, Margolick JB, Chmiel JS, Rinaldo C, Detels R. Effect of highly active antiretroviral therapy on survival among HIV-infected men with Kaposi sarcoma or non-Hodgkin lymphoma. Int J Cancer. 2002;98(6):916-22.

117. Giraldo G, Beth E, Haguenau F. Herpes-type virus particles in tissue culture of Kaposi's sarcoma from different geographic regions. J Natl Cancer Inst. 1972;49:1509-26.

118. Chang Y, Cesarman E, Pessin MS, Lee F, Culpepper J, Knowles DM, Moore PS. Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. Science. 1994 ;266:1865-9.

119. Stallone G, Infante B, Grandaliano G, Schena FP, Gesualdo L. Kaposi's sarcoma and mTOR: a crossroad between viral infection neoangiogenesis and immunosuppression. Transpl Int. 2008;21:825-32.

120. Patel RM, Goldblum JR, Hsi ED. Immunohistochemical detection of human herpes virus-8 latent nuclear antigen-1 is useful in the diagnosis of Kaposi sarcoma. Mod Pathol. 2004;17:456-60.

121. Patel RM, Goldblum JR, Hsi ED. Response to Pantanowitz et al. Regarding our paper on the utility of the detection of HHV-8 LNA-1 by

immunohistochemistry in the diagnosis of Kaposi's sarcoma. Mod Pathol. 2005;18:1011-2.

122. Mbulaiteye SM, Biggar RJ, Goedert JJ, Engels EA. Immune deficiency and risk for malignancy among persons with AIDS. J Acquir Immune Defic Syndr. 2003;32:527-33.

123. Barozzi P, Luppi M, Facchetti F, Mecucci C, Alù M, Sarid R, Rasini V, Ravazzini L, Rossi E, Festa S, Crescenzi B, Wolf DG, Schulz TF, Torelli G. Post-transplant Kaposi sarcoma originates from the seeding of donor-derived progenitors. Nat Med. 2003;9:554-61.

124. Cesarman E, Chang Y, Moore PS, Said JW, Knowles DM. Kaposi's sarcoma-associated herpesvirus-like DNA sequences in AIDS-related body-cavity-based lymphomas. N Engl J Med. 1995;332:1186-91.

125. Soulier J, Grollet L, Oksenhendler E, Cacoub P, Cazals-Hatem D, Babinet P, d'Agay MF, Clauvel JP, Raphael M, Degos L, et al. Kaposi's sarcoma-associated herpesvirus-like DNA sequences in multicentric Castleman's disease. Blood. 1995;86:1276-80

126. Dupin N, Fisher C, Kellam P, Ariad S, Tulliez M, Franck N, van Marck E, Salmon D, Gorin I, Escande JP, Weiss RA, Alitalo K, Boshoff C. Distribution of human herpesvirus-8 latently infected cells in Kaposi's sarcoma, multicentric Castleman's disease, and primary effusion lymphoma. Proc Natl Acad Sci U S A. 1999;96:4546-51.

127. Ditter, D. KSHV viral latent lifecycle, in: Damania B and Pipas J. (Eds) DNA Tumor Viruses, Springer, 2008.

128. Verma SC, Lan K, Robertson E. Structure and function of latency-associated nuclear antigen. Curr Top Microbiol Immunol. 2007;312:101-36.

129. Rainbow L, Platt GM, Simpson GR, Sarid R, Gao SJ, Stoiber H, Herrington CS, Moore PS, Schulz TF. The 222- to 234-kilodalton latent nuclear protein (LNA) of Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) is encoded by orf73 and is a component of the latency-associated nuclear antigen. J Virol. 1997;71:5915-21.

130. Friborg J Jr, Kong W, Hottiger MO, Nabel GJ. p53 inhibition by the LANA protein of KSHV protects against cell death. Nature. 1999;402:889-94.

131. Rigoni-Stern, D. Fatti statistici relative alle mallatie cancrosi che servirono de base alla poche cose dette dal cott. Giornale service propr.pathol.terap.ser. 1842;2:507-517.

132. zur Hausen H. Papillomaviruses in the causation of human cancers - a brief historical account. Virology. 2009;384:260-5.

133. Vonka V, Kanka J, Jelinek J, Subrt I, Suchanek A, Havrankova A, Vachal M, Hirsch I, Domorazkova, A., Savadova, H, et al. Prospective study on the relationship between cervical neoplasia and herpes simplex type-2 virus. I. Epidemiological characteristics. Int J Cancer 1984;33:49-60.

134. Vonk V, Kanka J, Hirsch I, Zavadova H, Kremar M, Suchankova A, Resakova D, Broucek J, Press M, Domorazkova E, Svoboda B, Havrankova A, Jelinek J.
Prospective study on the relationship between cervical neoplasia and herpes simplex type-2. II Herpes simplex virus type-2 antibody presence in sera taken at enrollment. Int J Cancer 1984;33:61-66.

135. Gissmann L, DeVilliers E-M, zur Hausen H. Analysis of human genital warts (condylomata acuminata) and other genital tumors for human papillomavirus type 6 DNA. Int J Cancer 1982;29, 143-146.

136. Gissmann L, Wolnik L, Ikenberg H, Koldovsky U, Schnurch HG, zur Hausen H.
Human papillomavirus type 6 and 11 sequences in genital and laryngeal papillomas and in some cervical cancers. Proc. Nat Acad Sci USA 1983;80:560-563.

137. Hildeshein A, Han CL, Brinton LA, Kurman JJ, Schiller JT. Human papillomavirus type 16 and risk of preinvasive and invasive vulvar cancer: results from a seroepidemiological case-control study. Cancer Epidemiol Biomark Prev 1997;6:807-813.

138. Hakim AA, Dinh TA. Worldwide impact of the human papillomavirus vaccine. Curr Treat Options Oncol. 2009;10:44-53.

139. Georgieva S, Iordanov V, Sergieva S. Nature of cervical cancer and other HPV - associated cancers. J BUON. 2009;14:391-8.

140. Madsen BS, Jensen HL, van den Brule AJ, Wohlfahrt J, Frisch M. Risk factors for invasive squamous cell carcinoma of the vulva and vagina--population-based case-control study in Denmark. Int J Cancer. 2008;122:2827-34.

141. Rubin MA, Kleter B, Zhou M, Ayala G, Cubilla AL, Quint WG, Pirog EC. Detection and typing of human papillomavirus DNA in pen ile carcinoma: evidence for multiple independent pathways of penile carcinogenesis. Am J Pathol 2001;159:1211-1218.

142. Bezerra AL, Lopes A, Santiago GH, Ribeiro KC, Latorre M R, Villa LL. Human papillomavirus as a prognostic factor in carcinoma of the penis: analysis of 82 patients treated with amputation and bilateral lymphadenectomy. Cancer 2001;91:2315-2321.

143. Lont AP, Kroon BK, Horenblas S, Gallee MP, Berkhof J, Meije, CJ, Snijders PJ. Presence of high-risk human papillomavirus DNA in penile carcinoma predicts favorable outcome in survival. Int J Cancer 2006;119:1078-1081.

144. International Agency for Research on Cancer (IARC), 1995. Monographs on the Evaluation of Carcinogenic Risks to Humans. Human Papillomaviruses. Vol. 64. 1995, Lyon, France.

145. Frisch M, Biggar RJ. Aetiological parallel between tonsillar and anogenital squamous-cell carcinomas. Lancet 1999;354:1442-1443.

146. Hoots BE, Palefsky JM, Pimenta JM, Smith JS. Human papillomavirus type distribution in anal cancer and anal intraepithelial lesions. Int J Cancer. 2009;124:2375-83.

147. Smith JS, Backes DM, Hoots BE, Kurman RJ, Pimenta JM. Human papillomavirus type-distribution in vulvar and vaginal cancers and their associated precursors. Obstet Gynecol. 2009;113:917-24.

148. Li N, Franceschi S, Howell-Jones R, Snijders PJ, Clifford GM. Human papillomavirus type distribution in 30,848 invasive cervical cancers worldwide: Variation by geographical region, histological type and year of publication. Int J Cancer. 2011;128:927-35.

149. Sjoeborg KD, Tropé A, Lie AK, Jonassen CM, Steinbakk M, Hansen M, Jacobsen MB, Cuschieri K, Eskild A. HPV genotype distribution according to severity of cervical neoplasia. Gynecol Oncol. 2010;118:29-34.

150. Howell-Jones R, Bailey A, Beddows S, Sargent A, de Silva N, Wilson G, Anton J, Nichols T, Soldan K, Kitchener H; Study Group Collaborators. Multi-site study of HPV type-specific prevalence in women with cervical cancer, intraepithelial neoplasia and normal cytology, in England. Br J Cancer. 2010;103:209-16.

151. Nash G, Allen W, Nash S. Atypical lesions of the anal mucosa in homosexual men. JAMA 1986;256:873-876.

152. Scheurlen W, Stremlau A, Gissmann L, Hohn D, Zenner HP, zur Hausen H.
Rearranged HPV 16 molecules in an anal and in a laryngeal carcinoma. Int J Cancer 1986;38:671-676.

153. Hill SA, Coghill SB. Human papillomavirus in squamous carcinoma of anus. Lancet 1986;2:1333.

154. Daling JR, Weiss NS, Hislop TG, Maden C, Coates RJ, Sherman KJ, Ashley RL. Beagrie, M, Ryan JA, Corey L,. Sexual practices, sexually transmitted diseases, and the incidence of anal cancer. N. Engl J Med 1987;317:973-977.

155. WHOCT, 2000. Pathology and Genetics of Tumours of the Digestive System. 2000 IARC Press, Lyon.

156. Syrjanen K, Syrjanen S, Lamberg M, Pyrhonen S, Nuttinen J. Morphological and immunohistochemical evidence suggesting human papillomavirus (HPV) involvement in oral squamous cell carcinogenesis. Int J Oral surg 1983;12:418-24.

157. Löning T, Ikenberg H, Becker J, Gissmann L, Hoepfer I, zur Hausen H. Analysis of oral papillomas, leukoplakias, and invasive carcinomas for human papillomavirus type related DNA. J Invest Dermatol. 1985;84:417-20.

158. Stelow EB, Jo VY, Stoler MH, Mills SE. Human papillomavirus-associated squamous cell Carcinoma of the upper aerodigestive tract. Am J Surg Pathol. 2010;34:e15-24

159. Shiboski CH, Schmidt BL, Jordan RC. Tongue and tonsil carcinoma: increasing trends in the U.S. population ages 20-44 years. Cancer. 2005;103:1843-9.

160. Herrero R, Castellsagué X, Pawlita M, Lissowska J, Kee F, Balaram P, Rajkumar T, Sridhar H, Rose B, Pintos J, Fernández L, Idris A, Sánchez MJ, Nieto A, Talamini R, Tavani A, Bosch FX, Reidel U, Snijders PJ, Meijer CJ, Viscidi R, Muñoz N, Franceschi S; IARC Multicenter Oral Cancer Study Group. Human papillomavirus and oral cancer: the International Agency for Research on Cancer multicenter study. J Natl Cancer Inst. 2003;95:1772-83.

161. Hennessey PT, Westra WH, Califano JA. Human papillomavirus and head and neck squamous cell carcinoma: recent evidence and clinical implications. J Dent Res. 2009;88:300-6.

162. Kreimer AR, Clifford GM, Boyle P. Human papillomavirus types in head and neck squamous cell carcinomas worldwide: a systematic review. Cancer Epidemiol Biomarkers Prev 2005;14:467-75.

163. D'Souza G, Kreimer AR, Viscidi R, Pawlita M, Fakhry C, Koch WM, Westra WH, Gillison ML. Case-control study of human papillomavirus and oropharyngeal cancer. N Engl J Med. 2007;356:1944-56.

164. Mannarini L, Kratochvil V, Calabrese L, Gomes Silva L, Morbini P, Betka J, Benazzo M. Human Papilloma Virus (HPV) in head and neck region: review of literature. Acta Otorhinolaryngol Ital. 2009;29:119-26.

165. Sturgis EM, Cinciripine PM. Trends in head an neck cancer incidence in relation to smoking prevalence: an emerging epidemic of human papillomavirus-associated cancers? Cancer 2007;110:1429-1435.

166. Rose Ragin CC, Taioli E. Second primary head and neck tumor risk in patients with cervical cancer – SEER data analysis. Head Neck 2008;30:58-66.

167. Gillison ML, D'Souza G, Westra W, Sugar E, Xiao W, Begum S, et al. Distinct risk factor profiles for human papillomavirus type 16-positive and human papillomavirus type 16-negative head and neck cancers. J Natl Cancer Inst 2008;100:407-20.

168. Watts DH, Koutsky LA, Holmes KK, Goldman D, Kuypers J, Kiviat NB, Galloway DA. Low risk of perinatal transmission of human papillomavirus: results from a prospective cohort study. Am J Obstet Gynecol 1998;178:365-73.

169. Koch A, Hansen SV, Nielsen NM, Palefsky J, Melbye M. HPV detection in children prior to sexual debut. Int J Cancer 1997;73:621-4.

170. Rosenquist K, Wennerberg J, Schildt EB. Oral status, oral infections and some lifestyle factors as risk factors for oral and oropharyngeal squamous cell carcinoma. A population-based case- control study in southern Sweden. Acta Otolaryngol 2005;125:1327-36.

171. Rajkumar T, Sridhar H, Balaram. Oral cancer in Southern India: the influence of body size, diet, infections and sexual practices. Eur J Cancer Prev 2003;12:135-43.

172. Frisch M, Biggar RJ. Aetiological parallel between tonsillar and anogenital squamous-cell carcinomas. Lancet 1999;354:1442-3.

173. Trottier H, Burchell AN. Epidemiology of mucosal human papillomavirus infection and associated diseases. Public Health Genomics. 2009;12:291-307,

174. Syrjanen S. Human papillomavirus (HPV) in head and neck cancer. J Clin Virol 2005;32(Suppl 1):S59-66.

175. Munoz N, Bosch FX, Castellsague X. Against which human papillomavirus types shall we vaccinate and screen? The international perspective. Int J Cancer 2004;111:278-85.

176. Gillison ML, Koch WM, Capone RB. Evidence for a casual association between human papillomavirus and subset of head and neck cancers. J Natl Cancer Inst 2000;92:709-20.

177. Havre PA, Yuan J, Hedrick L, Cho KR, Glazer PM. P53 Inactivation by HPV16 E6 results in increased mutagenesis in human cells. Cancer Res 1995;55:4420-4.

178. Solinas-Toldo S, Durst M. Lichter P. Specific chromosomal imbalances in human papillomavirus- transfected cells during progression toward immortality. Proc Natl Acad Sci USA 1997;94:3854-9.

179. Heselmeyer K, Schrock E, Du Manoir S, Blegen H, Shah K, Steinbeck R, et al. Gain of chromosome 3 q defines the transition from severe dysplasia invasive carcinoma of the uterine cervix. Proc Natl Acad Sci USA 1996;93:479-84.

180. Longworth MS, Laimins LA. The binding of histone deacetylases and the integrity of zinc finger- like motifs of the e7 protein are essential for the life cycle of human papillomavirus type 31. J Virol 2004;78-:3533-41.

181. Zhang B, Chgen W, Roman A. The E7 proteins of low- and high-risk human papillomaviruses share the ability to target the pRB family member p130 for degradation. Proc Natl Acad Sci USA 2006;103:437-42.

182. Chen Z, Storthz KA, Shilitoe EJ. Mutations in the long control region of human Papillomavirus DNA in oral cancer cells and their functional consequences. Cancer Res 1997;57:1614-9.

183. Reznikoff CA, Belair C, Savelieva E, Zhai Y, Pfeifer K, Yeager T, et al. Long-term genomestability and minimal genotypic and phenotypic alterations in HPV16 E7-, but not E6-,immortalized human uroepithelial cells. Genes Dev 1994;8:2227-40.

184. Mellin H, Dhlgren L, Munck-Wikland E, Lindholm J, Rabani H, Kalantari M, et al. Human papillomavirus type 16 is episomal and a high viral load may be correlated to better prognosis in tonsillar cancer. Int J Cancer 2002;102:152-8.

185. Van Tine BA, Dao LD, Wu SY, Sonbuchner TM, Lin BY, Zou N, Chiang CM, et al. Human papillomavirus (HPV) origin-binding protein associates with mitotic spindles to enable virus DNA partitioning. Proc Natl Acad Sci USA 2004;101:4030-5.

186. Dijkstra MG, Heideman DA, de Roy SC, Rozendaal L, Berkhof J, van Krimpen K, van Groningen K, Snijders PJ, Meijer CJ, van Kemenade FJ. p16(INK4a) immunostaining as an alternative to histology review for reliable grading of cervical intraepithelial lesions. J Clin Pathol. 2010;63:972-7.

187. Galgano MT, Castle PE, Atkins KA, Brix WK, Nassau SR, Stoler MH. Using biomarkers as objective standards in the diagnosis of cervical biopsies. Am J Surg Pathol. 2010;34:1077-87.

188. Cubilla AL, Lloveras B, Alejo M, Clavero O, Chaux A, Kasamatsu E, Monfulleda N, Tous S, Alemany L, Klaustermeier J, Muñoz N, Quint W, de Sanjose S, Bosch FX. Value of p16(INK)⁴(a) in the pathology of invasive penile squamous cell carcinomas: A report of 202 cases. Am J Surg Pathol. 2011;35:253-61.

189. Patel S, Chiplunkar S. Host immune responses to cervical cancer. Curr Opin Obstet Gynecol. 2009;21:54-9.

190. Pyeon D, Newton MA, Lambert PF, den Boon JA, Sengupta S, Marsit CJ, et al. Fundamental differences in cell cycle deregulation in human papillomavirus-positive and human papillomavirus negative head/neck and cervical cancers. Cancer Res 2007;67;4605-19.

191. Khan MJ, Castle PE, Lorincz AT, Wacholder S, Sherman M, Scott DR, Rush BB, Glass AG, Schiffman M. The elevated 10-year risk of cervical precancer and cancer in women with human papillomavirus (HPV) type 16 or 18 and the possible utility of type-specific HPV testing in clinical practice. J Natl Cancer Inst. 2005;97:1072-9.

192. Matsumoto K, Oki A, Furuta R, Maeda H, Yasugi T, Takatsuka N, Mitsuhashi A, Fujii T, Hirai Y, Iwasaka T, Yaegashi N, Watanabe Y, Nagai Y, Kitagawa T, Yoshikawa H; for Japan HPV And Cervical Cancer (JHACC) Study Group. Predicting the progression of cervical precursor lesions by human papillomavirus genotyping: A prospective cohort study. Int J Cancer. 2010 Aug 23. [Epub ahead of print]

193. Braakhuis BJ, Snijders PJ, Keune WJ, Meijer CJ, Ruijter-Schippers HJ, Leemans CR, Brakenhoff RH.Genetic patterns in head and neck cancers that contain or lack transcriptionally active human papillomavirus. J Natl Cancer Inst 2004;97:998-1006.

194. Smeets SJ, Braakhuis BJ, Abbas S, Snijders PJ, Ylstra B, van de Wiel MA, Meijer
GA, Leemans CR, Brakenhoff RH. Genome-wide DNA copy number alterations in head and neck squamous cell carcinomas with or without oncogene-expressing human papillomavirus. Oncogene 2006;25:2558-2564.

195. Weinberger PM, Yu Z, Haffty BG, Kowalski D, Harigopal M, Brandsma J, Sasaki C,
Joe J, Camp RL, Rimm DL, Psyrri A. Molecular classification identifies a subset of human papillomavirus-associated oropharyngeal cancers with favorable prognosis. J Clin Oncol 2006;24:736-747.

196. Kumar B, Cordell KG, Lee JS, Worden FP, Prince ME, Tran HH, Wolf GT, Urba SG,
Chepeha DB, Teknos TN, Eisbruch A, Tsien CI, Taylor JM, D'Silva NJ, Yang K,
Kurnit DM, Bauer JA, Bradford CR, Carey TE. EGFR, p16, HPV Titer, Bel-xL and p53, sex, and smoking as indicators of response to therapy and survival in oropharyngeal cancer. J Clin Oncol 2008;26:3128-3137.

197. Charfi L, Jouffroy T, de Cremoux P, Le Peltier N, Thioux M, Freneaux P, et al. . Two types of squamous cell carcinoma of the palatine tonsil characterized by distinct etiology, molecular features and outcome. Cancer Lett 2008;260:72-78.

198. Fakhry C, Westra WH, Li S, Cmelak A, Riodge JA, Pinto H, et al. Improved survival of patients with human papillomavirus -positive head and neck squamous cell carcinoma in a prospective clinical trial. J Natl Cancer Inst 2008;100:261-369.

199. Poiesz BJ, Ruscetti FW, Gazdar AF, Bunn PA, Minna JD, Gallo RC. Detection and isolation of type retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. Proc Natl Acad Sci U S A. 1980;77:7415-9.

200. Manns A, Blattner WA. The epidemiology of the human T-cell lymphotrophic virus type I and type II: etiologic role in human disease. Transfusion. 1991;31:67-75.

201. Mahieux R, Gessain A. The human HTLV-3 and HTLV-4 retroviruses: new members of the HTLV family. Pathol Biol (Paris). 2009;57:161-6.

202. Bittencourt AL, de Oliveira Mde F. Cutaneous manifestations associated with HTLV-1 infection. Int J Dermatol. 2010;49:1099-110.

203. Ehrlich GD, Andrews J, Sherman MP, Greenberg SJ, Poiesz BJ. DNA sequence analysis of the gene encoding the HTLV-I p21e transmembrane protein reveals inter- and intraisolate genetic heterogeneity. Virology. 1992;186:619-27.

204. Proietti FA, Carneiro-Proietti AB, Catalan-Soares BC, Murphy EL. Global epidemiology of HTLV-I infection and associated diseases. Oncogene. 2005;24:6058-68.

205. Tsuji Y, Doi H, Yamabe T, Ishimaru T, Miyamoto T, Hino S. Prevention of mother-to-child transmission of human T-lymphotropic virus type-I. Pediatrics. 1990 ;86:11-7.

206. Wiktor SZ, Pate EJ, Rosenberg PS, Barnett M, Palmer P, Medeiros D, Maloney EM, Blattner WA. Mother-to-child transmission of human T-cell lymphotropic virus type associated with prolonged breast-feeding. J Hum Virol. 1997 Nov-Dec;1(1):37-44.

207. 9: Hirata M, Hayashi J, Noguchi A, Nakashima K, Kajiyama W, Kashiwagi S, Sawada T. The effects of breastfeeding and presence of antibody to p40tax protein of human T cell lymphotropic virus type-I on mother to child transmission. Int J Epidemiol. 1992;21:989-94.

208. 10: Oki T, Yoshinaga M, Otsuka H, Miyata K, Sonoda S, Nagata Y. A sero-epidemiological study on mother-to-child transmission of HTLV-I in southern Kyushu, Japan. Asia Oceania J Obstet Gynaecol. 1992;18:371-7.

209. 11: Li HC, Biggar RJ, Miley WJ, Maloney EM, Cranston B, Hanchard B, Hisada M. Provirus load

in breast milk and risk of mother-to-child transmission of human T lymphotropic virus type I. J Infect Dis. 2004;190:1275-8.

210. 12: Takezaki T, Tajima K, Ito M, Ito S, Kinoshita K, Tachibana K, Matsushita Y. Short-term breast-feeding may reduce the risk of vertical transmission of HTLV-I. The Tsushima ATL Study Group. Leukemia. 1997;11 Suppl 3:60-2.

211. 13: Khabbaz RF, Darrow WW, Hartley TM, Witte J, Cohen JB, French J, Gill PS, Potterat J, Sikes RK, Reich R, et al. Seroprevalence and risk factors for HTLV-I/II infection among female prostitutes in the United States. JAMA. 1990;263:60-4.

212. 14: Osame M, Janssen R, Kubota H, Nishitani H, Igata A, Nagataki S, Mori M, Goto I, Shimabukuro H, Khabbaz R, et al. Nationwide survey of HTLV-I-associated myelopathy in Japan: association with blood transfusion. Ann Neurol. 1990;28:50-6.

213. 15: Gonçalves DU, Proietti FA, Ribas JG, Araújo MG, Pinheiro SR, Guedes AC, Carneiro-Proietti AB. Epidemiology, treatment, and prevention of human T-cell leukemia virus type 1-associated diseases. Clin Microbiol Rev. 2010;23:577-89.

214. 16: Lee R, Schwartz RA. Human T-lymphotrophic virus type 1-associated infective dermatitis: a comprehensive review. J Am Acad Dermatol. 2011;64:152-60.

215. 17: LaGrenade L, Hanchard B, Fletcher V, Cranston B, Blattner W. Infective dermatitis of Jamaican children: a marker for HTLV-I infection. Lancet. 1990;336:1345-7.

216. 18: La Grenade L, Manns A, Fletcher V, Derm D, Carberry C, Hanchard B, Maloney EM, Cranston B, Williams NP, Wilks R, Kang EC, Blattner WA. Clinical, pathologic, and immunologic features of human T-lymphotrophic virus type I-associated infective dermatitis in children. Arch Dermatol. 1998;134:439-44.

217. 19: Oliveira Mde F, Brites C, Ferraz N, Magalhaes P, Almeida F, Bittencourt AL. Infective dermatitis associated with the human T cell lymphotropic virus type I in Salvador, Bahia, Brazil. Clin Infect Dis. 2005;40:e90-6.

218. Bittencourt AL, Barbosa HS, Vieira MD, Farré L. Adult T-cell leukemia/lymphoma (ATL) presenting in the skin: clinical, histological and immunohistochemical features of 52 cases. Acta Oncol. 2009;48:598-604.

219. Gonçalves DU, Guedes AC, Proietti AB, Martins ML, Proietti FA, Lambertucci JR; Interdisciplinary HTLV-1/2 Research Group. Dermatologic lesions in asymptomatic blood donors seropositive for human T cell lymphotropic virus type-1. Am J Trop Med Hyg. 2003;68:562-5.

220. Brites C, Weyll M, Pedroso C, Badaró R. Severe and Norwegian scabies are strongly associated with retroviral (HIV-1/HTLV-1) infection in Bahia, Brazil. AIDS. 2002;16:1292-3.

221. Lenzi ME, Cuzzi-Maya T, Oliveira AL, Andrada-Serpa MJ, Araújo AQ. Dermatological findings of human T lymphotropic virus type 1 (HTLV-I)-associated myelopathy/tropical spastic paraparesis. Clin Infect Dis. 2003;36:507-13.

222. Tsukasaki K, Yamada Y, Ikeda S, Tomonaga M. Infective dermatitis among patients with ATL in Japan. Int J Cancer. 1994;57:293.

223. Hanchard B, LaGrenade L, Carberry C, Fletcher V, Williams E, Cranston B, Blattner WA, Manns A. Childhood infective dermatitis evolving into adult T-cell leukaemia after 17 years. Lancet. 1991;338:1593-4.

224. Mahé A, Meertens L, Ly F, Sow PS, Diop CT, Samb ND, Diop OM, Valensi F, Gessain A. Human T-cell leukaemia/lymphoma virus type 1-associated infective dermatitis in Africa: a report of five cases from Senegal. Br J Dermatol. 2004;150:958-65.

225. Tschachler E, Franchini G. Infective dermatitis: a pabulum for human T-lymphotrophic virus type I leukemogenesis? Arch Dermatol. 1998;134:487-8.

226. Gonçalves DU, Guedes AC, Carneiro-Proietti AB, Lambertucci JR. HTLV-I associated infective dermatitis may be an indolent HTLV-I associated lymphoma. Braz J Infect Dis. 200;4:100-2.

227. Marriott SJ, Semmes OJ. Impact of HTLV-I Tax on cell cycle progression and the cellular DNA damage repair response. Oncogene. 2005;24:5986-95.

228. Matsuoka M, Green PL. The HBZ gene, a key player in HTLV-1 pathogenesis. Retrovirology. 2009;6:71.

229. Maloney EM, Hisada M, Palmer P, Brooks K, Pate E, Wiktor SZ, Lagrenade L, Manns A. Human T cell lymphotropic virus type I-associated infective dermatitis in Jamaica: a case report of clinical and biologic correlates. Pediatr Infect Dis J. 2000;19:560-5.

230. Carvalho EM, Bacellar O, Porto AF, Braga S, Galvão-Castro B, Neva F. Cytokine profile and immunomodulation in asymptomatic human T-lymphotropic virus type 1-infected blood donors. J Acquir Immune Defic Syndr. 2001;27:1-6.

231. Van den Hove LE, Vandenberghe P, Van Gool SW, Ceuppens JL, Demuynck H, Verhoef GE, Boogaerts MA. Peripheral blood lymphocyte subset shifts in patients with untreated hematological tumors: evidence for systemic activation of the T cell compartment. Leuk Res. 1998;22:175-84.

232. Manns A, Miley WJ, Waters D, Hanchard B, Wharfe G, Cranston B, Williams E, Blattner WA. Prognostic significance of quantitative viral markers in adult T-cell leukemia/lymphoma. Blood. 1999;94:372-3.

233. Okayama A, Stuver S, Matsuoka M, Ishizaki J, Tanaka G, Kubuki Y, Mueller N, Hsieh CC, Tachibana N, Tsubouchi H. Role of HTLV-1 proviral DNA load and clonality in the development of adult T-cell leukemia/lymphoma in asymptomatic carriers. Int J Cancer. 2004;110:621-5.

234. Hisada M, Okayama A, Shioiri S, Spiegelman DL, Stuver SO, Mueller NE. Risk factors for adult T-cell leukemia among carriers of human T-lymphotropic virus type I. Blood. 1998;92:3557-61.

235. Shimoyama M. Diagnostic criteria and classification of clinical subtypes of adult T-cell leukaemia-lymphoma. A report from the Lymphoma Study Group (1984-87). Br J Haematol. 1991;79:428-37.

236. Fujino T, Nagata Y. HTLV-I transmission from mother to child. J Reprod Immunol. 2000;47:197-206.

237. Pawson R, Richardson DS, Pagliuca A, Kelsey SM, Hoque S, Breuer J, Newland AC, Mufti GJ. Adult T-cell leukemia/lymphoma in London: clinical experience of 21 cases. Leuk Lymphoma. 1998;31:177-85.

238. Santos JB, Farré L, Batista Eda S, Santos HH, Vieira MD, Bittencourt AL. The importance of flower cells for the early diagnosis of acute adult T-cell leukemia/lymphoma with skin involvement. Acta Oncol. 2010;49:265-7.

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REFERENCE VALUES OF CD4-LYMPHOCYTE COUNTS IN HIV SERONEGATIVE PREGNANT WOMEN IN BUEA, CAMEROON

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Abstract
Pregnancy is a physiologically immunocompromised state, during which alterations in T-lymphocyte subsets may occur. Reference values for CD4 counts in pregnancy have not been established particularly in sub-Saharan populations. This study aimed at describing expected ('normal') values of CD4 counts in healthy HIV-negative pregnant women so these could serve as reference for assessing the progress of HIV disease in HIV-infected pregnant women.
The study was conducted in antenatal clinics in the Buea Health District, Cameroon. All eligible women were interviewed using a standardized questionnaire. Whole blood samples collected were tested for HIV using Determine 1/2 and SD Bioline HIV-1/2 3.0 rapid tests. The CD4+ absolute counts were assessed using the Partec Cyflow Counter and the CD4 easy count kit. A total of 279 women were analysed. Their ages ranged from 15 to 47 years. A vast majority (95%) of participants were in the second or third trimester of gestation. Slightly less than half (43%) were primiparous. The CD4 cell count ranged from 321 to 1808 cells/µl . This distribution was approximately normal with a mean of 851cells/µl, a median of 831cells/µl , and a standard deviation of 254cells/µl . The expected ('normal') range, covering 95% of the sample was 438-1532 cells/µl. Participants with malaria parasitaemia tended to have a lower CD4 count (lower on average by 115 cells/µl, P<0.001). CD4 cell counts in HIV-negative pregnant women appear similar to those of the general population of HIV-negatives. These values can thus be used as references when assessing HIV-seropositive pregnant women.

Keywords: CD4 counts, HIV-negative, pregnancy

Introduction

CD4 counts are widely used as prognostic markers to assess the degree of immune impairment in HIV seropositive individuals and treatment decisions are based on this. They are also used to monitor antiretroviral therapy (ART)[1,2,3,4,5]. Indeed the most recent WHO guidelines on HIV treatment recommend the use of CD4 counts to determine the need for combination antiretroviral therapy in HIV-positive patients, including pregnant women. In 2005, a predictive values of CD4+ lymphocyte count in adult populations in Nigeria published [6]. Before then, reference values of CD4+-cells were not available from West African populations. Rather, values from publications based on studies in Western countries were largely employed for clinical decision making [6]. Evidence in literature suggest significant geographical and racial differences in these parameters; for example, lower CD4+-cell counts have been recorded for Asians compared to Caucasians [7] and studies in African populations have shown mean CD4+cell counts in healthy Ethiopians that are markedly lower than those in Ugandans and Tanzanians [6].

Reference values for CD4 counts in pregnancy are even less known particularly in sub-Saharan populations. Pregnancy is a physiologically immunocompromised state, during which alterations in T-lymphocyte subsets may occur [8,9]. Normal pregnancy has been described as an immune–suppressed state that is induced so that the foetus would not be rejected by the mother's immune system. However, more recent evidence indicates that the immunology of both pregnancy and the sex steroid hormones is exceedingly complex and not fully understood [9]. The foetus is likely protected not by immune suppression in the mother but by changes in Human Leukocyte Antigen (HLA) expression in the trophoblast.

Despite uncertainties on the variation of CD4 counts during pregnancy, CD4 counts are being used to assess the need for treatment in pregnant HIV-positive women in Cameroon and most other resource limited settings. In practice, the CD4 threshold for starting ART in pregnant women is the same as in non-pregnant populations. We hypothesized that this practice may be erroneous if it were confirmed that lower CD4 counts were to be expected in HIV-negative pregnant women. If the latter were true then the CD4 count threshold for initiating antiretroviral therapy in pregnancy may need to be lower than that in non-pregnant populations. There is therefore a need to establish expected ('normal') values of CD4 counts in healthy HIV-negative pregnant women so these could serve as reference for assessing the degree of progress in HIV disease in HIV-infected pregnant women.

Materials and Methods

This study was conducted in antenatal clinics (ANCs) in the Buea Health District of Cameroon. Two clinics, the Buea Road Health Centre (BRHC) and the Provincial Hospital Annex Buea (PHAB) were used. A previous survey of the ANCs in the

health district showed that these two ANCs had the highest number of pregnant women for prenatal care. Pregnant women attending these two ANCs were eligible and those who consented were included in the study. All eligible and consenting women were interviewed using a standardized questionnaire. After which about 5ml of venous blood was collected by venipuncture from each participant into EDTA tubes. These samples were transported from collection sites to the Faculty of Health Science Laboratory, University of Buea, in a cooler containing ice.

Once in the laboratory, the samples were tested immediately for HIV using Determine HIV1/2 (Abbott Co. Ltd. , Japan) and SD Bioline HIV-1/2 3.0 rapid tests (Standard Diagnostics, INC., USA) This was done in conformity with the WHO recommendation Strategy 2 ; which states that HIV can be diagnosed using two rapid tests [10] and this is the diagnostic strategy used in HIV Treatment Centers in Cameroon. The CD4+ absolute counts were done using the Partec Cyflow Counter and the CD4 easy count kit (Partec GmbH, Germany).

The samples were also tested for malaria using a fluorescent microscope (Partec GmbH, Germany). 10ul of whole blood from the EDTA tubes was placed onto the dried-in reagent spot on the test slides. The slides were tilted in all directions in order to distribute the drop of blood. Slides were covered with a cover slip and incubated for 5 minutes. The slides were then placed under the Cyscope. The UV light was switched on and the prescence or not of parasites observed.

Data Analysis

Data collected were entered into an MS Access interface on Epi-info. Statistical analysis was conducted using STATA version 9 (STATA Corp., College Station, Texas, USA). Participants' demographic characteristics and the variations in the CD4 cell counts in the whole sample were analysed. Variations in mean CD4 cell count (and its variance) by participant characteristics were then compared using Student's t-tests (for characteristics with two categories) and ANOVA tests (for characteristics with more than 2 categories). A priori, the statistical significance level was set at 0.05 while any difference in CD4 count of 100 or more was to be considered meaningful.

Ethical considerations

Ethical clearance for this study was obtained from the Regional Delegation of Public Health, Buea, Cameroon. Participation in this study was voluntary. A signed consent form was sought from all participants. All procedures were standard and only involved minimal risk to the participants. Study results were returned to patients and incorporated into their care by their respective providers.

Results

A total of 307 women were approached. Of these, 12(3.9%) participants did not consent to HIV testing. Sixteen (5.2%) of the remaining 295 women tested positive for HIV. All HIV-positive cases were excluded from this analysis. The age of participants ranged from 15 to 47 years with a mean (+/- standard deviation) of 26 years (+/- 5.56). Table 1 summarises the participants' characteristics. A vast majority (95%) of participants were either in the second or third trimester of gestation. Slightly less than half (43%) were primiparous. Questions on the means of accommodation (used as indicators of socio-economic status) revealed that 15% were living with relatives.

The CD4 cell counts ranged from 321 to 1808 cells/µl of blood. The distribution of CD4 counts is shown in Figure 1. This distribution was approximately normal with a mean of 851cells/µl , a median of 831cells/µl , and a standard deviation of 254cells/µl . The 2.5^{th} percentile and 97.5^{th} percentiles were estimated to be 438cells/µl and 1532cells/µl respectively, suggesting an expected ('normal') range, covering 95% of the sample, of 438 to1532 cells/µl .

Table 2 shows the variations in the mean CD4 cell counts by participants' characteristics. Overall, there was no substantial (nor statistically significant) variation in CD4 count with characteristics other than malaria. The mean CD4 cell count (SD) in malaria negative pregnant women was 927(275) cells/µl as compared to 818(236) cells/µl in malaria positive women.

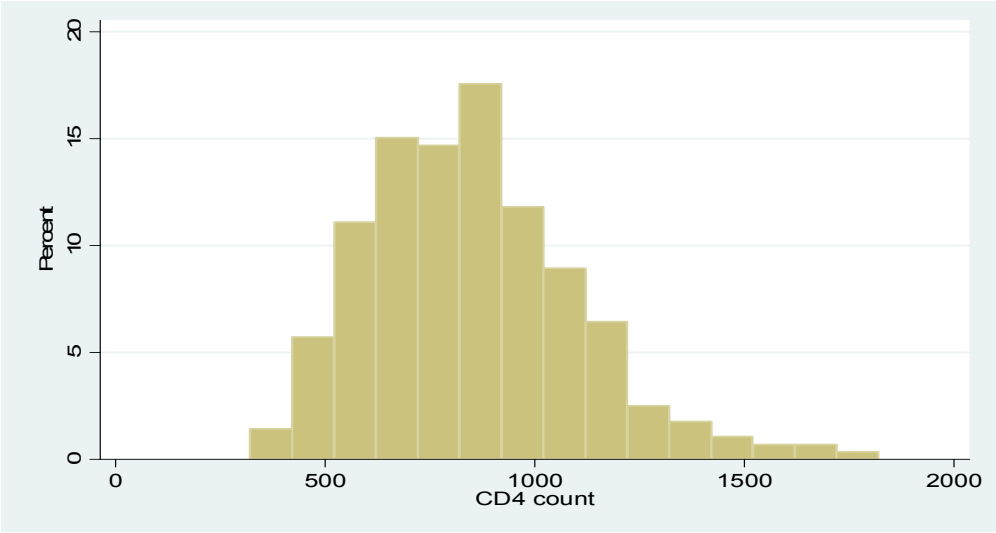
Figure 2 depicts the CD4 count distribution by malaria status. Malaria positive women still had a significantly lower CD4 cell count than malaria negative women: an average difference of 115 cells/µl ($P < 0.001$), even after controlling for other participant characteristics.

TABLE 1: SUMMARY OF PARTICIPANTS' CHARACTERISTICS

Characteristics	Number (%)*
Period of gestation	
1st Trimester	15 (5.38)
2nd Trimester	149 (53.41)
3rd Trimester	115 (41.22)
Gravidity	
1	119 (42.65)
2	52 (18.64)
3	46 (16.49)
4	34 (12.19)
≥5	28 (10.05)
Marital Status	
Single	61 (22.18)
Married	184 (66.91)
Divorced	9 (3.27)
Widow	21 (7.64)
Education	
No Education	9 (3.23)
Primary	86 (30.82)
Secondary	123 (44.09)
Tertiary	61 (21.86)
Means of accommodations	
Living with relative	42 (15.11)
Renting	187 (67.27)
Living in your own house	49 (17.63)

*percentage based on number of respondents

FIGURE 1: CD4 COUNT DISTRIBUTION IN 279 HIV-NEGATIVE PREGNANT WOMEN IN BUEA



Discussion

This study aimed at describing reference values of CD4 counts in HIV-negative pregnant women in Buea, South West Region of Cameroon, these CD4 values were found to be normally distributed and similar to values described for non-pregnant HIV negative women in the literature. The study also established that demographic and socio-economic characteristic did not influence CD4 counts in pregnancy.

The mean CD4 values found in this population were within the range of values reported in other HIV-negative populations. The mean CD4 count was higher, by 80 cells/µl, than the 771 (250) cells/µl , reported by Aina et al in 2005 in a population of HIV-negative adults in neighbouring Jos, Nigeria [6]. In the Central African Republic, the mean CD4 value in non-pregnant females was 940cells/µl [3] a value which was higher, by 89 cells/µl, than that observed in our study of pregnant women. In Botswana mean CD4 T-lymphocyte counts (+/- SD) in HIV-seronegative non-pregnant females was

827(+/- 245), [Bussmann *et al*, 2004]. Normal values for CD4 and CD8 T lymphocyte subsets in Shanghai was 727/540 cells / ul Jiang *et al,* 2004. In another study, mean CD4 T cell values (+/- standard deviation) for adult populations in other non-African population were; Chinese 812(+/- 255), Malaysian 856 (+/- 308), Indian 958(+/-296) and Hong Kong 812(+/-256) [5].

Similar to our findings, other studies have found little or no relationships between socio-demograhic characteristics and CD4 count levels. A study [6] found no association between gestational ages with CD4 counts. In another investigation carried out on peripheral blood lymphocyte subsets in adolescents, age had no effect on the CD4 + cell counts in the female participants and was found to have no correlation with CD4 lymphocyte count [12].

In this study malaria positive pregnant women tended to have significantly lower CD4 counts. This has also been reported in non-pregnant adult populations [13], and in HIV and malaria positive adults[Chalwe *et al,* 2009]. In these studies the CD4 count in malaria positive participants was lower by about 300cells/µl and more than 400cells/µl respectively. These lower CD4 counts in malaria could be a result of malaria infection impairing the normal functioning of the immune system [10,15]. Pregnant women are susceptible to malaria during pregnancy. *Plasmodium falciparum* sequesters in the placenta. Parasitized cells in the placenta express unique variant surface antigens (VSA) and lack of immunity to these pregnancy-specific variant surface antigens explains some of the pregnancy-associated malaria susceptibility. Peripheral blood T-cell responses may be decreased in malaria and this may be due to trafficking of memory T-cells out of the circulation [16].

FIGURE 2: CD4 COUNT BY MALARIA STATUS

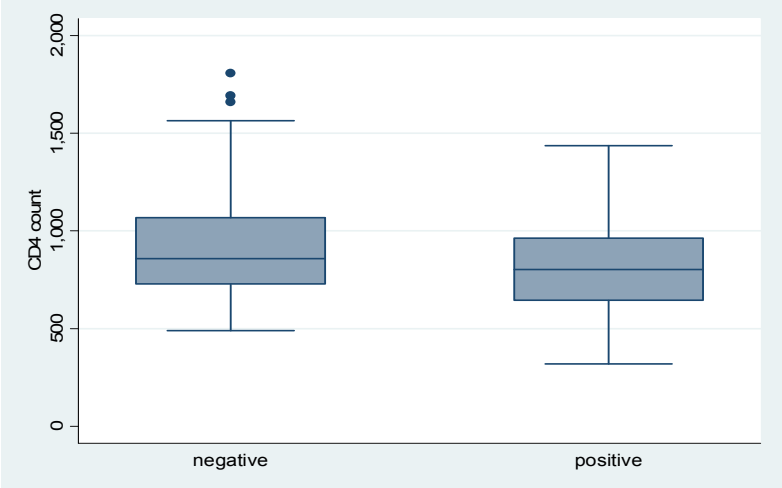


TABLE 2: VARIATIONS IN CD4 LYMPHOCYTE COUNTS N HIV-NEGATIVE PREGNANT WOMEN

Characteristics	Level	N	Mean CD4	SD	P-value	Adjusted mean difference in CD4	95% CI	P-value
Age	25 or less > 25 years	139 137	846 856	275 233	0.75	21 Ref	-55,97	0.58
Gestation	1st Trimester 2nd Trimester 3rd Trimester	15 149 115	837 842 865	324 242 259	0.75	Ref 37 60	- 101,174 -79,199	 0.60 0.40
Gravidity	Primiparous Not primiparous	119 160	841 858	267 244	0.59	Re 27	-49,103	0.49
Marital Status	Married Not Married	184 91	850 862	242 277	0.71	Ref 37	-38,112	0.33
Education	Less than secondary Secondary or more	95 184	848 853	273 244	0.88	03 Ref	-64,71	0.93
Accommodation	Living with relative Renting Living in own house	42 187 49	840 852 856	227 244 314	0.95	Ref 35 42	 -63,132 -79,162	 0.49 0.50
Phone ownership	No Yes	61 218	826 858	257 253	0.39	Ref 36	-43,115	0.37
Malaria status	Negative Positive	85 194	927 818	275 236	<0.001	115 Ref	48,181	0.001

***adjusted for all listed characteristics, obtained using linear regression. Ref=reference level in the comparison of means**

If confirmed that pregnant malaria positive women tended to have a lower CD4 count, the malaria status would need to also be taken into account when assessing the need for antiretroviral therapy in an HIV-positive woman. Given the cross-sectional nature of the study, we could not establish the long-term evolution of CD4 counts in these malaria positive women. It will be interesting to know whether the low-CD4 counts are only transient and increase after malaria treatment. In which case, in assessing HIV-positive women, a repeat CD4 assay may be needed in the pregnant women who are also malaria positive, before deciding if they need to be placed on antiretroviral therapy.

This study's findings may however be hampered by some limitations. First, the study was conducted in only one health district and may thus not be representative of the whole country. Secondly, HIV serostatus was determined using rapid tests only – some cases of acute HIV may have been misdiagnosed as being HIV-negative. We suspect that this would have resulted only in a slight underestimation of the mean CD4 counts as the cases of acute HIV would not yet have started depleting their CD4 counts.

Conclusion

Most studies have reported mean CD4 values of normal adult populations and non pregnant females. Reference values for HIV-negative pregnant women are rare in the literature. In this study we report reference values of mean CD4 T-cell counts of pregnant women in Buea, Cameroon. These values were similar to those of the general population of non-pregnant HIV-negative women. Knowledge of these values will be clinically useful for the monitoring of HIV-seropositive pregnant women.

References

1. Dayama, A,, Pandit ,D., Mudaliar, S., Bharadwaji, R. (1994). A pilot study on CD4 and CD8 cell counts in Healthy HIV seronegative pregnant women. *AIDS* 8 (6): 843-7.
2. Anglaret, X., Diagbouga, S., Mortier, E., Meda, N., Verge-Valette, V., Sylla-Koko, F., Cousens, S, Laruche, G., Ledru, E., Bonard, D., Dabis, F., Van de Perre, P. (1997). CD4+ T-lymphocyte counts in HIV infection: are European Standards applicable to African Patients? *J Acquir Immune Defic Syndr Hum Retroviro*, 14 (4):361-7.
3. Menard, D., Mandeng, M. J., Tothy, M. B., Kelembho, K., Gresenguet, G., Talarmin, A. (2003). Immunohaematological Reference Ranges for Adults from the Central African Republic. *Clinical and Diagnostic Laboratory Immunology*. 10(3): 443-

4. Bussmann, H., William, W. C., Masupu, K. V., Peter, T., Gaolekwe, S. M., Kim, S., Reich, A. M., Ahn, S., Wu, Y., Thior, I., Essex, M., and Richard, M. (2004). Low CD4+ T-lymphocyte values in Human Immunodeficiency Virus-Negative Adults in Botswana. Clin *Diagn* Lab *Immunol* 11(5): 930-935.
5. Ching, W. J., Tan, G. B., and Kuperan, P. (2004). Establishment of Adult Peripheral Blood Lymphocyte Subset Reference Range for Asian Population by Single-Platform Flow Cytometry: Influence of Age, Sex and Race and Comparison with other Published Studies. *Clinical and Diagnostic Laboratory Immunology* 11(1): 168-173.
6. Aina, O., Dadik, J., Charurat, M., Amangaman, P., Gurumd, S., Mang, E., Guvit, R., Ndam, L., Datong, P., Daniyam, C., Kanki, P., Abimiku, A. (2005). Reference Values of CD4 T lymphocytes in Human Immunodefficiency Virus-Negative Adult Nigerians. *Clinical and Diagnostic Laboratory Immunology* 12(4): 525-53.
7. Wotring, L. L., Montgomery, J. P., Mokotoff, E. D., Markowitz, N., Lawrence, R. C. (2005). Pregnancy and Other factors Associated with Higher CD4+ T-cell Counts at HIV Diagnosis in Southeast Michigan 1992-2002. *MedGenmed* .7(1): 1.
8. Dayama, A., Pandit, D., Mudaliar, S., Bharadwaji, R., Shrotri, A. N. and Joshi, S. (2003). A pilot study on CD4 and CD8 cell count in healthy HIV seronegative pregnantwomen. *Indian J.Med. Res* 117: 198-200.
9. Anastos, K. (2007). Pregnancy Does Not Accelerate HIV in Women. Good News For women living with HIV. *The Journal of Infectious Diseases* 196: 971-973.
10. Cheesebrough, M. (2007). District Laboratory Practice in Tropical Countries, part 2, 2nd Edition, Cambridge University Press, London.
11. Jiang, W., Kang, L., Lu, H-Z., Pan, X., Lin, Q., Pan, Q., Xue, Y., Weng, X., and Tang, Y-W. (2004). Normal Values for CD4 Lymphocyte Subsets in Healthy Chinese Adults from Shanghai. *Clinical and Diagnostic Laboratory Immunology*,11(4) :811-813.
12. Rudy, B. J., Wilson, C. M., Durako, S., Moscick, A-B., Muenze, L., and Douglas, S. D. (2002). Peripheral Blood Lymphocyte Subsets in Adolescents: a Longitudinal Analysis from the REACH Project. *Clinical and Diagnostic Laboratory Immunology* 9(5): 959-965.
14. Chalwe, V., Vangeertruyden, J-P., Mukwamataba, D., Menten, J., Kamalamba, J., Mulenga, M., D'Alessandro, U. (2009). Increased Risk for Severe malaria in HIV-1 infected Adults , Zambia. *Emerging Infectious Diseases* 15(5).
15. Kfutwah, A. , Mary, J. Y. , Lemen, B. , Leke, R. , Rousset, D.. (2009). *Plasmodium falciparum* Infection Significantly Impairs Placental Cytokine Profile in HIV infected Cameroonian Women'. *PLos ONE* 4(12):e8114.doi:10. 1371/journal.pone.0008114

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CYTOMEGALOVIRUS IN IMMUNOSUPPRESSED PATIENTS: A SILENT AND POTENTIAL KILLER.

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SUMMARY
Cytomegalovirus (CMV) is a recognized cause of morbidity and mortality among immunocompromised individuals. This review will concentrate on understanding the pathogenesis, clinical manifestations and laboratory diagnostic options for CMV infection.

KEY WORDS- REVIEW, CYTOMEGALOVIRUS, IMMUNOSUPPRESSED

INTRODUCTION

Human Cytomegalovirus (CMV) is an enveloped DNA virus, a potential killer or a lifelong silent killer (1). CMV infections are common and are usually asymptomatic however clinically ignificant infections are encountered frequently in pregnant women, neonates and immunocompromised patients (2). CMV is an important cause of orbidity and mortality among immunosuppressed patients. After a primary infection, which usually occurs during childhood, CMV reactivation occurs when the host immune system is compromised, either through infection such as, HIV immaturity(neonate) or through other iatrogenic means following organ transplantation, the virus is able to exert its full pathogenic potential (3)
CMV is one of the commonest viral opportunistic infection in persons with Acquired Immune Deficiency Syndrome (AIDS). Clinical disease due to CMV has been recognized in up to 5-25% of patients with AIDS (4). Prior to the advent of Highly Active Antiretroviral Therapy (HAART) for HIV infection, CMV retinitis was the most common cause of blindness in adults with AIDS (5). CMV is the most common cause of congenital malformation resulting from viral intrauterine infection in developed countries (6).

Virus associated leucopenia is common in solid organ transplant recipients, also seen are obliterative bronchiolitis in lung transplant ,graft atherosclerosis after heart transplantation and Cytomegalovirus-related rejection of renal allografts[2] . Clinical trials have shown that preventive approaches utilizing antiviral agents have lead to a reduction in the rates of CMV infection and disease and indirect sequelae associated with CMV. Currently, prophylaxis or periodic monitoring and antiviral therapy targeted towards patients with viral replication are routinely employed at many transplant centers.
The present article is meant to highlight the important features of the Cytomegaloviruses and also to increase the level of awareness on their potential to cause life threatening infections among the immunocompromised patients.

STRUCTURE OF CMV

Cytomegalovirus is a DNA virus belonging to the family Herpesviridae and the subfamily, Beta Herpesviridae which also includes the Roseolovirus and the Muromegalovirus. CMV is morphologically indistinguishable from other human Herpes viruses (7). Surface projections of envelope are distinct and spikes are dispersed evenly over the entire surface. The nucleocapsid is isometric, and surrounded by the tegument that consists of globular material which is frequently asymmetrically distributed and may be variable in amount (8). The nucleocapsid is sometimes penetrated by stain (although an intact envelope impermeable is to stain). Viral nucleocapsid is angular and has an icosahedral symmetry. There are 162 capsomeres per nucleocapsid, and capsomeres are hexagonal in cross section with a hole running half way down the long axis. The viral core consists of a fibrillar spool on which DNA is wrapped while the ends of the fibres are anchored to the underside of the capsid shell. Incomplete virus particles often present, and they represent capsids lacking the envelope.
CMV is inactivated by a number of physical and chemical treatments including; heat (56.5^{0}c for 30 minutes), low PH, ether, ultraviolet light and cycles of freezing and thawing[8]. CMV virions contain one molecule of linear double stranded DNA. Total genome length is 120,000-220,000 .Molecular virological techniques have been used to study variation among CMV strains. DNA strains have been shown to have similar but distinctive fragment

migration patterns. Antigenic heterogeneity among CMV strains has been detected in cross neutralization and other serologic assays; however evidence for distinct serotypes is limited (9-11).

EPIDEMIOLOGY OF CMV

CMV is ubiquitous, being found universally throughout all geographic locations and socio economic groups, and infects between 50% and 85% of adults in the United States by 40 years of age (12). CMV infection is more widespread in developing countries and in areas of low socio economic condition (13). In the developing countries, very young children are often virtually universally CMV antibody positive (14). The early acquisition and high prevalence of CMV antibody has been ascribed to low socio economic conditions, poor hygiene, and overcrowding (14). However reports from studies carried out on the people of the New Guinea highland villages with primitive standards of hygiene and who often lived apart from childhood until marriage as well as studies carried out on people of the New Hebrides and Solomon island who lived in crowded population but are scrupulously clean, the prevalence of CMV infection was higher in the latter than the former group. It was therefore concluded that close personal contact, rather than poor hygiene was more important for the acquisition and spread of CMV. The vehicle of transmission of CMV has been shown to include; saliva, breast milk , intrauterine infection, urine, droplet infection , blood transfusion , organ transplantation , sexual intercourse and formites among others (6) .

PATHOGENESIS OF CMV

An understanding of the process of CMV viral replication will provide insights into its molecular mechanisms of infection and pathogenesis (15). Following infection, or contact with the virus, the CMV DNA enters the nucleus of the host cells and begins the process of replication and shedding, leading to the release of new virions (viral particles) into the blood and other body fluids. This replication cycle, takes approximately 24 hours, and consist of 3 phases ; an immediate early phase (4 hours)-during which regulator proteins are made followed by an early phase (8 hours) – during which viral DNA polymers are made and a late phase (12hrs) – during which structural proteins and new progeny viral particles are assembled .

In an immunocompetent host, most of the virus is destroyed by CMV –specific cytotoxic T-cells, and the infectious process becomes asymptomatic. The presence of asymptomatic CMV infection is based on the detection of CMV in body fluids or on seroconversion from a negative to a positive status. The latter is defined as the appearance of IgM or a fourfold rise in the level of CMV specific IgG antibodies.

In immunocompromised patients who are not receiving prophylactic treatment against CMV, symptomatic CMV infection; CMV disease and organ involvement may result (16). There are various mechanisms by which CMV disease may occur and these includes; direct cellular loss through viral infection, both of individual cells, and of larger foci because of propensity to spread, leading to coalescing cells. This close cell-cell interaction protects virus from antibody inactivation. Systemic hypoxic- Ischemic insult due to systemic hypotension and immunologic injury, involving antigen antibody complex deposition may also be involved in organs such as the kidney

Eventually, CMV attains a latent state and persistent infection within T- cells, endothelial cells and monocyte-derived macrophages ensues (16). Cell mediated immunity which is required for resolution of symptoms, also contributes to symptoms while the role of antibodies are limited. Suppression of cell mediated immunity as seen in HIV infection allows recurrence of symptoms and can result in exacerbation of disease. The virus also has ability to induce immunosuppression in the body (9). The degree of morbidity induced by CMV is influenced by the degree of immunosuppression. In transplant recipients, there are two factors that influence the degree of morbidity due to CMV infection; the type and extent of immunosuppression, and the type of transplant. Morbidity is highest among bone marrow transplant (BMT) patients, and lowest among kidney transplant patients.

Multiple mechanisms of immune evasion for CMV could relate to the pathogenic role of the virus. Recently, the expression of immune evasion genes US3, US6, and US11 of CMV in the blood of solid organ transplant recipients has been investigated[11].

PATHOLOGY OF CMV

CMV produces characteristic cytopathic changes on infected cells which appear large, rounded and contains ground glass appearing inclusion bodies in the cytoplasm. These infected cells are the hallmark of CMV infection and indicates the presence of CMV in a sample. The nuclear inclusion has the appearance of an owl's eyes because it is typically surrounded by a clear halo that extends to the nuclear membrane (17).

CLINICAL MANIFESTATIONS OF CMV

Following a first exposure to CMV, the virus lies dormant in the body for life and can be reactivated. Usually a reactivated infection causes few or no symptoms. CMV disease may manifest in any of the under listed ways.

A. Congenital infection:

Congenital CMV infection is defined as the isolation of CMV from the saliva or urine of the neonate within 3 weeks of birth (18). The virus may also be transmitted from the mother to the infant in breast milk[19] . CMV is now the commonest cause of

congenital infection and affects around 0.3%-1% of all live births[20]. A total of 5-10% of congenitally infected infants have symptoms at birth while fatal disease occurs in 20% of these infants (20). Ninety percent of the symptomatic survivors have long term sequelae while 15% of the asymptomatic survivors also have long term sequelae. CMV is now the second commonest cause of mental retardation after Downs's syndrome and causes more cases of congenital damage than Rubella (21) .

Cytomegalic inclusion disease refers to any group of diseases caused by cytomegalovirus infection, and marked by characteristic inclusion bodies in enlarged infected cells. The classic disease is congenital, being acquired *in utero* from the mother and typically presents with intrauterine growth retardation, jaundice, hepatosplenomegaly, thrombocytopenia and encephalitis, with or without microcephaly. It is often difficult to differentiate, on clinical grounds between the several agents that may cause intrauterine infection. The severe thrombocytopenia, hepatitis, preumonitis and myocarditis attributed to the virus may be life threatening. CNS involvement may lead to seizures, focal neurological signs, and mental retardation. Unlike rubella, there is no evidence that CMV is teratogenic. Most of the damage is caused by destruction of target cells once they have been formed, and unlike rubella, the fetus can be damaged by infection during any stage of pregnancy. In 20% of cases (1% of those who are congenitally infected), the infection is so severe that mortality occurs during infancy, while the rest are likely to sustain serious abnormalities for the rest of their lives. Brain damage is by far the commonest abnormality on follow up, and thus may manifest as microcephaly, mental retardation and seizures. The spectrum of brain damage varies from mild to severe. Optic atrophy, deafness, and blindness may also be present (22).

The effects of congenital CMV infection are as follows ; CNS abnormalities – microcephaly, mental retardation, spasticity, epilepsy, periventricular calcification, eye- choroidoretinits, and optic atrophy, ear – sensorineural deafness, liver – hepatosplenomegaly and jaundice which is due to hepatitis., lung – pneumonia, heart – myocarditis, haematological – Thrombocytopenic purpura, haemolytic anaemia, neutopenia and lymphocytosis. Late sequelae include damage to the enamel forming organ of the teeth, resulting in yellow discoloration of the teeth and brittleness. This occurs in 40% of infants (22).

B. PERINATAL INFECTION

Despite the continued excretion of high titers of virus in the urine for many months, the vast majority of perinatally infected infants do not develop acute symptoms, although few cases of infantile pneumonitis have been reported (21, 23). This appears to be an exceedingly rare event.

C. POSTNATAL INFECTION

The incubation period for CMV is thought to be 4-8 weeks. Primary CMV infection in the postnatal period is usually mild or asymptomatic. Occasionally, primary infection may be accompanied by the syndrome of infectious mononucleosis, with atypical lymphocytosis. This is similar to the syndrome produced by Epstein barr virus (EBV) except that lymphadenopathy is uncommon, and Paul-Bunnell test is negative. CMV- induced mononucleosis can be symptomatically indistinguishable from EBV-induced mononucleosis (24). Malaise, fever of up to 39.4^{o}C, chills, sore throat, headache, and fatigue can be the predominant features of both viruses. Many of the same clinical manifestations that are typical of EBV-induced mononucleosis (e.g. lymphadenopathy, splenomegaly, pharyngeal erythema) also can occur with CMV, although less frequently. Presentation of acute cytomegalovirus infection in an immunocompetent host may be in form of mononucleosis.

Patients with mononucleosis may present with nonspecific skin rashes such as; generalised maculopapular, urticarial, and scarlatiniform rashes. These rashes are not a direct cause of CMV proliferation within the skin, but are the result of an immunologic response to the virus. The classic hypersensitivity drug rash which is associated with ampicillin therapy given to patients with EBV-induced mononucleosis also can occur with CMV-induced mononucleosis. The post perfusion syndrome is essentially CMV mononucleosis acquired by blood transfusion (25). Sometimes the hepatitis picture predominates so that a diagnosis of non-non-B hepatitis is made.

D. CMV INFECTION IN THE IMMUNOCOMPROMISED PATIENTS

Primary CMV infection in immunocompromised individuals is far less likely to be asymptomatic. These patients develop spiking pyrexia, which resolves within a few days (25). Some may develop a viraemia with septicemia - like syndrome in the presence or absence of hepatitis. Pneumonitis may develop, which is associated with grave prognosis .The virus may disseminate to involve the retina, causing CMV retinitis. CMV retinitis is the commonest occular opportunistic infection, and the most common cause of visual loss in people with AIDS (23). CMV may disseminate to the gut, where it may cause an asymptomatic infection or ulceration or haemorrhage by the erosion of nearby blood vessels. In patients with CMV - induced immuno suppressive syndrome the patient becomes unable to deal with opportunistic infections such as Pseudomonas. AIDS patients may develop low grade encephalopathy and CMV adenitis. In addition, kaposi sarcoma has been associated with

past CMV infection. CMV is by far the most common infection in bone marrow transplant recipients. Infection by this virus occurs in over one-half of patients. The virus is an immunomodulator and CMV disease exacerbates an ongoing immunosupression in transplant recipient, thereby increasing the risk for bacterial and fungal infection such as *Norcadia asteriodes, Mycoplasma*, and *Pneumocystis carini*.

LABORATORY DIAGNOSIS OF CMV INFECTION

CMV should be suspected if a person has symptoms of infectious mononucleosis but has negative test results for mononucleosis and Epstein Barr virus, or negative heterophile antibody test and shows signs of hepatitis, but has negative test result for hepatitis A, B, and C (26).

MICROSCOPY

Electron microscopy – Virions in the urine of congenitally infected infants may be visualized by electron microscopy in up to 80% of cases (27, 28). The pseudoreplica method of electron microscopy can be used to detect the virus in urine and oral specimens of congenitally infected infants. Positive results can be obtained from almost all specimens that have infectivity titers of > 10^4 /ml of urine.

Tissue immunofluorescence – Infected tissue cells may be stained by specific anti CMV antibody. This is based on the principle that viral specific antibody conjugated to fluorescent labeled is allowed to react with the cells obtained from the clinical specimen. Preparations from bronchoalveolar lavage specimens can also be examined in this manner, and results of high sensitivity and specificity are possible. Cytomegalic inclusions can be recognized from biopsy materials by the typical 'owl's eyes appearance" (29).

SEROLOGICAL DIAGNOSIS OF CMV INFECTION

ENZYME LINKED IMMUNOSORBENT ASSAY

A variety of tests are available for serologic diagnosis of CMV infection. The choice of method to be used depends on; factors such as the patient population, cost of test, turnaround time, equipment needs and ease of performance. Several commercial kits are available for detection of CMV IgG and IgM antibodies in body fluids. ELISA has replaced other traditional methods for detecting antibodies to CMV. The ELISA is based on the principle that Cytomegalovirus IgG and IgM specific antibodies are detected by addition of diluted patients 's sera to wells coated with purified CMV antigen. The IgG and IgM antibody, if present, binds to the antigen. An enzyme conjugate is added to bind the antigen-antibody complex, if present. A substrate to the enzyme is added, and a hydrolytic reaction between the enzyme and its substrate results in a change in the colour of the end product, whose intensity can be measured at 450 nm and is directly proportional to the amount of IgG specific antibody in the patient' s sera.

The main advantages of the ELISA are that; it is rapid, sensitive and specific. They are also useful in detection of CMV antibodies in urine. The limitation of ELISA is that they have low sensitivity when used to detect CMV antibodies in urine; this is because CMV is complexed to beta 2 microglobulin in the urine and may result in false positive results[30].

PASSIVE LATEX AGGLUTINATION TEST

This provides a simple and rapid means of detecting antibodies to CMV in human sera and plasma. This method is highly sensitive and specific, and it may serve to determine the immune status of patient or blood donor populations. The procedure detects both IgG and IgM antibodies but does not differentiate between the two classes of immunoglobulins (30, 31).

COMPLEMENT FIXATION

The complement fixation (CF) test has been used for many years in clinical laboratories and is suitable for detecting rise in antibody titers. The CF test with glycine - extracted antigen is more sensitive for distinguishing sero-positive from sero-negative specimens.

ANTIGEN DETECTION TESTS

CMV ANTIGENEMIA TEST

This test is based on the detection of pp 65, a structural protein expressed on the surface of infected polymorphonuclear leucocytes (32). The number of infected leucocytes present has been reported to correlate with the severity of infection. The main advantage of this test is that it is very rapid such that a result can be available within the same day.

Detection of early antigen fluorescent foci (DEAFF)

This is a method used for the early diagnosis of CMV infection. The specimen is inoculated into cell culture which is examined 24 hours later by immunofluorescence for expressed CMV encoded early proteins. The monoclonal antibodies must be able to cover most, if not all strains of CMV. Rapid culture methods other then the DEAFF tests are also available.

VIRAL CULTURE

Human CMV has been cultured in human cells only and previous claims that it could be grown in other animal cells is yet to be substantiated. Human fibroblast cells best support the growth of CMV and therefore can be used for diagnostic purposes.

Acceptable fibroblast cultures include those prepared from human embryonic tissues or

foreskins and serially passaged diploid human fetal lung strains such as W1-38, MRC-5, or IMR-90. The time of appearance and extent of cytopathic effect depends on the amount of virus present in specimens. CPE may develop by 24 hours and progress rapidly to involve most of the monolayer. More commonly foci of CPE, consisting of enlarged, rounded refractile cells appear during the first week and progresses slowly to surrounding cells.

MOLECULAR TECHNIQUES

POLYMERASE CHAIN REACTION (PCR)

The use of PCR in the diagnosis of CMV infection has been widely studied. The PCR is based on the use of thermostable DNA polymerase to extend oligonucleotide primers complimentary to the viral DNA genome target. Consecutive cycles of denaturation, annealing and extension results in an experimental accumulation of target DNA. PCR offers the advantages of being rapid and sensitive. However, its inherent sensitivity poses a problem since latent CMV genomes which are present in practically all sero-positive individuals, may be detected. In several studies, the sensitivity of the assay was increased by amplifying gene fragments from both the immediate early and late CMV genes or by using nested primers to a single gene fragment.

PCR has been used successfully to detect CMV DNA in a variety of clinical specimens from organ transplant recipients, patients with AIDS, and infants with congenital infection. The utility of PCR for the continued surveillance of immunocompromised patients and for evaluating the therapeutic efficacy of antiviral drugs has also been demonstrated.

HYBRIDIZATION

Molecular dot blot Hybridization techniques have been described for detection of the CMV genome in urine and peripheral blood leucocytes. Hybridization depends on the detection of a signal generated after the interaction of a labeled probe with the target nucleic acid. This occurs when the sequence of nucleic probe is sufficiently similar to that of the target nucleic acid and a duplex is formed and held together by hydrogen bonds in nucleic pairing. Biotinylated or horseradish peroxidase labeled CMV specific DNA probe kits are commercially available. In situ hybridization has the advantage of being rapid and easy to read by light microscope.

TREATMENT

In the management of CMV disease, four different strategies can be utilized; antiviral therapy, prophylaxis, pre-emptive and suppressive treatment. Ganciclovir, a nucleoside analogue of guanine is a potent inhibitor of CMV replication in vitro. Valganciclovir, a prodrug of ganciclovir has also been found to be useful in treatment of CMV infection. The use of both drugs has been associated with myelosuppression which is often dose dependent. Foscarnet, a pyrophosphate analogue with in vitro activity against all human Herpes viruses as well as HIV has also been useful in treatment of CMV retinitis in AIDS patients. Nephrotoxicity and electrolyte imbalance are the most common toxicity associated with foscarnet. Foscarnet may be used as a second line therapy in CMV patients with ganciclovir resistance.

Oral ganciclovir has been licensed for CMV prophylaxis in patients with advanced AIDS. Routine prophylaxis however has not become standard in most HIV care settings, mainly due to the high cost of prophylaxis, potential toxicity and the inconvenience of taking 12 capsules per day.

PREVENTION AND CONTROL

Optimal prevention of CMV disease would be vaccination. However, until today there is no effective vaccine available. The Towne strain reduced the severity of disease without affecting the infection rate. Studies are currently going on to determine an alternative approach which is the use of subunit recombinant or DNA vaccines (32).

REFERENCES

1. Hodinka, RL and Friedman, HM. Human Cytomegalovirus. In: Murray, PR, Baron, EJO, Pfaller, MA, Tenover, FC, Yolken, RH. Manual of Clinical Microbiology. 6th ed. ASM;2005; **556**: 566-569.
2. Brooks, GF, Caroll, KC, Butel, JS, Morse, SA. Herpes viruses In: Jawetz, Melnick & Adelberg" s Medical Microbiology. 24th ed .2007; 428-451.
3. Emery, V.C. Investigation of CMV disease in immunocompromised patients. *J Clin Pathol*. 2001; 54: 84-88.
4. 4Murray, PR, Rosenthal, KS, Kobayashi, Pfaller, MA. Human Herpesviruses. Medical Microbiology. 14th ed. Mosby Inc.2000; 475-498.
5. Vandkova, Z, Dvorak, P. Cytomegalovirus Infection in Immunocompetent and imunocompromised individuals- a review. *Curr Drug Targets Immune Endocr Metabol Disord*.2001; 179-187.
6. Stagno S. Reynolds DW., Pass RF and Alford C.A (1993): Breast milk and the risk of CMV infection. *N Engl J Med*. 1993;**302**:1073-6.
7. Krench, U. Jung, M. and Hung, F.: The acquisition of Cytomegalovirus antibodies

in a normal population: A serological study .*J. Hyg*. (Camb) 1997;**63**:798-89.

8. Tsaparas Y.F., Brigden M.L. Marthias R. Thomas E. Proportion positive for Epstein barr virus, Cytomegalovirus, Toxoplasma, Human herpesvirus 6, Human Immunodeficiency Virus types 1&2 in heterophile negative patients with an absolute lymphocytosis. *Arch. Pathol. Lab.Med* .2003; **124**:1324-30

9. Cicognac, Pollsky B . Cytomegalovirus infection in Bone marrow transplant patients . *Infect Med*.1993; **11**:256-262.

10. Patel R. Syndman DR, Rubin RH., Ho, M Pescovitz M, *et al* Serological findings in cytomegalovirus infection. *Clin Infect Dis* .2009; 61:1279-1289.

11. Patel, R., Snydman D.R Rubin R.H., Ho M, Pescovitz M. *et al*. Cytomegalovirus prophylaxis in solid organ transplant recipients. *Clin. Dis*.**;** 2009**; 33**.suppl 1:532-7.

12. Leinikki, P., Heinonem, K and Pettay, O. Incidence of cytomegalovirus infections in early childhood *Scand J. Inf. Dis.1996*. **4**:1-5.

13. Cabau, N., Coignard S, Labadie, M.D. *et al* Sero-epidemiology of cytomegalovirus infections during adolescence in urban communities. *Arch. Dis. Child* .**54**:286-90.

14. Bello, C.S.S. Cytomegalovirus infection in the Gambia M.D. Thesis. Ahmadu Bello University, Zaria, Nigeria. 1984

15. Bale JF, Zummerman B, Clud G.A, Pass F.F. Cytomegalovirus transmission in child care homes. *Arch. Pediatr Adolescence Med*. 1999;**153**:75-9.

16. Wingard, J.R, Piantadosis, S., Burn W.H. Pathogenesis of Cytomegalovirus infection after marrow transplantation . *Infect Dis*.1999; **153**:478-88.

17. Euright, H., Haake, R., Weisdorf, D., *et al* Risk factors to Cytomegalovirus infection. *J Infect Dis.* 2001;**55**, 1339-46.

18. Meyers J. D ,Flournoy, N. Clinical syndromes cytomegalovirus infection after human marrow transplantation. *J Infect Dis.* 1996; **153**,478:88.

19. Goodwich, J.M., & Bowden, R. Organ invovelment and changes in Cytomegalovirus infectious. *Clin Infect Dis* .1994;**19**, 287-98.

20. Boeckh, M., Gooley, T.A., & Bowden, R.A. Symptoms of Cytomegalovirus induced mononucleosis. *J Infect Dis*.2001;**178**, 1153-7.

21. Meyers, J.D., & Dandliker, P.S (1998): Symptoms and signs of Cytomegalovirus infections. *New Engl J Med* .1998**;318**. 70-75.

22. Atkinson, K. & Downs, D. (1995) Classical presenting features of infectious mononucleosis. *Brit J Haematol* . 1995**; 79**; 57-62.

23. You, JC, Dimopoulos, M.A. Presentation and complication of cytomegalovirus in Bone marrow transplant patient. *Euro J Haematol.2001;* **47**:371-6.

24. Goodrich, J.M. & Mori, M. Opportunistic infections among. Bone marrow transplant recipients. *New Engl J Med* 2004;.**325**,1601-7.

25. Canpolat C, Culbert, S. Presenting features of cytomegalovirus infection in paediatric allogeneic bone marrow transplant recipients. *J Med Virol*.1997; **25**: 179-188.

26. Greger B, Vallbracht A, Kurth J. Diagnostic tests in infectious mononucleosis. *J Clin Microbiol* 2003; **26**, 2531-5.

27. Vander B.J.W, Speich R. Introduction to Cytomegalovirus infection and disease. *Clin Dis*. 2001; 33 Supp 1:S 32-7.

28. Hilt, D.C & Buchhotz, D cytomegalovirus infection: diagnosis and management approaches. *J Infect Dis*.2001; **152**:1172-81.

29. Bedayi PL, Risichella I.S. Strumia, R., Gilli P. *et al,* serologic & Haematologic changes induced by acute cytomegalovirus infectious. *J Infect Dis* . 1997; **178**; 1791-3.

30. Conti, D. J & Freed B.M. Patterns of enzyme abnormities associated with Cytomegalovirus induced mononucleosis. *Ann Int Med* . 2006; **120**; 375-6.

31. Kletzmayr, J. & Kotzmann, H. Laboratory Diagnosis of Cytomegalovirus infection. *Journal of the American society of virology 2006;* **7;** 325-30.

32. Einsele, H, Ehninger, G., Hebart, H. Cytomegalovirus pp 65 antigenaemia based diagnostic option. *J Med Virol*.2005; **14;** 32-8.33.

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DETECTION OF INFLUENZA A VIRUS IN PIGS IN LAGOS, NIGERIA

RUNNING TITLE: INFLUENZA A VIRUS IN NIGERIA

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ABSTRACT
This study detected and subtyped strains of influenza virus from pigs in Lagos, South-western Nigeria. A total of 116 (58 nasal and 58 throat) samples from healthy pigs were analysed from two different sites in Ayedoto farm at Ojo Local Government between June and September, 2010 using reverse transcription polymerase chain reaction (RT-PCR). Influenza virus type A 31(26.7%) was detected. Subtyping was done using RT-PCR with H1, H3 and H5 primers and only subtypes H1 [5(16.1%)] and H5 [5(16.1%)] were detected. No positive detection was made for subtype H3. This research work is the first documented detection of influenza A virus in pigs in Lagos, Nigeria and demonstrates the need for a sustainable surveillance mechanism of swine and other influenza viruses to be able to prevent influenza epidemic in the environment.
Keywords: Subtype, Influenza A, Pig, Lagos

INTRODUCTION

The types of influenza virus found in pigs are known as swine influenzagenerally called swine flu or swine-origin influenza virus (S-OIV) (1, 2). Influenza virus belongs to the genus Orthomyxovirus in the family Orthomyxoviridae which consists of influenza A, B and C viruses and has an envelope, single-stranded, negatively sensed RNA, eight separate segments and pleomorphic appearance with an average diameter of 120nm (3, 4).

Influenza A virus causes swine influenza which is an acute, highly contagious respiratory disease (5) with classical aetiological types that include influenza A subtypes H1N1, H1N2, H2N3, H3N1 and H3N2 and rarely influenza C while influenza B has not been reported in swine (6, 7, 4).This is because type A group continually undergoes antigenic shift and drift unlike B and C which are relatively stable (8, 4). Generally, 16 haemagglutinin (H or HA) and 9 neuraminidase (N or NA) subtypes have been identified. This means pigs can also be infected with other subtypes as they play a substantially important role in the ecology of influenza A virus and can act as a "mixing vessel". When co-infections among human, avian or swine influenza viruses occur within a specific host, a new subtype can be produced by antigenic re-assortment (9, 10).

The virus usually spreads via aerosols produced by an infected person when coughing, sneezing or spitting or through contaminated hand to eye, nose or mouth either from fomites or direct personal contact such as hand-shake(11, 12).

In Nigeria, influenza viruses have caused a significant amount of morbidity in the general population but the incidence of excess mortality is unknown.Until the recent advancement in the field of molecular virology, the detection of influenza virus has always been by isolation method (13). This study therefore evaluates the detection of influenza A virus in order to establish that different strains can co-circulate among local pigs in Lagos which can cause possible intermittent infection in man.

MATERIALS AND METHODS

Study site

The study subjects were collected from pigs at two different sites in Ayedoto farm settlement, Agric, Ojo Local Government, Lagos between June and September, 2010.

Sample population, collection and preservation

A total of 58 (58 nasal and 58 throat=116 samples) apparently healthy land race pigs aged between 2-30 months old were the subject for this study. Nasal and throat swab samples that contained adequate numbers of ciliated and columnar epithelial cells were collected from land race pigs. Each swab was transferred into commercially available sterile cryovials containing 2ml aliquot of Dulbecco's modified eagle medium transport medium with antibiotic to prevent desiccation, death and bacterial growth. They were conveyed to the laboratory in coolers with dry ice packs immediately after collection. The samples were kept at 4^{o}C for extraction the next day (< 24hrs) since freezing and thawing reduce the ability to recover virus. Aliquots of samples were frozen at -70^{o}C.

Extraction Process

This study extracted RNA genetic material by diatomaceous sand method using Qiagen kit (Germany). 140ul of sample was added to 560ul of lysis buffer with carrier RNA by dispensing each into sterile Eppendorf vial followed by addition of 100 mg diatomaceous sand (Sigma-Aldrich, USA) in order to trap and bind the RNA (5). 560ul of absolute ethanol was added and mixed by pulse vortexing until homogeneous mixture was obtained. Incubation was done at room temperature for 20 minutes with vortexing at 5 minutes interval. The mixture was then spun at 13,000 rpm for 1 minute while the supernatant was decanted. Serial washings using two kinds of washing buffer (AW1 and AW2: 500ul each) were carried out before each centrifugation at 13, 000 rpm for 1 minute and separation of supernatant. 400 ul of acetone was later added with another vortexing and centrifugation at full speed 13,000 rpm for 1 minute. The supernatant was decanted while the pellet was drained and dried at 56 ^{o}C for 15 minutes. Elution was done with a 100ul of PCR water. Final vortexing and centrifugation was carried out at 13,000 rpm for 2 minutes. The supernatant was then separated and stored in RNA 1.5ul Eppendorf tube at -80^{o} C before amplification.

Master Mix Preparation and PCR Amplification

A one step process was used for both reverse transcription and PCR amplification treatment process with Qiagen (Germany) one step kit. The master mix used for one reaction before the addition of 5ul of suspected influenza RNA extract for the synthesis of complementary DNA (cDNA) include: 5ul Rnase free water, 5ul 5x RT buffer, 5ul Q-solution, 1 uldNTP-mix, 1.5 ul each of forward and reverse primer and 1 ul enzyme mix.

The RT-PCR mixture was then incubated at 50^{o}Cfor 30 minutes for reverse transcription followed by 45 cycles run in a thermo cycler (Eppendorf, Germany) PCR machine. Denaturation – 95^{o} C for 15 minutes; Activation – 95 ^{o} C for 30 seconds; Annealing – 55 ^{o} C for 30 seconds; Elongation – 72 ^{o} Cfor 30 seconds; and Extension– 30 ^{o} C for 30 seconds (14, 15, 5).

Agarose Gel Electrophoresis Process

Identification of amplicons was carried out by agarose gel electrophoresis method with 2% agarose gel (Peolab, Germany). 2 ul of SYBR gel stain (Invitrogen, USA) was added. The gel was later poured into a casting block with inserted comb. It was allowed to solidify for 15 minutes. The comb was gently removed whilethe gel was placed inside an electrophoresis tank containing TAE buffer 50x (Genaxxon, Germany). 1ul of 6x loading dye (Fermentas, EU) plus 5ul of amplicons were loaded per lane onto the gel. 3ul of a 100bp DNA ladder (Invitrogen, USA) was loaded along with the samples as marker. Electrophoresis was carried out in a Westburg electrophoresis machine (Biometra, Netherland) for 30 minutes by 3-4cm at 130 volts. Ultra violet (UV) source safe imager trans illuminator (Invitrogen, USA) was used to illuminate and viewed the gel pictures before they were taken by Biodoc analyze 2.0(Biometra, Germany). The correct RNA amplification was indicated by correct size and a comparison with positive and negative controls of influenza A (figure 1) and subtypes H1 and H5 (figure 2).

RESULTS

A total of 116 (58 nasal, 58 throat) samples from 58 pigs were studied. Influenza A virus 31(26.7%) was detected using RT-PCR (Table 1).The 31(26.7%) positive influenza A virus detected were typed with influenza A subtypes H1 [5(16.1%)] and H5 [5(16.1%)] results obtained. No positive detection was made for subtype H3 (Table 2).

TABLE 1: DISTRIBUTION OF INFLUENZA A VIRUS USING RT-PCR METHOD

INFLUENZA TYPE	NO OF SAMPLES TESTED	NO OF POSITIVES (%)
A	116	31(26.7%)

TABLE 2: DISTRIBUTION OF SUBTYPES OF INFLUENZA A VIRUS USING H1, H3 AND H5 PRIMERS

INFLUENZA A SUBTYPE	NO OF SAMPLES TESTED	NO OF POSITIVES (%)
H1	31	5(16.1%)
H3	31	-
H5	31	5(16.1%)

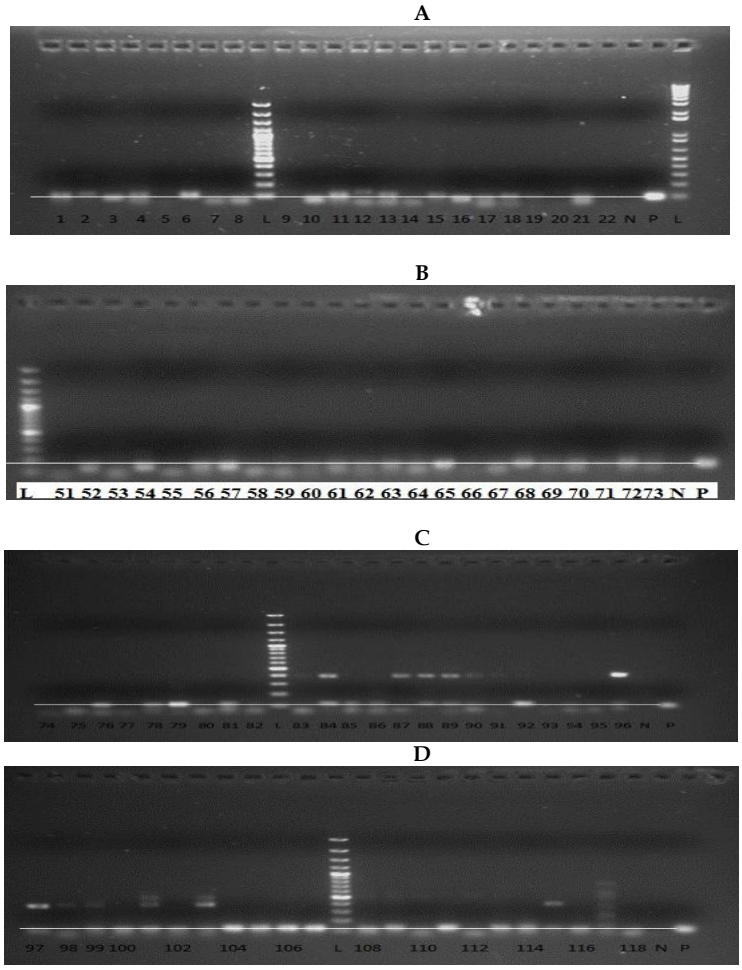

Figure 1:Amplicons of Influenza A virus separated by Agarose Gel Electrophoresis (2%) for 30 minutes by 3-4cm at 130 volts:Panel A has seven total no of positives as shown on lane 1, 4, 11, 12, 13, 17 and 18 when compared with the positive control (P) on last lane as ruled by the straight line passing through the center; Panel B has fivetotal no of positives (57, 65, 68, 70 and 72); Panel C. No of positives = 6 (76, 78, 79, 81, 85 and 86);Panel DNo of positives = 13 (100, 101, 102, 104, 105, 106, 107, 108, 109, 111, 113, 114 and 116).

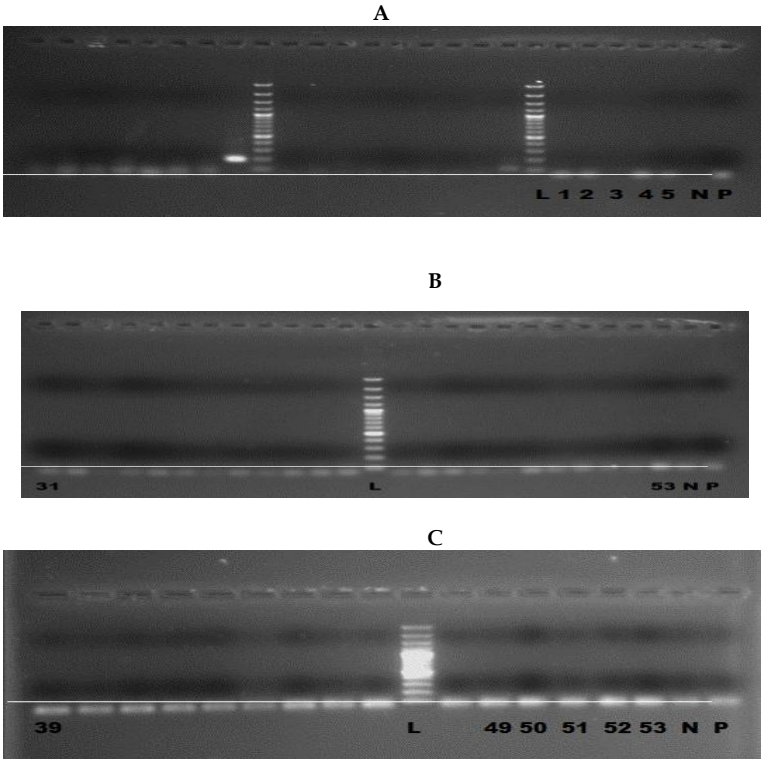

Figure 2:Amplicons ofHaemagg1utinin (H) 1 and Haemagglutinin (H) 5 protein genes of subtypes of Influenza A virus separated by Agarose Gel Electrophoresis (2%) for 30 minutes by 3-4cm at 130 volts:PanelA.Subtype H1; No of positives = 4 (1, 2, 4 and 5); PanelB. Continuation of SubtypeH1; No of positives = 1 (53);Panel C.SubtypeH5; No of positives = 5 (49, 50, 51, 52 and 53). Negative (N) and Positive (P) controls are as shown on second to the last and last lane respectively. 100 base pair Ladder (L) was used as the marker as shown on each gel panel.

DISCUSSION

Influenza virus is notoriously known for its unique ability to cause recurrent influenza epidemics and global pandemics during which acute febrile respiratory illness occurs explosively. There are two qualities of influenza virus that account for much of its spread. First is the ability to emerge and circulate in avian or porcine reservoirs by either genetic re-assortment or direct transmission and subsequently spread to human at regular intervals. Second, is the fast and unpredictable antigenic change ofimportant immune target once the virus is established in human (16).

The high number of positive results to influenza A virus 31(26.7%) detected in this study is characterized by its continuous antigenic drift and shift, making it more genetically diverse with high prevalence in the subject (8,4). This is further supported by the work of (17) which showed that influenza A virus mutates at a rate 2-3 times faster than type B which is relatively stable.

Only sub types H1 [5(16.1%)] and H5 [5(16.1%)]detected in this study disagree with the work of (1) and (5) that detected subtype H3 in pigs. (18) and (19) also detected and worked on both H1 and H3 Thailand and European subtypes in pigs respectively. No positive detection of H3 in this study may be due to H3 primer mismatch to local strains of influenza A in Nigeriasince foreign primer (Qiagen, Germany) were used which produced unclassified bands.

The detection of H5 agrees with the work of several authors since H5 subtypes are commonly found in birds (20). Another reason is that birds are being reared side by side with the pigs at the site of this study. Subtyping in this study however did not include the use of neuraminidase (N) primers due to cost and in availability in Nigeria.

The ability of RT-PCR to detect influenza virus types agree with the work of (21) that RT-PCR is capable of detecting the virus even when the viral genomes are present in low level and is generally more sensitive in

the detection of influenza virus than any other method including serology and culture (22).

REFERENCES

1. Adeola OA, Adeniji JA, Olusaga BO. Isolation of influenza A viruses from pigs in Ibadan, NigeriaVet. Ital. 2009; 45(3): 383-90
2. Faix DJ, Sherman SS, Waterman SH. Rapid-test sensitivity for novel swine origin influenza A (H1N1) virus in humansN Engl J Med. 2009; 361: 7
3. Gurtler, L. Virology of human influenza. In:Kamps, B. S., Hoffmann, C., Preiser, W. (eds) Influenza report 2006. Flying Publisher, Wuppertel 2006:87-91
4. Fowotade A, Agbede O, Nwabuisi C, Fadeyi A. A review of swine influenza: an emerging pandemicNigHospPract. 2009; 4: 3-4
5. Sreta D, Kedkovid R, Tuamsang S, Kitikoom P, Thanawongnuwech R. Pathogenesis of swine influenza virus (Thai isolates) in weanling pigs: an experimental trial Virol. 2009; 6: 34
6. Shin JY, Song MS, Lee EH. Isolation and characterization of novel H3N1 swine influenza viruses from pigs with respiratory diseases in Korea J of ClinMicrobiol. 2006; 44(11): 3923-7
7. Vincent AL, Lager KM, Ma W. Evaluation of haemmaglutinin subtype 1 swine influenza viruses from the United StatesVet Microbiol. 2006; 12-22
8. Brooks, G. F., Carol, K. C., Butel, J. S., Morse, S. A. Orthomyxoviruses (Influenza viruses). In: Jawetz, Melnick,Adelberg's Medical Microbiology. McGraw Hill, New York 2007
9. Easterday, B. C. Animal influenza. In:Kilbourne, E. D.(ed) The influenza viruses and influenza. Academic Press, Orlando 1975: 449-481
10. Webster R, Ox N, Stohl K. WHO manual on animal influenza diagnosis and surveillance. World Health Organization 2002: 15-67
11. Hall CB. The spread of influenza and other respiratory viruses: complexities and conjuctures. Clin Infect Dis. 2007; 45(3): 353-9
12. Weber TP, Stalianakis, NI. Inactivation of influenza viruses in the environment and modes of transmission: a critical review. J of Infect. 2008; 57(5): 361-73
13. Vanzyl, G. Laboratory findings. In:Kamps, B. S., Hoffman, C., Preiser, W. (eds) Influenza report 2006. Flying Publisher, Wuppertal 2006: 150-159
14. Boom R, Sol, CJ. Rapid and simple method for purification of nucleic acids. J of ClinMicrobiol. 2006; 28: 495-503
15. Cox, N. J., Neumann, G., Donis, R. O.,Kawaoka, Y. Orthomyxoviruses: Influenza. In: Microbiology and Microbial Infections: Virology. 10thedn. Edward Arnold Publisher, Washington 2005:634-680
16. Behrens, G and Stoll, M. Pathogenesis and Immunology. In:Kamps, BS, Hoffman C, Preiser W.(eds) Influenza report 2006. Flying Publisher, Wuppertal 2006
17. Nobusawa F, Sato K. Comparison of the mutation rates of human influenza A and B. J Virol.2006; 80 (7): 3675-8
18. Damrongwatanapokin S, Pinychon W, Parchariyanon S,Damrongwatanapokin T. Serological study and isolation of influenza A virus infection of pigs in Thailand. Proceedings of the 19th IPVS Congress, Denmark 2006
19. Van Reeth K, Gregory V, Hay A, Pansaert M. Protection against European H1N2 swine influenza virus in pigs previously infected with H1N1 and/ or H3N2 subtypes. Vaccine. 2003; 21: 1375-1381
20. Olofson S, Kumlin U, Dimock K, Arnberg N. Avian influenza and sialic acid receptors: More than meets the eye? Lancet Infect Dis. 2005; 5: 184-8
21. Landolt, GA, Karasin, AL, Phillips L, Olsen, CW. Comparison of the pathogenesis of two genetically different H3N2 influenza A viruses in pigs. J of ClinMicrobiol. 2003; 941:1936-1941
22. Zambon M, Hays J, Webster A, Newman R, Keene O. Diagnosis of influenza in the community: relationship of clinical diagnosis to confirmed virological, serological or molecular detection of influenza. Arch. InternMed. 2001; 161: 2116-22

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TRENDS IN THE RESISTANCE PATTERN OF BACTERIAL PATHOGENS OF OTITIS MEDIA IN IBADAN, NIGERIA.

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ABSTRACT
Otitis media, an inflammation of the middle ear, is a common illness in childhood, and also one of the most frequent reasons for outpatient antimicrobial therapy. This study was undertaken to determine the bacterial etiology of otitis media in our environment and their pattern of antibiotic susceptibility. Between November 2009 and March 2011, ear swabs collected from 132 patients with clinical diagnosis of acute otitis media and chronic suppurative otitis media were subjected to bacteriological analysis. The bacterial pathogens isolated were tested against ten antibiotics using standard bacteriologic techniques.

A total of 142 isolates were recovered from the 132 patients involved in this study. The most frequently isolated organism in acute otitis media and chronic suppurative otitis media was *Pseudomonas aeruginosa*, (43.7%), followed by Klebsiella species (31.0%), Proteus species (14.1%), *Escherichia coli* (7%), *H.influenzae* (2.8%) and *Staphylococcus aureus* (1.4%). Generally, high resistance rates were recorded against many of the antibiotics tested. However, ciprofloxacin demonstrated the highest susceptibility rates for *P.aeruginosa* (77.4%) and Klebsiella species (59.1%).All the pathogens demonstrated nil susceptibility towards cefixime except *E.coli* where the susceptibility rate was 40%.In conclusion, determination of the susceptibility pattern of bacterial pathogens of otitis media is of utmost importance to its effective management.

Key words: Otitis media, bacterial pathogens, resistance pattern

INTRODUCTION

Otitis media (OM) is the inflammation of the middle ear, which may be caused by bacteria, fungi or viruses. There are three types of otitis media, and these are, acute purulent otitis media, otitis media with effusion and chronic suppurative otitis media (1).Acute bacterial infection of the middle ear normally originates from an upper respiratory tract infection with the bacteria entering by the auditory (Eustachian) tube, which is the principal portal of entry of pathogens into the ear (2).

Chronic suppurative otitis media (CSOM) is defined as a chronic inflammation of the middle ear and mastoid cavity, which presents with recurrent ear discharges through a tympanic perforation(3).Effusion of OM may be serous, mucoid or purulent (4). Otitis media may be associated with infection or may be sterile. The sterile variety is usually called serous OM and is often attributed to allergy, but may also occur from numerous other potential sources including radiation treatment or virus (5,6).

Otitis media is very common in childhood and is almost always accompanied by a viral upper respiratory infection (URI) with a peak incidence between 4–7 years of age (7). Seventy–five percent of children experience at least one episode by their third birthday. Almost half of these children will have 3 or more ear infections during their first 3 years. The reason for the higher frequency in these populations is the anatomic differences in skull base and Eustachian tube and biologic susceptibility (8). Although OM is primarily a disease of infants and young children, it can also affect adults (9). Furthermore, the incidence is higher in males than in females (10).

The significant risk factors in OM include socioeconomic status, cultural, seasonal, and age factors, as well as family history of middle ear disease (11).

The most common bacterial pathogens of OM are *Streptococcus pneumoniae*, *Hemophilus influenzae* and *Moraxella catarrhalis* (12). Other pathogens responsible for OM are *Staphylococcus aureus*, *Escherichia coli,* Klebsiella species, *Pseudomonas aeruginosa* and Proteus species (13). The types of pathogens involved in OM have also been found to be dependent on geographical location (14). The aim of this study was therefore to determine the bacterial etiology of OM in this environment and their susceptibility pattern to the available antibiotics.

MATERIALS AND METHODS

This is a laboratory-based study carried out between November 2009 and March 2011 at the Medical Microbiology Laboratory of University College Hospital (UCH), Ibadan, Nigeria.

One hundred and thirty-two ear swabs which were collected from patients with clinically diagnosed acute otitis media and chronic suppurative otitis media at the otolaryngology clinic of UCH were included in this study. Bacterial pathogens of acute otitis media and CSOM were isolated and identified from these aural swabs using standard bacteriological methods. They were subsequently subjected to antimicrobial susceptibility testing according to the recommendation of the Clinical and Laboratory Standards Institute (CLSI) for disc diffusion tests (15). The susceptibility pattern of the bacterial pathogens were determined towards the following antimicrobial agents, ciprofloxacin, gentamycin, pefloxacin, ofloxacin, ceftriaxone, cefuroxime, ceftazidime, amikacin, amoxycillin/clavulanate, and cefixime. The diameters of zones of inhibition around the bacterial colonies were measured with a calibrated ruler and interpreted as susceptible or resistant using an interpretive chart (15).

RESULTS

Aural swabs obtained from 132 patients with clinical diagnosis of CSOM and OM were included in this study. Seventy(53%) of these patients were males while 62(47%) were females. Among these, 80(60.6%) were diagnosed as CSOM while 52(39.4%) were diagnosed as OM. The age distribution of the patients are demonstrated in Table 1.

Table 1.Distribution of otitis media by age and sex

Age (Years)	No (%)
< 10	62 (47.0)
10 – 20	30 (22.7)
21 – 40	18 (13.6)
41 – 60	8 (6.1)
> 60	6 (4.6)
*Unspecified	8 (6.1)
Total	132 (100.0)
Sex	
Male	70 (53.0)
Female	62 (47.0)
Total	132 (100.0)

***Study patients gave estimated ages which may not be accurate, hence classified as "unspecified".**

A single pathogen was demonstrated in 120(90.9%) patients, two in 10 (7.6%) and no growth in 2(1.5%). The double pathogens were demonstrated in 4 (80%) patients with CSOM and 1(20%) with OM.

Pseudomonas aeruginosa was the most frequently isolated in both CSOM and OM while *S.aureus* 2 (1.4%), was the least commonly isolated. The frequency of isolation of the bacterial pathogens in CSOM and OM are demonstrated in Table 2.

Table 2. Distribution of bacteria pathogens in otitis media.

Bacterial Pathogens	CSOM	OM	Total No (%)
Klebsiella species	24	20	44 (31.0%)
Pseudomonas aeruginosa	40	22	62 (43.7)
Proteus species	12	8	20 (14.1)
Escherichia coil	8	2	10 (7.0)
Staphylococcus aureus	2	-	2 (1.4)
Hemophilus influenzae	4	-	4 (2.8)
Total	90	52	142 (100.0)

CSOM – Chronic suppurative otitis media

OM – Acute otitis media.

From 132 patients with CSOM and OM, a total of 142 bacterial pathogens were isolated. Of these, 140(98.6%) were Gram–negative bacilli and 2 (1.4%),Gram–positive cocci.*Pseudomonas aeruginosa* 62(44.3%) and Klebsiella species 44(31.4%) were the most prevalent among the Gram-negative pathogens. *S.aureus* was the only Gram-positive pathogen isolated.

Of the ten antibiotics tested against these bacterial pathogens, ciprofloxacin had the highest susceptibility rates for *P. aeruginosa* (77.4%) and Klebsiella species (59.1%). Cefixime demonstrated nil (0%) susceptibility to all the pathogens except *E.coli* where the susceptibility rate was 40%. The antibiotic susceptibility profiles of the various pathogens isolated are shown in Table 3.

Table 3. Antibiotic susceptibility pattern of bacterial pathogens of otitis media

Antibiotics	Susceptibility pattern of isolates					
	Pseud N = 62	Kleb N = 44	Proteus N = 20	*E.coli* N = 10	*S.aureus* N = 2	*H.inf* N = 4
	No (%)	No (%)	No (%)	No (%)	No (%)	No (%)
Ciprofloxacin	48(77.4)	26(59.1)	6(30.0)	4(40.0)	2(100.0)	2(50.0)
Gentamycin	40(64.5)	20(45.5)	10(50.0)	2(20.0)	2(100.0)	2(50.0)
Pefloxacin	24(38.7)	8 (18.2)	2(10.0)	4(40.0)	0(0.0)	0(0.0)
Ofloxacin	36(58.1)	26 (59.1)	10(50.0)	0(0.0)	2(100.0)	2(50.0)
Ceftriaxone	16(25.8)	20 (45.5)	14(70.0)	4(40.0)	0(0.0)	2(50.0)
Cefuroxime	4(6.5)	16 (36.4)	4(20.0)	4(40.0)	0(0.0)	4(100.0)
Ceftazidime	42(67.7)	10 (22.7)	6(30.0)	4(40.0)	0(0.0)	0(0.0)
Amikacin	26(41.9)	4(9.1)	0(0.0)	2(20.0)	0(0.0)	0(0.0)
Augmentin	0(0.0)	12(27.3)	0(0.0)	0(0.0)	2(100.0)	4(100.0)
Cefixime	0(0.0)	0(0.0)	0(0.0)	4(40.0)	0(0.0)	0(0.0)

Augmentin-Amoxycillin/clavulanate

DISCUSSION

In this study, *P.aeruginosa* (43.7%) was found to be the most prevalent bacterial pathogen of OM and CSOM in this environment, followed by Klebsiella species (31%), *Proteus mirabilis* (14.1%), *Escherichia coli* (7%), *H. influenzae* (2.8%) and *S.aureus* (1.4%). The preponderance of *P.aeruginosa* in this study is in agreement with a study conducted by Kawo et al in the Northern part of Nigeria (16). In contrast, however, in a study conducted in Iran, the most frequently isolated pathogen of OM was *S. aureus* followed by *P.aeruginosa* and Proteus species (17). In the United States of America, Israel and Eastern Europe, the commonest bacterial pathogens of OM were *Streptococcus pneumoniae*, *H.influenzae* and *Moraxella catarrhalis* (14).

In the present study, the peak incidence was found in the age group 0 – 10 years (47%), followed by 10–20 years (22.7%), and a decline with advancement in age (Table 1). This has been attributed to the shorter, wider, and horizontal Eustachian tube in children than in adults, offering greater opportunities for pathogens to ascend from nasopharynx to the sterile middle ear cavity (18).

The frequency of double causative agents (7.6%) was much lower than single causative agent (90.9%). This contradicts the report of Alsaimary where the frequency of double causative agents was higher than single causative agent (19).The incidence rate of otitis media is higher in males (53%) than females (47%) which agrees with the finding of another study elsewhere (10).

Many antimicrobial agents have been used for the treatment of OM infection and these include penicillin, cephalosporins, vancomycin and azithromycin. However, bacterial resistance to these antimicrobials has become an increasing problem in the treatment of otitis media (19). In this study, the most effective antimicrobial agent against *P.aeruginosa*, the most prevalent etiological agent of OM in this environment, was ciprofloxacin (77.4%), followed by ceftazidime (67.7%), gentamycin (64.5%), ofloxacin(58.1%) and amikacin(41.9%). The high susceptibility demonstrated towards ciprofloxacin is similar to what was reported in another study where *P.aeruginosa* demonstrated 100% susceptibility to ciprofloxacin(17).

Generally, in this study, high resistance rates were recorded towards a number of antibiotics commonly used in this environment, and these included pefloxacin, ceftriaxone, cefuroxime, amikacin, amoxycillin/ clavulanate and cefixime.

This may be attributed to the inappropriate and indiscriminate use of these antibiotics in our environment. This calls for judicious use of antibiotics and periodical monitoring of the antibiotic resistance pattern in this region.

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REFERENCES

1. Berman S.Classiification and criteria of otitis media. Clin Microbiol Infect 1997; (suppl)3:1-4.
2. Atlas RM. Microbiology Fundamentals and Applications. Second edition Macmillan Publishing Company 1998 pp 669,638.
3. Howard D. Intercultural commucation and conductive hearing loss. J First People Child Family Rev. 2009; 3(4): 97.
4. Abera B, Biadeglegne F. Epidemiology of otitis media. Inter J Paed Otolaryngol 2008; 72(6): 787-792.
5. Ahlo OP, Oja H, Koivu M, Sorri M. Risk factors for chronic otitis media with effusion in infancy, each acute otitis media episode induces a high but transient risk. Arch otolaryngol Head Neck Surg 1995; 121: 839 – 843.
6. Daly KA. Epidemiology of otitis media. Otolaryngol Clin North Am. 1991; 24:775–782.
7. Bluestone CD, Klein JO. Otitis media in infants and children, 2nd W.C.Saunders companyPhiladelphia.1995;1-3/39-68/73-101/145-231.
8. Chan KH, Swarts JD, Rudoy R, Dever GJ,Yuji M. Otitis media in the republic of Palau. A case-series study. Arch otolaryngol Head Neck Surg 1993; 119:425–428.
9. GateGA.Cost–effectiveness considerations in otitis media treatment. Otolaryngology Head and Neck Surg 1996; 114 (4): 525 – 530.
10. Paradise JL, Rochette HE, Colborn DK, Bernard BS, Smith OG, Kurs–Lasky M et al. Otitis media in 2253 Pittsburg area infants; Prevalence and risk factors during the first two years of life. Pediatr 1997; 99; 318–333.
11. Rotowa NA, Montefiore D, Adeyemi-Doro FAB. An in-vitro study in ciprofloxacin and other antimicrobials against Gram–negative bacteria isolated from patients in Ibadan, Nigeria. Afr J Med Med Sc 1989; 18: 63–67.
12. Kilpi T, Herva E, Kaijalainem T, Syrjnen R, Takala AK. Bacteriology of acute otitis media in a cohort of Finnish children followed for the first two years of life. Pediatr Infect Dis J 2001; 20: 654 – 662.
13. Klein J. Otitis media. Clin Inf Dis 1994; 823–833.
14. Jacobs MR, Dagan R; Appelbaum PC, Burch DJ. Prevalence of antimicrobial–resistant pathogens in middle ear fluid; multinational study of 917 children with acute otitis media. Antimicrob Agents Chemother 1998; 42: 589 – 595.
15. Clinical and Laboratory Standards Institute (CLSI). Performance Standards for Antimicrobial Susceptibility Testing. 7th ed Wayne (PA); CLSI; 2007.

16. Kawo AH, Daiyah BM, Yusha'u M. Susceptibility patterns of bacterial pathogens associated with otitis media at Murtala Muhammed Specialist Hospital, Kano, Nigeria. Int J Pharm App Sc 2010; 1: 74–78.

17. Ettehad G, Refahi S, Nemmati A, Pirzadeh A, Daryani A. Microbial and antimicrobial susceptibility patterns from patients with chronic otitis media in Ardebl. Int J Trop Med 2006; 1(2): 62 – 65.

18. Bluestone CD, Klein JO. Microbiology. In: Bluestone CD, Klein JO, eds. Otitis media in infants and children. 3rd ed. Philadelphia, PA: W.B. Saunders 2001, pp 79–1014.

19. Alsaimary IE, Alabbasi AM, and Najim JM. Antibiotics susceptibility of bacterial pathogens associated with otitis media. J Bact Res 2010; 2(4): 41–50.

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DETECTION OF AMP C BETA LACTAMASES IN CLINICAL ISOLATES OF *ESCHERICHIA COLI* AND *KLEBSIELLA*

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ABSTRACT
Detection of AmpC-mediated resistance in Gram negative organisms poses a problem due to misleading results in phenotypic tests. There are no recommended guidelines for detection of this resistance mechanism and there is a need to address this issue as much as the detection of extended spectrum beta lactamases (ESBLs) since both may co-exist and mask each other. Several methods have been used to detect the presence of AmpC β-lactamase production in some isolates but most of these methods are not reliable. There is a need for a reliable method of evaluating the presence of AmpC β-lactamases in clinical isolates. A total of 81 consecutive non repetitive clinical isolates of *Escherichia coli* (n=40) and *Klebsiella* spp. (n=41) were screened for AmpC production by disc diffusion method using cefoxitin (30 µg) disc and confirmed by inhibitor based test using boronic acid as inhibitor. A total of 16 *E.coli* isolates (40%) and 16 *Klebsiella* isolates (39.02%) screened harbored AmpC enzymes, of which 43.75% of *E.coli* and 56.25% of *Klebsiella* isolates co-produced ESBL enzymes. Pure AmpC production was observed in 56.25% of *E.coli* and 43.75% of *Klebsiella* isolates. The inhibitor based test was useful in identifying cefoxitin susceptible AmpC producers and could also effectively differentiate ESBL from AmpC producing isolates.

KEY WORDS: ESBL, antibiotic susceptibility, clinical samples, β-lactam disks.

INTRODUCTION

AmpC β-lactamases have gained importance since the late 1970s as one of the mediators of antimicrobial resistance in Gram negative bacilli. These enzymes are cephalosporinases capable of hydrolyzing all β-lactams to some extent (1). AmpC β-lactamases are two types – plasmid-mediated and chromosomal or inducible AmpC. Chromosomal AmpC enzymes are seen in organisms such as *Citrobacter freundii*, *Enterobacter cloaca*, *Morganella morgani*i, *Hafnia alvei* and *Serratia marcescens* and are typically inducible by β-lactam antibiotics such as cefoxitin and imipenem but poorly induced (if at all) by the third or fourth generation cephalosporins (2). The most common cause of AmpC over expression in clinical isolates is a mutation in *ampD* leading to AmpC hyperinducibility or constitutive hyperproduction (3).

Plasmid-mediated AmpC β-lactamases have been found worldwide but are less common than extended-spectrum β-lactamases (ESBLs), and in *E.coli*, they appear to be less often a cause of cefoxitin resistance than an increased production of chromosomal AmpC β-lactamase (4). Most strains with plasmid-mediated AmpC enzymes have been isolated from patients after several days of hospitalization, but recently, AmpC-producing isolates in cultures from long-term care facilities, rehabilitation centers, and outpatient clinics have been reported (5;6). Risk factors for bloodstream infections caused by AmpC-producing strains of *Klebsiella pneumoniae* include long hospital stay, care in an intensive care unit (ICU), central venous catheterization, need for an indwelling urinary catheter, and prior administration of antibiotics, especially broad-spectrum cephalosporins and β-lactamase inhibitor combinations, and are thus similar to risk factors for infection by ESBL-producing *K.pneumoniae* strains (7;8).

For clinical microbiologists, detection of AmpC-mediated resistance in Gram negative organisms poses a problem because the phenotypic tests may be misleading resulting in misreporting and treatment failures. There are presently no CLSI or other approved criteria for AmpC detection (9). Organisms producing enough AmpC β-lactamase will typically give a positive ESBL screening test but fail for the confirmatory test involving increased sensitivity with clavulanic acid (10). Screening with cefoxitin disc is recommended for initial detection. However, it does not reliably indicate AmpC production. Some of the phenotypic tests include the three-dimensional tests (11), double-disk test (12) and Etest strips with gradient of cefotetan or cefoxitin on one half and the same combined with a constant concentration of cloxacillin on the other half (13).

This study was undertaken to detect the presence of AmpC β-lactamases in clinical Gram negative isolates by inhibitor based method using boronic acid (BA) (14) and also assess if this test could be used to differentiate between ESBLs and AmpC producers.

MATERIALS AND METHOD

A total of 40 *E.coli* isolates and 41 *Klebsiella* isolates were obtained from clinical samples from Federal Medical center, Owerri, Nigeria. Of the 40 *E.coli* isolates, 10 isolates each were obtained from urine samples, wound, stool and high vaginal swab (HVS). Of the 41 *Klebsiella* isolates, 16 were obtained from urine, 12 from wound while 13 isolates were from stool samples.

ANTIBIOTIC SUSCEPTIBILITY TEST

Antibiotic susceptibility testing was performed using Kirby Bauer method on Mueller-Hinton agar according to CLSI guidelines (15). The antibiotics tested (in µg) were cefoxitin (30), ceftazidime (30), gentamicin (5), ciprofloxacin (5), ampicillin (10), amikacin (30), cefepime (30), cefotaxime (30) and imipenem (10). *E.coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 700603 were used as control strains.

ESBL SCREENING

Isolates were tested for ESBL production using the method described previously (16). Briefly, disks containing standard 30 µg of aztreonam, ceftazidime and cefriaxone were placed 15 mm (edge to edge) from an amoxicillin-clavulanic acid disk (20 and 10 µg, respectively) on inoculated Mueller-Hinton agar. Inoculated media were incubated overnight at 35 °C. An enhanced zone of inhibition between any one of the β-lactam disks and the disk containing clavulanic acid was interpreted as evidence for the presence of an ESBL.

AMP C β-LACTAMASE PRODUCTION TEST

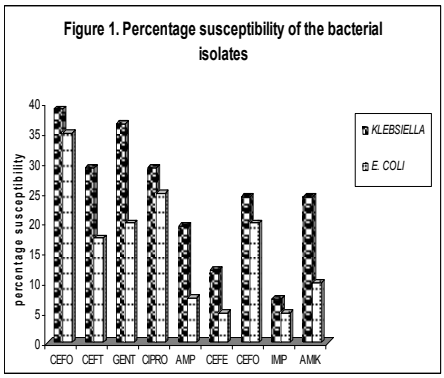
All isolates were tested for AmpC β-lactamase production on disks containing boronic acid (14). A disk containing 30 µg of cefoxitin and another containing 30 µg of cefoxitin with 400 µg of boronic acid were placed on Mueller-Hinton agar. An organism demonstrating a zone diameter around the disk containing cefoxitin and boronic acid ≥5 mm than the zone diameter around the disk containing cefoxitin alone was considered an AmpC producer.

STATISTICAL ANALYSIS

Data obtained from this study were analyzed using a two-way analysis of variance (ANOVA) and values for P≤0.05 were considered statistically significant.

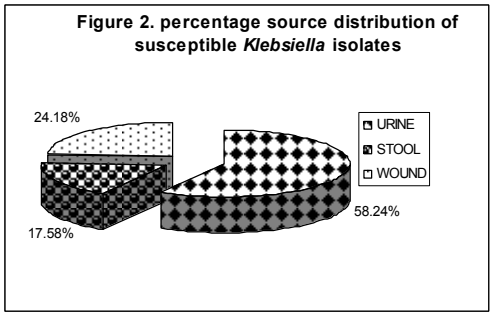
RESULTS

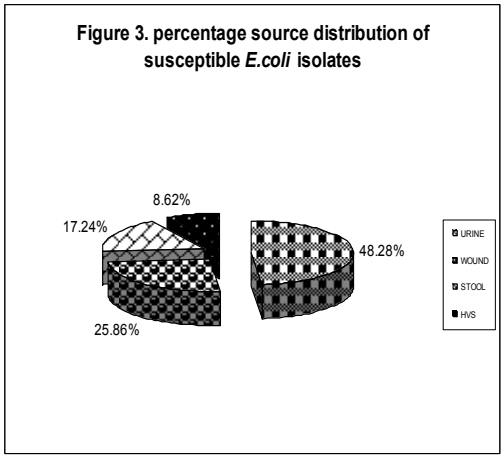
All the isolates were susceptible to at least one of the antibiotics. Most of the *E.coli* isolates were susceptible to cefoxitin, gentamicin, ceftazidin, ciprofloxacin, cefotaxime and amikacin, while majority of them were resistant to imipenem (figure 1). The *Klebsiella* species were less susceptible to ampicillin, cefepine, imipenem and amikacin but the majority were susceptible to cefoxitin, ciprofloxacin and cefotaxime. *Klebsiella* species were more susceptible to the antibiotics tested than the *E.coli* isolates.



KEY: CEFO- cefoxitin, CEFT- ceftazidin, GENT- gentamicin, CIPRO- ciprofloxacin AMP- ampicillin, CEFE- cefepine, CEFO- cefotaxime IMIP- imipenem , AMIK- amikacin

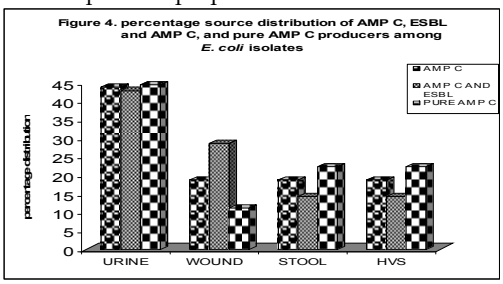
It was observed that most of the susceptible isolates were from urine samples (58.24% and 48.28% for *Klebsiella* and *E.coli*, respectively), while the least were from stool (17.58%) and HVS (8.62%) samples for *Klebsiella* and *E.coli* isolates respectively (figures 2 and 3).The number of *Klebsiella* isolates got from urine samples were more than *E.coli* isolates. The percentage prevalence of susceptible isolates from urine samples was significantly higher than those of the other samples (p≤0.05).



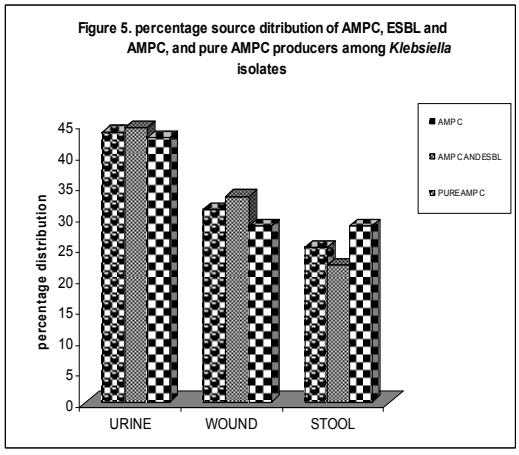


AmpC beta lactamase was detected in 16(40%) of the *E.coli* isolates. Of these isolates, 7(43.75%) were in combination with ESBL while 9(56.25%) were

pure AmpC producers. The percentage prevalence of these isolates from urine samples was significantly higher than those from other samples ($p \leq 0.05$). Wound samples had the least percentage distribution of the isolates which are pure AmpC producers (11.11%) (Figure 4). In each case, the number of pure AmpC producers was more than those with combined ESBL and AmpC production except in the wound sample where the number of combined ESBL and AmpC producers was more than the pure AmpC producers.



Sixteen (39.02%) out of 41 *Klebsiella* isolates were AmpC producers. Of this number, 9(56.25%) were both ESBL and AmpC producers while 7(43.75) were pure AmpC producers. Most of these isolates were also from urine samples (figure 5).The number of isolates with combined ESBL and AmpC production were more than the number of pure AmpC producers in both urine and wound samples but in the stool sample, the number of pure AmpC producers was more than those with combined ESBL and AmpC production.



DISCUSSION

Currently, CLSI documents do not indicate the screening and confirmatory tests that are optimal for detection of AMP C beta lactamases (17). However, several studies have been done on various test methods namely, the three dimensional test (11), modified double disk test (18), AmpC disk test (19), inhibitor based method employing inhibitors like boronic acids (14), broth microdilution method (14) and cefoxitin agar method (20). In spite of many phenotypic tests, isoelectric focusing (21) and genotypic characterization (22) are considered gold standards as the results with the phenotypic tests can be ambiguous and unreliable. In the present study, inhibitor based method with boronic acid was employed for AMP C β-lactamase screening.

A study by Manchanda and Singh, (23) discovered that few of their isolates were susceptible to cefoxitime, all of the cefoxitin-susceptible isolates that harboured an AmpC β-lactamase had MICs of cefoxitin <2 mg/L using the broth dilution method. Bauernfeind et al. (33) isolated a clinically significant strain of *K. pneumoniae* that harboured a novel type of AmpC β-lactamase and that also demonstrated a low level of activity against cephamycins (cefoxitin MIC 4 mg/L). This is at variance with the present study where majority of the isolates were found to be susceptible to cefoxitime. In 2002, Barlow and Hall found *ampC* alleles from the chromosomes of two β-lactam-sensitive *C. freundii* strains isolated in the 1920s, before the clinical use of antibiotics (24). Cefoxitin resistance in AmpC non-producers could be due to some other resistance mechanism(s). Lack of permeation of porins as one of the resistance mechanisms has been reported (25). Hernandez-Alles et al. (26) have demonstrated that interruption of a porin gene by insertion sequences is a common type of mutation that causes loss of porin expression and increased cefoxitin resistance in *K. pneumoniae*. AmpC production in cefoxitin-susceptible isolates may have a mechanism similar to that of ESBLproducing organisms that appear susceptible to ceftazidime by the disc diffusion method. These data indicate that although screening methods that use cefoxitin in standardized methods to detect AmpC-harbouring isolates are useful, they are not perfect. The results in the present study showed that screening should include all the clinical isolates showing resistance to any of the cephalosporins and/or aztreonam, irrespective of their cefoxitin susceptibility status.

AmpC beta lactamase was detected in 16(40%) of the *E.coli* isolates. Of these isolates, 7(43.75%) were in combination with ESBL while 9(56.25%) were pure AmpC producers. The percentage prevalence of these isolates from urine samples was significantly higher than those from other samples ($p \leq 0.05$). Wound samples had the least percentage of susceptible isolates (11.11%). Sixteen (39.02%) out of 41 *Klebsiella* isolates were AmpC producers. Of this number, 9(56.25%) were both ESBL and AmpC producers while 7(43.75) were pure AmpC producers. This prevalence was higher when compared with the reports from other parts of the world (27; 28; 29; 30).

It has been stated that the AmpC beta lactamases when present along with ESBLs can mask the phenotype of the later (31). In this study, it was found that both of these enzymes were equally expressed suggesting a possible low level expression of AmpC enzymes. However, in all

these AmpC producers, chromosomal derepressed and plasmid mediated enzymes were not distinguished as this requires genotypic confirmatory tests. Sixteen of the forty-one *Klebsiella* isolates screened were AmpC producers suggesting the presence of plasmid mediated mechanism as these species do not harbor chromosomal AmpC genes (31). The susceptibility of ceftazidime and cefotaxime serves as a poor marker for the identification of AmpC enzymes (14). It is known that plasmid mediated AmpC enzymes can sometimes appear falsely susceptible to these drugs (32). There are newer Ambler class C (ACC) type of enzymes which have relatively a lower activity to cefoxitin (33) and hence appear susceptible. In the present study, some of the AmpC producing isolates were susceptible to the third generation cephalosporins and cefoxitin by the disc diffusion method. This indicates the probable presence of such ACC enzymes. This needs to be confirmed by molecular methods.

The inhibitor based confirmatory method appears promising for AmpC detection as it increased the sensitivity of the test by picking up additional AmpC producers. Boronic acid has been reported to be effective inhibitor of class C beta lactamases (34; 35; 36). In addition, it can also differentiate ESBL enzymes from the AmpC enzymes.

To conclude, a mixed type of drug resistance mechanisms seem to operate in the isolates tested. There is need for a correct and reliable phenotypic test to identify AMP C beta lactamases and ESBL producers. Inhibitor based method using boronic acid appears to be effective in discriminating this type of resistant isolates.

REFERENCES

1. Hanson ND. AMP C beta lactamases: what do we need to know for the future? J. Antimicrob. Chemother. 2003; 52: 2-42.

2. Philippon A, Arlet G, Jacoby GA. Plasmid-determined AMP C-type beta lactamases. Antimicrob. Agents Chemother. 2002;46: 1-11.

3. Schmidtke AJ, Hanson ND. Model system to evaluate the effect of *ampD* mutations on AMP

   C-mediated β-lactam resistance. Antimicrob. Agents Chemother. 2006;50: 2030-2037.

4. Jacoby AG. AMP C β-lactamases. Clin. Microbiol. Rev. 2009; 22(1):161-182.

5. Hanson D, Moland ES, Hong SG, Propst K, Novak DJ, Cavalieri SJ. Surveillance of community-based reservoirs reveals the presence of CTX-M imported AMP C, and OXA-30 β-lactamases in urine isolates of *Klebsiella pneumoniae* and *Escherichia coli* in a US community. Antimicrob. Agents Chemother. 2008; 52: 3814-3816.

6. Migliavacca R, Nucle, E, D'Andrea MM, Spalla M, Giani T, Pagani L. Acquired AMP C type beta-lactamases: an emerging problem in Italian long-term and rehabilitation facilities. New Microbiol. 2007; 30: 295-298.

7. Pai H, Kang CI, Byeon JH, Lee KD, Park WB, Kim HB, Kim EC, Oh MD, Choe KW. Epidemiology and clinical features of bloodstream infections caused by AMP C-type-β-lactamase-producing *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. 2004; 48: 3720-3728.

8. Yan JJ, Ko WC, Wu JJ, Tsai SH, Chuang CL. Epidemiological investigation of bloodstream infections by extended spectrum cephalosporin-resistant *Escherichia coli* in a Taiwanese teaching hospital. J. Clin Microbiol. 2004; 42: 3329-3332.

9. Doi Y, Paterson DL. Detection of plasmid-mediated class C β-lactamases. Int. J. Infect. Dis. 2007; 11: 191-197.

10. Bell JM, Chitsaz M, Turnidge JD, Barton M, Walters LJ, Jones R. Prevalence and significance of a negative extended-spectrum β-lactamase (ESBL) confirmation test result after a positive ESBL screening result for isolates of *Escherichia coli* and *Klebsiella pneumoniae*: result from SENTRY Asia-pacific surveillance program. J. Clin. Microbiol. 2007; 45: 1478-1482.

11. Thomson KS, Sanders CC. Detection of extended-spectrum β-lactamases in members of the family *Enterobacteriaceae*: comparison of double-disk and three-dimensional tests. Antimicrob. Agents Chemother. 1992; 36: 1877-1882.

12 Ruppe E, Bidet P, Verdet C, Arlet G, Bingen E. First detection of the Ambler Class C 1 AMP C β-lactamase in *Citrobacter freundii* by a new, simple double-disk synergy test. J Clin. Microbiol. 2006; 44: 4204-4207.

13. Bolmstrom A, Engelhardt A, Bylund L, Ho P, Karlson A. Evaluation of two new Etest strips for AMP C detection. Abstract D-0451.*46th interscience conference on antimicrobial agents and chemotherapy*. 2006.

14. Coudron PE. Inhibitor-based methods for detection of plasmid-mediated AMP C beta-lactamases in *Klebsiella* spp., *Escherichia coli* and *Proteus mirabilis*. J. Clin. Microbiol. 2005; 43: 4163-4167.

15. Clinical Laboratory Standard Institute. *Performance standards for antimicrobial disk susceptibility tests*. 8th ed. Approved standard M2-A8. Wayn, Pa: Clinical Laboratory Standards. 2003.

16. Akujobi CO, Ogbulie JN, Alisi SC. Occurrence of extended spectrum β-lactamases in *Escherichia coli*

isolated from piggery farms in Imo State, Nigeria. World J. Microbiol. Biotechnol. 2008; 24: 2167-2170.

17. Hemalatha V, Padma M, Uma Sekar Vinodh, TM, Arunkumar AS. Detection of AMP C beta lactamases production in *Escherichia coli* and *Klebsiella* by an inhibitor based method. Indian J. Med. Res. 2007; 126: 220-223.

18. Pitout JD, Reisbig MD, Venter EC, Church DL, Hanson ND. Modification of the double-disk test for detection of enterobacteriaceae producing extended-spectrum and AMP C beta-lactamases. J. Clin. Microbiol. 2003; 41: 3933-3935.

19. Black J, Moland ES, Thomson KS. A simple disk test for detection of plasmid-mediated AMP C production in Klebsiella. In: *Program and abstracts of the forty-second interscience conference on antimicrobial agents and chemotherapy*; Abstract D-534, Washington, DC, USA:American Society for Microbiology; P. 140. 2002.

20. Nasim K, Elsayed S, Pitout JDD, Conly J, Church DL, Gregson DB. New method for laboratory detection of AMP C beta-lactamases in *Escherichia coli* and *Klebsiella pneumoniae*. J. Clin. Microbiol. 2004; 42: 4799-4802.

21. Mathew M, Harris AM, Marshall MJ, Ross GW. The use of analytical isoelectric focusing for detection and identification of beta-lactamases. Gen. Microbiol. 1975; 88: 169-178.

22. Perez-Perez FJ, Hanson ND. Detection of plasmid-mediated AMP C beta-lactamase genes in clinical isolates by using multiplex PCR. J. Clin. Microbiol. 2002; 40: 2153-2162.

23. Manchanda V, Singh NP. Occurrence and detection of AmpC β-lactamases among Gram-negative clinical isolates using a modified three-dimensional test at Guru Tegh Bahadur Hospital, Delhi, India. J. Antimicrob. Chemother. 2003; 51:415-418.

24. Barlow M, Hall B G. Origin and evolution of the AmpC beta-lactamases of Citrobacter freundii. Antimicrob. Agents Chemother. 2002; 46:1190–1198.

25. Pangon B, Bizet C, Bure A, Pichon F, Philippon A, Ragnier B. In vivo selection of a cephamycin-resistant, porin-deficient mutant of K. pneumoniae producing a TEM-3 betalactamase. J. Infect. Dis. 1989; 159: 1005–1006.

26. Hernandez-Alles S, Benedi V J, Martinez-Martinez L, Pascual A, Aguilar A, Tomas J M. Development of resistance during antimicrobial therapy caused by insertion sequence interruption of porin genes. Antimicro. Agents Chemother. 1999; 43: 937–9.

27. Liu PYF, Gur D, Hall LMC, Livermore DM. Survey of the prevalence of beta lactamases among 1000 Gram negative bacilli isolated consecutively at the Royal London Hospital. J. Antimicrob.Chemother. 1992; 30: 429-447.

28. Coudron PE, Moland ES, Thomson KS. Occurrence and detection of AMP C beta lactamases among *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis* isolates at a veteran medical center. J.Clin. Microbiol. 2000; 38: 1791-1796.

29. Singhal S, Mathur T, Khan S, Upadhyay DJ, Chugh S, Gaind R. Evaluation of methods for AMP C beta-lactamases in Gram negative clinical isolates from tertiary care hospitals. Indian J. Med Microbiol. 2005; 23: 120-124.

30. Manchanda V, Singh P, Shamweel A, Eideh HK, Thukral SS. Molecular epidemiology of clinical isolates of AMP C producing *Klebsiella pneumoniae*. Indian J. Med. Microbiol. 2006; 24: 177-181.

31. Thomson KS. Controversies about extended-spectrum and AMP C beta-lactamases. Emerg. Infect. Dis. 2001; 7: 333-336.

32. Black JA, Thomson KS, Buynak JD, Pitout JDD. Evaluation of beta-lactamase inhibitors in disk tests for detection of plasmid-mediated AMP C beta-lactamases in well-characterized clinical strains of *Klebsiella* spp. J. Clin. Microbiol. 2005; 43: 4168-4171.

33. Bauernfeind A, Schneider I, Jungwirth R, Sahly H, Ullmann U. A novel type of AMP C beta-lactamase, ACC-1, produced by a *Klebsiella pneumoniae* strain causing nosocomial pneumonia. Antimicrob. Agents Chemother. 1999; 43: 1924-1931.

34. Beesley T, Gascoyne N, Knott-Hunziker V, Petursson S, Waley SG, Jaurin B. The inhibition of class C beta-lactamases by boronic acids. Biochem. J. 1982; 209: 229-233.

35. Liebana E, Gibbs M, Clouting C, Barker l, Crifton-Hardley FA, Pleydell E. Characterization of beta-lactamases responsible for resistance to extended-spectrum cephalosporins in *Escherichia coli* and *Salmonella enterica* strains from food-producing animals in the United Kingdom. Microb. Drug Resist. 2004; 10: 1-9.

36. Powers RA, Blazquez J, Scott Weston G, Morosini M, Baquero F, Shoichet Bk. The complexed structure and antimicrobial activity of a non-beta-lactam inhibitor of AMP C beta-lactamase. Protein Sci. 1999; 8: 1330-2337.

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ASSESSMENT OF THE DIAGNOSTIC POTENTIAL OF CLINOTECH TB SCREEN TEST FOR DIAGNOSIS OF PULMONARY TUBERCULOSIS IN NIGERIA

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ABSTRACT

The Clinotech TB Screen test, a 3rd generation multi-antigen rapid chromatographic immunoassay for detection of IgG antibodies in serum against recombinant protein antigens 38kDa, 16kDa and 6kDa, was assessed for its diagnostic potential for diagnosis of active pulmonary TB in routine TB control programme in Abia State, Nigeria. The overall sensitivity and specificity of Clinotech TB Screen test were 24.1% and 87.8% (95% Confidence intervals [CI]: 14.7-33.5% and 80.6-95.0%) respectively. The positive and Negative Predictive Values (PPV and NPV) were 79.2% and 37.5% respectively. The performance of the test was inferior to that of the sputum smear microscopy which had a sensitivity of 50.0% (95% CI, 39.0%-61.0%) and specificity of 92.3(95% CI: 86.4-98.2%). In 37 culture positive smear positive PTB cases, Clinotech TB Screen test was positive in 18(48.65%). The rapid test showed a very low degree of sensitivity in smear –negative culture positive PTB cases; detecting just one (2.38%) out of 42 cases. These results indicate that the diagnostic value of Clinotech TB Screen test for routine diagnosis of PTB in this setting is limited.

Key words: Tuberculosis, serological tests, immunochromatographic tests, rapid TB tests

INTRODUCTION

Diagnosis is a key element in the global plan to control TB (1). Rapid and accurate diagnosis is critical to effective TB care and interruption of transmission (2). However, lack of simple, rapid and accurate diagnostic tests remains one of the greatest constraints for effective control of TB, especially in developing countries where the highest burdens of TB are found and facilities for performing traditional laboratory diagnosis are inadequate or not available (3). Furthermore, the existing conventional diagnostic methods such as sputum smear microscopy (SSM), culture and chest radiography have technical and operational characteristics that limit the quality and the scope of their applications in developing countries (4).

Rapid Diagnostic tests (RDTs) employing immunological principles to detect antibodies have recently been developed. These tests are attractive because they are easy to use, rapid, and relatively inexpensive and without the need for sophisticated laboratory infrastructure (5). They hold out a great promise of providing the developing countries with an invaluable way to quickly and cheaply diagnose infectious diseases (6).

Many antigens of *Mycobacterium tuberculosis* have been isolated, purified and tested for their potential for development of rapid serological tests (7). Some of these antigens have been employed in developing commercial serological TB tests (8). Clinotech TB Screen from Clinotech Diagnostics and Pharmaceuticals, Inc, Canada, is one of such tests being distributed in several developing countries including Nigeria. Evaluation of several rapid TB serological tests showed that the tests have variable performance in different epidemiological settings (5). We conducted the present study to assess the diagnostic performance characteristics of Clinotech TB Screen for diagnosis of pulmonary tuberculosis in Abia State, Nigeria.

MATERIALS AND METHODS

Study Participants.
The study participants were recruited from TB suspects, defined as patients with cough of at least 3 weeks' duration referred to the Leprosy and Tuberculosis Referral Hospital, Uzuakoli, Bende Local Government Area (LGA) and Aba South LGA Health Office, Aba, two major directly observed treatment short course (DOTS) centres within the Abia State TB Control Programme, to undergo sputum smear

microscopy investigation for pulmonary tuberculosis (PTB) between November 2008 and February 2010. Eligible study participants were TB suspects 15 years and above who were not on antituberculosis treatment at the time of recruitment. The study included 150 TB suspects who met the inclusion criteria and were prospectively enrolled as study participants. Informed consent for participation was obtained from the study participants. The study protocol was approved by the Ethical Committee of the Federal Medical Centre (FMC), Umuahia, Abia State.

Three sputum samples, one spot sample on the first day of patient visit, an early morning sample brought from home the next day, and the second spot sample collected when the patient brought the early morning specimen, were obtained from each study participant. Venous blood samples were also collected from the TB suspects for serological TB tests. The blood samples were collected into anticoagulant free plastic tubes from which the sera were later separated.

Sputum smear microscopy and sputum culture

For sputum smear microscopy, direct sputum smears were made from the sputum samples and stained by the Ziehl-Neelsen staining technique for acid-fast bacilli (AFB). For culture, the sputum samples were decontaminated using modified Petroff"s alkali method and concentrated by centrifugation at 3,000rpm for 30 minutes. The processed sputum samples were cultured on Lowenstein-Jensen (LJ) medium and incubated at 37^{o}C and examined for growth daily for the first 7 days and twice weekly thereafter up to 8th week until a definite result was obtained. The slants that did not showed any growth after 8 weeks of incubation were recorded as culture negative.

Clinotech TB Screen test

The Clinotech TB Screen test was manufactured by Clinotech Diagnostics and Pharmaceuticals, Inc, Canada and distributed in Nigeria by Clinotech Nigeria Limited, Lagos. The Clinotech TB Screen test is a 3rd generation chromatographic immunoassay (CIA) using direct binding double sandwich antibody (Ab-Ag-Ab) principle for direct qualitative detection of TB antibody in human serum or plasma. The test is a multi-antigen test containing recombinant *M. tuberculosis* antigens 38kDa, 16kDa and 6kDa conjugated to colloidal gold particles and immobilized on the test membrane.
The test was done according to the manufacturer's instructions contained in the package insert. The test device was removed from the pouch and placed on a clean, dry level surface. Using the plastic pipette provided in the test pouch, serum was drawn up to the 100µl mark on the pipette and applied into the sample application well. The test result was read at 15 minutes after sample application and interpreted according to the manufacturer's instructions.

RESULTS

Clinotech TB Screen test was compared with sputum smear microscopy in 150 TB suspects. The sputum samples of 52(37.14%) of the patients were positive for AFB. Clinotech TB Screen test was positive in 26 (50.00%) of the 52 smear positive cases and 6 (6.12%) of the 98 smear negative cases. In total the Clinotech TB Screen was positive for anti-TB antibodies in 32 (22.86%) of the total study participants. The ability of the test to detect the smear-positive cases according to AFB grading is shown in Table 1. The ability of the Clinotech TB Screen test to identify the culture positive PTB cases is shown in Table 2. In 37 culture and smear positive PTB cases, the test was positive in 18 (48.65%) and in 1 (2.38%) of 42 culture-positive but smear-negative PTB cases.

TABLE 1: PERFORMANCE OF CLINOTECH TB SCREEN TEST IN SPUTUM SMEAR POSITIVE PTB PATIENTS IN ABIA STATE

AFB Grade	No. (%) Positive by SSM	No. (%) Positive by Clinotech TB Screen test
Scanty (1-9)[a]	6/52 (11.54)	1/6 (16.67)
1+ (10-99)[b]	14/52 (26.92)	9/14 (64.29)
2+ (1-10)[c]	16/52 (30.77)	9/16 (56.25)
3+ (>10)[d]	16/52 (230.77)	7/16 (43.75)
TOTAL	52 (100)	26/52 (50.00)

[a] 1-9 AFB/100HPF (high power field)
[b] 10-99 AFB/100HPF
[c] 1-10 AFB/HPF
[d] >10AFB/HPF
SSM- Sputum smear microscopy

The overall performance of the test against a reference standard of sputum culture is presented in Table 3. The sensitivity and specificity were 24.1% and 87.8% respectively. The positive and negative predictive

values were 79.2% and 37.5% respectively. The combination of the results of Clinotech TB Screen test with those of smear microscopy resulted in the improvement of the sensitivity from 24.1% to 51.4% but with a corresponding decrease of specificity from 87.8% to 80.9%.

TABLE 2: DETECTION OF CULTURE POSITIVE PTB CASES BY CLINOTECH TB SCREEN TEST

Diagnostic category	No. of cases	No. Positive (%)	No. Negative (%)	No. (%) with low signal result (Faint)
Culture positive, smear positive (Cx +ve, Sm +ve)	37	18(48.65)	19 (51.35)	1 (5.55)
Culture positive, smear negative (Cx +ve, Sm –ve	42	1 (2.38)	41 (97.62)	0 (0)
Culture positive, smear positive (*NTM)	8	3 (37.50)	5 (62.25)	0 (0)
Culture positive, smear negative (NTM)	4	2 (50.00)	2 (50.00)	0 (0)

*NTM - Nontuberculous mycobacteria

TABLE 3. SENSITIVITY, SPECIFICITY, PPV AND NPV OF CLINOTECH TB SCREEN TEST IN REFERENCE TO SPUTUM CULTURE

Diagnostic tests	Sputum culture						
	Positive	Negative	Total	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
Clinotech TB Screen test							
Positive	19	5	24	24.1 (14.7-33.5)[a]	87.8 (80.6-95.0)	79.2 (70.2-88.2)	37.5 (26.8-48.2)
Negative	60	36	96				
Total	79	41	120				

[a]95% CI- 95% Confidence Interval
PPV- Positive Predictive value
NPV- Negative Predictive Value

DISCUSSION

We have assessed the diagnostic potential of Clinotech TB Screen test for diagnosis of active pulmonary TB in routine TB control programme in Abia State, Nigeria. The performance of the test was inferior to that of the sputum smear microscopy. While the sputum smear microscopy had a sensitivity of 50.0% (95% Confidence interval [CI], 39.0%-61.0%), the Clinotech TB Screen test had a sensitivity of 24.1%

(95% CI: 14.7%-33.5%). In 37 culture positive smear positive PTB cases, Clinotech was positive only in 18(48.65%). The rapid test showed a very low degree of sensitivity (2%), detecting just one of 42 cases of smear –negative culture positive PTB cases in this study. The overall sensitivity and specificity of Clinotech TB Screen test were 24.1% and 87.8% (95% CI: 80.6-95.0%) respectively. The positive and Negative Predictive Values (PPV and NPV) were 79.2% and 37.5% respectively. These values indicate that the diagnostic value of Clinotech TB Screen test for routine diagnosis of PTB in this setting is limited.

Previous evaluations of serological TB tests in Nigeria showed variable performance of low to moderate sensitivities. The ELISA-based Pathozyme TB EIA kits (Pathozyme-TB Complex Plus, Pathozyme Myco A, Myco M and Myco G were evaluated in Lagos by Enwuru *et al*. (9). These kits employed 38kDa and 16kDa antigens as in Clinotech TB Screen test. The authors reported sensitivities of 4% for Myco A, 24% for Myco M and 76% for Myco G with specificity of 100% for Myco G in smear and culture positive category. The performance of Clinotech TB Screen test in our study appeared to be lower than that of Myco G which is the kit in that series that was directed at detecting IgG antibodies. Ohanu *et al*. (10) reported the evaluation of the SmartCheck TB immunochromatographic test in a hospital-based study in Enugu, Nigeria. They used sputum smear microscopy as reference standard and compared the test with X-ray. The sensitivity was reported as 89.5% and the specificity was 77.7%. It is difficult to make a direct comparison of our results with this study because we used culture as reference standard and because the identity of mycobacterial antigen used in the SmartCheck TB was not stated in the paper. Our results are similar to those of Kassa-Kelembho *et al*. (11) who reported a sensitivity of 20.6% and specificity of 90.3% for SDHO MTB test in Bangui, Central African Republic.

Poor diagnostic performance of rapid TB tests has been reported in several previous studies. In a study conducted by Pottumarthy *et al*. (12) to compare the accuracy of seven antibody detection tests for diagnosis of tuberculosis in New Zealand among immigrants from countries with a high prevalence of tuberculosis, the sensitivity ranged from 16% to 57%. The two immunochromatographic tests, ICT Tuberculosis and Rapid Test TB, included in that study had sensitivity of 41% and 25% respectively. The corresponding specificities were 96% and 87% respectively. The results of laboratory –based evaluation of 19 commercially available rapid diagnostic tests for TB sponsored by Special Programme for Research and Training in Tropical Diseases (TDR) between 2005 and 2006 showed that the sensitivity of the tests ranged from 0.97% to 59.7%; specificity ranged from 53% to 98.7% (13). Clinotech TB Screen test was not included in the TDR study because the manufacturer declined to participate in the study. The evaluation of this test is reported for the first time in this study. Anderson *et al.* (14) evaluated three ELISA based TB antibody tests, InBios Active Tb Detect, IBL *M. tuberculosis* and Anda Biologics TB which utilized newer mycobacterial antigens: Mtb81, Mtb8, Mtb48, DPEP (MPT32) in addition to 38-KDa protein, and two additional proprietary antigens. The sensitivity of InBios Active Tb Detect was 83.3%, IBL *M. tuberculosis* had sensitivity of 5.6% and Anda Biologics TB had 83.3% with corresponding specificities of 98.9%, 100% and 72% respectively.

Although we found the Clinotech TB Screen test to be a rapid and easy to use test, the low sensitivity of the test makes it unsuitable for use as a stand alone test for routine diagnosis of TB in this setting.

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REFERENCES

1. Raviglione, M.C. The new Stop TB Strategy and the Global Plan to stop TB, 2006-2015. *Bulletin of the World Health Organization* 2007; 85 (5): 327
2. Pai, M., Ramsay, A. and O'Brien, R. Evidence-Based Tuberculosis Diagnosis. *PLOS Medicine* 2008; 5(7): e156.doi:10.137/journal.pmed.0050156
3. WHO/TDR *Diagnostics for Tuberculosis: Global demand and Market Potential*. 2006; WHO, Geneva, Switzerland
4. Perkins, M.D. and Kritski, A.L. Diagnostic testing in the control of tuberculosis. *Bulletin of the World Health Organization* 2002; 80 (6) 512-513.
5. Steingart, K. R., Henry M., Laal, S., Hopewell, P.C., Ramsay, A., Menzies, D., Cunningham, J., Weldingh, K. and Pai, M. Commercial serological Antibody Detection Tests for the Diagnosis of Pulmonary Tuberculosis: A systematic Review. *PLOS Medicine* 2007; 4(6): 1041- 1060, e202.doi:10.1371/journal.pmed.0040202
6. Perkins, M.D., Roscigno, G. and Zumla, A. Progress towards improved tuberculosis diagnostics for developing countries. *Lancet*. 2006; 367: 942 - 943

7. Perkins, M.D., Conde, M.B., Martins, M. and Kritski, A.L. Serologic diagnosis of tuberculosis using a simple commercial multi antigen assay *Chest* .2003; 123: 107 - 112.
8. Steingart, K. R., Dendukuri, N., Henry, M., Schiller, I., Nahid, P., Hopewell, P. C., Ramsay, A., Pai, M. and Laal, S. Performance of purified antigens for serodiagnosis of pulmonary tuberculosis: a meta-analysis. *Clinical and Vaccine Immunology* .2009;16(2): 260 – 276
9. Enwuru, C.A., Idigbe, E.O., Ezeobi, N.V., Oparaugo, C.T., Udensi, K. U., Onyewuche, J.I. and Ibiam, J. Comparative Study on Specific and Early Detection of Pulmonary Mycobacteria Complex using smear and culture methods and serological Pathozyme EIA kits. *African Journal of Clinical and Experimental Microbiology*. 2004; 5(2): 182 - 188.
10. Ohanu, M. E., Okonkwo, P. O., Aghaji, M., Njeze, N., Nwokocha, A. and Ele, E. Diagnosis of Tuberculosis: Experience with use of an Immunochromatographic Assay in a Nigerian Hospital. *Journal of College of Medicine*. 2004; 9(1): 42 – 44.
11. Kassa-Kelembho, E., Kassa, E., Zandanga, G., Service Y-B., Ignaleamoko, A. and Talarmin, A. Poor performance of a Novel Serological Test for Diagnosis of Pulmonary Tuberculosis in Bangui, Central African Republic *Clinical and Vaccine Immunology*. 2006; 13(6): 702 - 703.
12. Pottumarthy, S., Wells, V.C. and Morris, A.J. A comparison of seven tests for serological diagnosis of tuberculosis *Journal of Clinical Microbiology*. 2000; 38: 2227-2231
13. WHO/TDR. *Laboratory-based evaluation of 19 commercially available rapid diagnostic tests for tuberculosis (Diagnostics Evaluation Series, No. 2)* .2008; WHO, Geneva, Switzerland.
14. Anderson, B. L., Welch, R. J. and Litwin, C. M.. Assessment of three commercially available serologic assays for detection of antibodies to *Mycobacterium tuberculosis* and identification of active tuberculosis. *Clinical and Vaccine Immunology*. 2008; 15(11): 1644 – 1649.