(i)

TITLE:

SOME ASPECTS OF HEPATOCELLULAR CARCINOMA AND LIVER
CIRRHOSIS IN KENYA.

A DISSERTATION SUBMITTED IN PART-FULFILMENT FOR THE
DEGREE OF MASTER OF MEDICINE (PATHOLOGY)
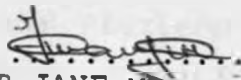UNIVERSITY OF NAIROBI

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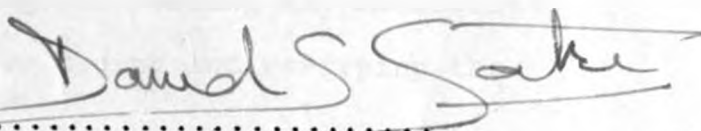## DECLARATION

This work is original and has not to my knowledge been submitted for a degree in any other University.

DR.JANE. MWANGI

This dessertation has been submitted for examination with my approval as the University supervisor

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ACKNOWLEDGEMENTS

CONTENTS

LIST OF FIGURES, PLATES AND TABLES

Abbreviations

HBCAg   - Hepatitis B core antigen
HBSAg   - Hepatitis B surface antigen
HBV   - Hepatitis B virus
HCC   - Hepatocellular carcinoma
PAP   - Peroxidase-anti-Peroxidase

SUMMARY

    The main aim of this study was to examine the role played by Hepatitis B Virus in the causation of hepato-cellular carcinoma and liver cirrhosis in Kenya.

    Liver biopsy samples were stained by the orcein histochemical method and peroxidase-anti-peroxidase immunohistochemical method to check for the presence of Hepatitis B surface antigen and Hepatitis B core antigen. The study included 108 cases of hepatocellular carcinoma, 35 cases of liver cirrhosis and 40 normal liver biopsies.

    Analysis of the results revealed the following important facts:

1) Only 2.5% of normal livers had HBSAg.

2) In contrast 33% of hepatocellular carcinoma and 26% of liver cirrhosis cases had HBSAg.

3) Background cirrhosis was found in 52% of HCC cases.

4) Positivity of HBSAg is more demonstrable in open liver biopsies than in needle biopsies;about 44% positivity as opposed to 10% in cirrhosis respectively.

5) Cases of liver cirrhosis show a higher positivity for HBCAg than HCC cases; 14% and 11.5% respectively. More cases of cirrhosis were positive for both HBSAg and HBCAg than HCC

6) This study also documents that viral antigens are randomly distributed in non-neoplastic tissue and therefore its demonstration is proportional to the amount of tissue examined.

7) Survival of the antigen in tissue depends on the type and concentration of fixative, fixation time and size of specimen. The antigen is destroyed or altered at temperatures above 60°C.

8) The orcein histocehmical stain is as sensitive as the Peroxidase-anti-Peroxidase immunohistochemical stain in the detection of HBSAg.

    Current views on the aetiology of hepatocellular carcinoma are discussed and preventive measures suggested.

1.   <u>INTRODUCTION</u>

Hepatocellular carcinoma is among the ten most common cancers in the world and one of the most prevalent cancers in developing countries. It is estimated that there are at least 250,000 new liver patients every year[1]. Mortality is 100% and the incidence continues to rise both in developing and developed countries. Hepatocellular carcinoma (HCC) is the commonest of the malignant tumours of the liver. Other primary liver malignancies are rare and include cholangiosarcoma, angiosarcoma and hepatoblastoma.

There is much geographical variation in the incidence of HCC. In tropical Africa, South East Asia and the Western Pacific regions rates of 12 - 150 per 100,000 have been reported[2,3,4]. It is however relatively uncommon in the developed countries of Europe, America and Australia. In Kenya HCC is the third commonest solid cancer in males after skin cancer and lymphoma. The male:female ratio is 9:1 and the peak age incidence is 35-45 yr[5]. This is in contrast to the developed countries where the peak age incidence is 65-75 yr. There is a striking worldwide correlation between the prevalence of Hepatitis B surface antigen (HBSAg) chronic carriers and the incidence of hepatocellular carcinoma. Epidemiological data from case control and cohort studies and several laboratory investigations indicate that there is a consistent and specific causal association between Hepatitis B virus (HBV) and hepatocellular carcinoma and that upto 80% of such cancers are attributable to the virus.[1]. Hepatitis B virus is thus second only to tobacco among known human associated carcinogens.

Hepatitis B virus was discovered by Blumberg in 1965[6]. It belongs to a group of animal viruses known as hapadnaviridae and measures 42nm in diameter. It consists of an envelop and nucleocapsid containing a circular DNA molecule, DNA polymerase, a DNA linked protein and a protein kinase. The envelop carries the HBSAg while the capsid carries the hepatitis B core antigen (HBCAg).

Hepatitis B surface antigen is a conformational antigen being
a dimer of two major proteins linked by disulphide bridges.

In a study carried out by Beasley et al[7] in Taiwan,
the relative risk of developing HCC for HBSAg carriers was
217 times as compared to non-carriers. The mortality rate
due to HCC in HBSAg carriers was 51% compared to 2% in the
control population. Among the non-carriers 90% had and HBV
marker (anti HBs and/or anti HBc antibodies). The high
incidence of HCC is therefore clearly related to the HBSAg
chronic carrier state and not to HBV infection per se.

In a prospective study on patients with chronic liver
disease, Kubo et al[8] found that HBSAg positive patients
developed HCC earlier than did antigen negative patients.

In tropical Africa and Far East Asia chronic HBV
carriers represent 10% or more of the population. This
is associated with a high incidence of HCC in these regions.

Hepatocarcinogenesis has long been postulated to be
related to cell regeneration and the subsequent malignant
transformation that ensues. Anthony et al[9] demonstrated
in Ugandan patients with cirrhosis that hepatocellular
swelling and dysplasia were closely associated with the
presence of HBSAg. They found dysplasia in 64.5% of 124
patients with HCC as compared to 1% in controls. Dysplasia
was considered as a precancerous lesion and HBV infection
was postulated to preceed malignant change in liver cells.
Farther evidence in support of the carcinogenic role of
HBV has been obtained from the induction of HCC experimentally
in woodchuk by inoculation with woodchuk hepatitis virus
at birth. This virus belongs to the same family with
HBV. Hepatitis B virus DNA sequences integrated into
HBSAg positive liver tissue have been demostrated by Southern
blotting technique. In chronic carriers free viral DNA,
without integration is detected while in the early stage
of tumour growth both free and integrated HBV DNA sequences
are found.
Brechot et al[10] have demonstrated integration of HBV
DNA in over 90% of HBSAg positive HCC tumour tissue.
Similar results were obtained by Shafritz and Kew in 1981,
Chen et al in 1982 and Hino et al in 1984[11,12,13].

In some cases of HCC without HBV serological markers
intergrated HBV DNA was found. Studies based in Kenya
have shown a close association between cirrhosis, HCC
and the presence of HBV markers in serum[14]. Integrated
HBV DNA was detected in 41% and episomal DNA in 18% of
HBSAg positive HCC liver tissue[15].

Cirrhosis is a chronic disease of many different
aetiologies resulting from long-continued loss of liver
cells, with a persistent inflammatory reaction accompanied
by fibrosis and compensatory reparative hyperplasia.

The high risk for malignancy associated with cirrhosis
varies with geographical location, aetiology and morphology.
It is highest when cirrhosis follows HBV infection. Gibson
et al calculated the approximate risk for HBSAg carriers
to develop cirrhosis as 16 and for these to develop HCC as
50[16].

Several studies using immunofluorescent techniques,
immunoelectron microscopy, histochemical and immunohistochemical
methods have established that the 20 nm particles comprising
HBSAg found in the sera of infected persons may be found
in hepatocyte cytoplasm of infected individuals[17-25].

Shikata and associates reported a staining method for HBSAg
in paraffin sections using orcein stain[21]. Shikata's
staining technique is based on the presence of disulphide
bonds in HBSAg.

Immunoperoxidase methods for detection of HBV antigens
have also been developed.[18]
These are based on antibody-linked histochemically
demonstrable enzymes such as horseradish peroxidase, A
sandwich reaction is involved in which antibody to the
target antigen (primary antibody of rabbit source),
antibody to the primary antibody (link antibody from goat)
and peroxidase-anti-peroxidase (PAP) reagent are added
sequentially with interposed washing steps. Because the
PAP reagent is linked to a rabbit antibody it will be linked
by the link antibody (goat anti-rabbit) to the primary antibody
and tissue antigen. Horseradish peroxidase acts on the substrate
diaminobenzidene to give a brown product.

Fig. 1

SCHEMATIC PRESENTATION OF MECHANISM OF PAP FOR HBSAg

| HBSAg | Rabbit anti HBSAg (Primary ab) | Goat anti-rabbit | Rabbit anti-HRP | HRP |

Rabbit immunoglobulin G

Goat anti-rabbit immunoglobulin G
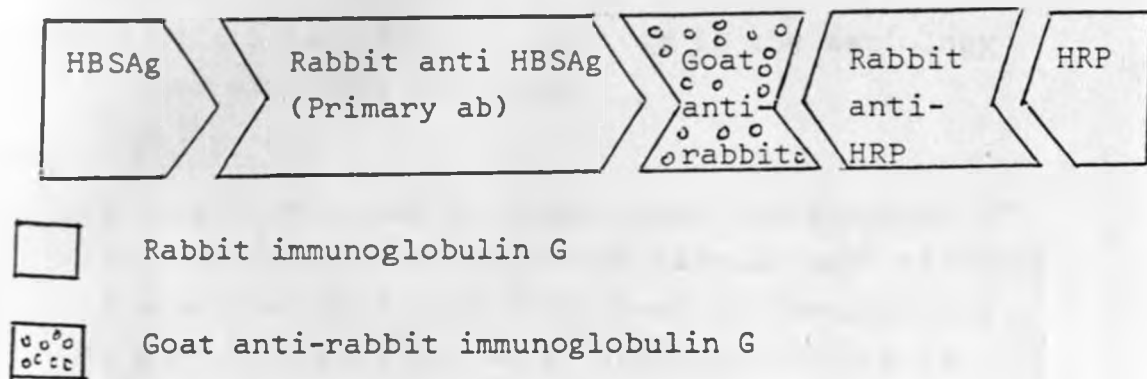
2.1   HYPOTHESIS:

1) Hepatitis B infection is involved in the aetiology of hepatocellular carcinoma in Kenya.

2) Hepatitis B infection is involved in the aetiology of liver cirrhosis in Kenya.

2.2   OBJECTIVES:

This study will seek to demonstrate the presence of HBSAg and HBCAg in liver tissue samples with cirrhosis and hepatocellular carcinoma fixed in formalin and embedded in paraffin. Liver samples reported as histologically normal will serve as controls.

2.3   SPECIFIC OBJECTIVES:

1) To compare HBSAg and HBCAg positivity in hepatocellular carcinoma, cirrhosis and normal liver tissue.

2) To examine the level of HBCAg positivity in HBSAg positive cirrhosis and heptocellular carcinoma.

3) To compare HBV components positivity in hepatocellular carcinoma with cirrhosis and that without associated cirrhosis.

4) To compare HBV components positivity in needle biopsies and open biopsies or autopsy specimens.

3.   MATERIALS AND METHODS

Liver tissues processed at the Kenyatta National Hospital
Department of Human Pathology in the period 1980 - 1984
were examined. Previous records were used to identify those
specimens on which a diagnosis of hepatocellular carcinoma,
cirrhosis or normal were made. Paraffin blocks of these
tissues were retrieved from the store, cut into 5um thick sections,
de-waxed and stained variously with Haematoxylin and Eosin,
orcein and by immunoperoxidase techniques.

The standard Haematoxylin and Eosin staining method
was used[26]. See Appendix 1. These sections were examined
to confirm the previous histological diagnosis. The orcein
stain according to Shikata et al[21] was used to demonstrate
HBSAg - Appendix 2.

Immunoenzymatic staining kits for HBSAg and HBCAg from
Dakopatts were used for immunological detection of the
respective antigens. These kits are designed to demonstrate
antigens primarily in formalin-fixed paraffin embedded
tissues. See Appendix 3 for methodology.

4.   RESULTS

A total of 183 specimens were included in the study.
These consisted of 108 cases of hepatocellular carcinoma,
35 cases of liver cirrhosis and 40 cases of histologically
normal livers.

4.1   Staining characteristics

The orcein stain showed up HBSAg as dark brown homogenous
or granular intra-cytoplasmic material. The antigen assumed
various forms including dense spherical bodies, linear
bodies, sickled bodies and sometimes just a brown dusting.
In some instances the stained material was vacuolated.

The immunoperoxidase HBSAg stain consistently identified
the same antigen as the orcein stain. The antigen stained
as brown homogenous or granular material in the cytoplasm
against a light blue background or negative cells. This
indicates that the two methods have similar sensitivity.
The orcein stain was taken up by elastic tissue, red blood
cells and dense nuclei or nuclear material. These could
however be clearly distinguished from the HBSAg in hepatocytes.

Hepatitis B core antigen was found expressed
predominantly in the nuclei of hepatocytes although variable
amounts were occasionally seen in the perinuclear cytoplasm.
Positive nuclei stained reddish-brown against the light
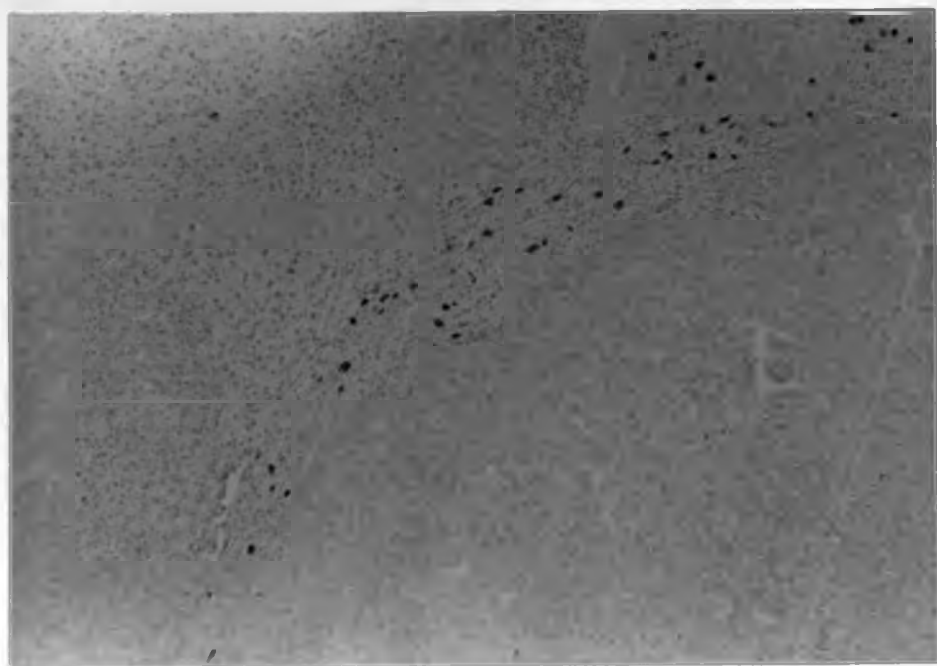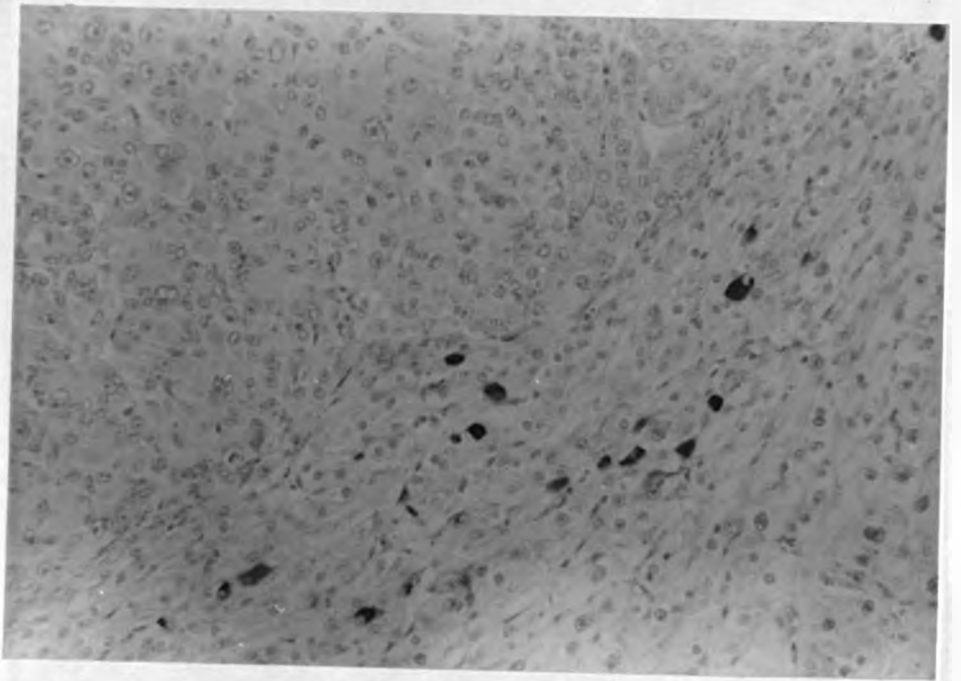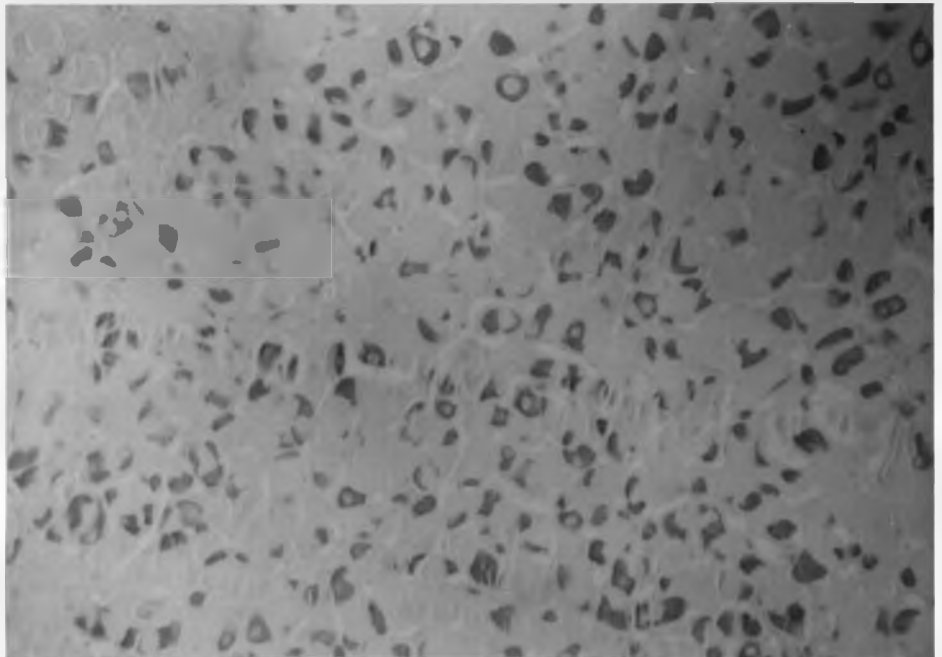blue staining cytoplasm and clear negative nuclei.

Plate 1: Immunoperoxidase stain for HBSAg
X 200

Plate 2: Immunoperoxidase stain for HBSAg

X 400

Plate 3: Orcein stain for HBSAg

x 400
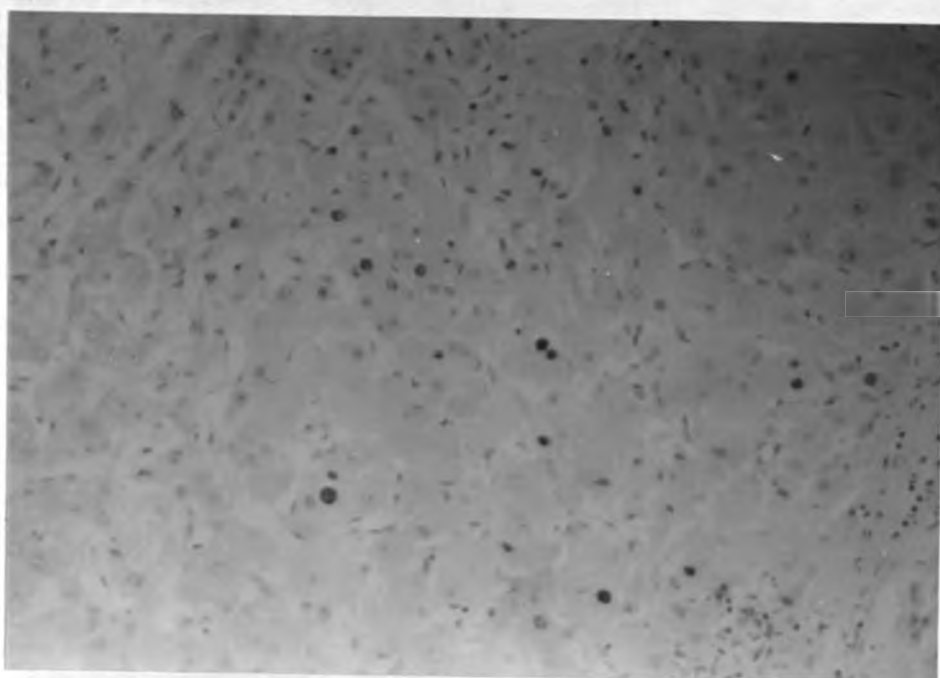
Plate 4: Immunoperoxidase stain for HBCAg

X 400

4:2   ANALYSIS OF RESULTS

The results were analysed using the Chi Square test and significance determined at 5% level of significance.

Positivity for HBV components in HCC liver biopsies with some non-neoplastic tissue and HCC liver biopsies consisting wholly of tumour tissue was examined. It can be seen from table 1 that of the 108 cases of hepatocellular carcinoma 56 consisted wholly of neoplastic tissue and no antigens were detected in these specimens. In the 52 cases with some non-neoplastic tissue antigen was detected only in the non-neoplastic parts. There were no HBV antigens in the tumour itself.

Only one of the forty control liver specimens was positive for HBSAg. None was positive for HBCAg. Hepatocellular carcinoma had a 33% HBSAg positivity rate while that for cirrhosis was 26%. This difference between HCC and cirrhosis was not statistically significant. Hepatitis B core antigen was more frequent in cirrhosis (14%) than in HCC (11.5%) but this was again not statistically significant. These findings are depicted in table 2.

Table 3 shows that Hepatitis B core antigen was present in relatively higher amounts in HBSAg positive cirrhosis (55.5%) than in HBSAg positive HCC (35%) but the difference was not statistically significant.

Hepatitis B virus component positivity in HCC with associated cirrhosis was analysed in table 4. Twenty seven (51.9%) of the 52 cases of HCC with some non-neoplastic tissue had associated cirrhosis. Seven of these were positive for HBSAg (26%) and two had HBCAg (7.4%). Twenty five of the 52 HCC cases had no associated cirrhosis. Eight of these were positive for HBSAg (32%) while four (16%) were HBCAg positive. The differences were not statistically significant.

Tables 5 and 6 compare the positivity for HBV components in needle biopsies and open biopsies or autopsy specimens with hepatocellular carcinoma and cirrhosis. A significant difference was observed in the detection of HBSAg from needle and open biopsies for both HCC and cirrhosis. This difference was not evident for the detection of HBCAg.

TABLE 1 :

COMPARISON OF HBV COMPONENT POSITIVITY IN HCC BIOPSIES WITH SOME

NON-NEOPLASTIC TISSUE AND HCC BIOPSIES CONSISTING WHOLLY OF TUMOUR

HBV COMPONENT POSITIVITY	HCC WITH SOME NON-NEOPLASTIC TISSUE (n=52)	HCC WITHOUT NON-NEOPLASTIC TISSUE (ALL TUMOUR) (n=56)
HBSAg	17/52	0/56
HBCAg	6/52	0/56

TABLE 2:

COMPARISON OF HBV COMPONENT POSITIVITY IN HCC AND CIRRHOSIS:

HBV COMPONENT POSITIVITY	HCC n=52	CIRRHOSIS n=35	X^2	P
HBSAg	17/52(33%)	9/35(26%)	0.486	P=0.5(not significant)
HBCAg	6/52(11.5%)	5/35 (14%)	0.002	P=0.9(not significant)

(5% level of significance.)

TABLE 3:

COMPARISON OF HBCAg POSITIVITY IN HBSAg POSITIVE

HCC AND CIRRHOSIS:

	HBSAg POSITIVE		x^2	P
	HCC(n=17)	(n=9) CIRRHOSIS		
HBCAg	6/17 (35%)	5/9(55.5%)	0.334	0.5 P 0.6 (not significant)

(5% LEVEL OF SIGNIFICANCE)

TABLE 4:

COMPARISON OF HBV COMPONENTS POSITIVITY IN HCC WITH AND

WITHOUT ASSOCIATED CIRRHOSIS:

HBV COMPONENT POSITIVITY	HCC WITH CIRRHOSIS (n=27)	HCC WITHOUT CIRRHOSIS (n=25)	x^2	P
HBSAg	7/27(26%)	8/25(32%)	0.36	P=0.6 (Not significant)
HBCAg	2/27(7.4%)	4/25(16%)	0.94	P=0.3 (Not significant)

TABLE 5:

COMPARISON OF POSITIVITY FOR HBV COMPONENTS IN NEEDLE BIOPSIES AND OPEN

BIOPSIES OR AUTOPSY SPECIMENS WITH HEPATOCELLULAR CARCINOMA.

HBV COMPONENT POSITIVITY	NEEDLE (n=28)	OPEN (n=24)	x^2	P
HBSAg	5/28(17.9%)	12/24(50%)	4.7	P=0.03(Significant)
HBCAg	2/28(7.1%)	4/24(12.5%)	0.4	P=0.52(not significant

TABLE 6:

COMPARISON OF HBV POSITIVITY IN NEEDLE BIOPSIES AND OPEN BIOPSIES OR AUTOPSY SPECIMENS WITH CIRRHOSIS:

HBV COMPONENT POSITIVITY	NEEDLE (n=20)	OPEN (n=16)	x^2	P
HBSAg	2/20(10%)	7/16(43.7%)	3.75	P=0.05(significant)
HBCAg	1/20(5%)	4/16(25%)	1.535	P=0.2(not significant)

5.1   DISCUSSION

The relationship between HCC and HBV is implicit in
the world-wide epidemiological data which show a highly
significant excess of HBV markers in patients with HCC
when compared with normal controls. In tropical Africa
and Far East Asia there is a very high incidence of both
the HBV carrier status and HCC. The great excess of
HBV detected in HCC, 33% and cirrhosis, 26% in contrast to
2.5% in normal liver unequivocally confirms the close
and specific association between HBV and HCC and cirrhosis.
A similar association has been found by other workers
based in Kenya such as Bagshawe et al[27], Bowry[28],
Greenfield[15] and Wankya[29].

Hepatitis B core antigen was detected less frequently
than HBSAg. All tissues positive for HBCAg were also
positive for HBSAg.

The core antigen is believed to be an indicator of
active virus replication. It has been found more
frequently in association with episomal viral DNA while
HBSAg alone is found when viral DNA integrates into the
host genome[30]. No significant difference was noted
in the level of HBCAg in HCC and cirrhosis.

In the present study 52% of HCC was associated with
cirrhosis. Higher figures for HCC associated with cirrhosis
have been found in Europe and America[30]. It is
postulated that in areas with a high incidence of HCC
the virus is acquired at birth and HCC develops without
preceeding cirrhosis in many cases. The high rate of
HBSAg in both HCC and cirrhosis suggests that the virus
is involved in the aetiology of both conditions.

Viral antigens were very randomly distributed in
the non-neoplastic liver tissue. In several instances
screening of one block revealed no antigen while
another block from the same case showed antigen in
small groups of hepatocytes. The number of positive
cells varied from a few individual cells randomly
dispersed in the lobules to groups of clustered
positive hepatocytes. The random distribution of
antigens necessitates the examination of multiple samples
for each case.

This study has shown a significant difference in HBV
component detection between needle and open biopsies.
Similar observations have been made by Okuda et al[21]
and Nayak et al[24].

Studies from India and South East Asia detected
HBSAg positivity rates of above 70%.

The lower level of HBV component positivity obtained
in this study could be due to various factors:
(1) The level of 33% HBSAg positivity in HCC compares
well with that of 44% obtained by Greenfield[15] by
detection of HBV DNA in HCC tissue. This study was
based in Kenya. These figures imply that factors
other than HBV may play more significant roles in the
aetiology of cirrhosis and HCC in Kenya than has been
previously assumed. Aflatoxin and non- A- non B viruses
could be important factors.
(2) The quantity of tissue examined in each case:
More than 50% of the samples were needle biopsies and
in the majority only one paraffin block was available for
examination. It has been shown by various workers
that the level of HBV component positivity obtained is
directly proportional to the amount of tissue examine
due to the random distribution of antigens. Many studies
quoted in the literature have been performed using only
autopsy material from which adequate tissue could be
obtained[11, 12, 24, 25].
(3) Survival of antigen in tissue depends on the
type and concentration of fixative, fixation time and
the size of specimen to be fixed[26, 31]. Neutral
phosphate-buffered 10% formalin is recommended and
the tissue should not remain in formalin for longer
than 24 hours. Rapid high temperature processing destroys
antigenicity and temperatures above $60^{o}C$ must be avoided.
This was a retrospective study using paraffin blocks
prepared in 1980-1984. The conditions of formalin
preparation, duration of tissue fixation and processing
were not standardized. It is therefore likely that
some antigenicity may have been lost during fixation with
unbuffered formalin which is our standard fixative or
during processing if excessively high temperatures were
inadvertently used.

5.2   CONSTRAINTS

Several constraints were encountered during the course of this study.

Many workers have shown that a sizable amount of tissue needs to be examined before the HBV status of the specimen in question can be determined[11,12,22-26]. In this study only one paraffin block was available for most cases and this may not have been representative of the whole liver. Some blocks consisted wholly of neoplastic tissue and could not be included in the analysis of results.

This being a retrospective study the conditions of tissue fixation and processing were not standardized and some antigens may have been lost or altered.

Reagents for the immunoperoxidase method are very expensive so it was not possible to test all the samples by this method. It was however observed that the less expensive orcein stain is as sensitive as the immunoperoxidase stain in detection of HBSAg. Unfortunately there is no substitute for detection of HBCAg.

5.3   AETIOLOGY OF HCC

Latest evidence from both human and animal studies suggests that HCC results from an interaction of initiating (genotoxic) and promoting (epigenetic) factors in a genetically susceptible host. Hepatitis B virus may initiate a carcinogenic process early in life by promotional insertion, turning on a cellular oncogene, to form stage I cells which are held in a non-proliferative state by inhibiting chalones produced by adjacent normal cells. Aflatoxin or other promoting factors like alcohol, tobacco and nitrosamines may then convert the initiated stage I cells to stage II cells through inhibition of intercellular communication. Stage III cells are then activated to produce clones of promoter independent HCC cells, stage IV. Mutation of chalone receptor genes, alcohol, tobacco and hepatotropic viruses may serve as activators. Certain factors like age at exposure to antigen, immune status, hormonal status and genetic make-up play an important but as yet unspecified role[30,32,33].

Chemical carcinogens have been observed to cause HCC in protein deficient experimental animals without an intervening cirrhosis. Protein deficiency may be synergistic in promoting HCC in man probably through alterations of immune mechanisms[32]. This may farther account for the high incidence of this tumour in the developing countries.

The antigens of HBV have consistently been found in the non-tumourous liver of patients with HCC but hardly any is seen in the tumour itself. It is possible that viral replication becomes increasingly defective during neoplastic transformation with eventual loss of viral antigens. It has also been observed that positivity for HBV decreases as the patients age increases suggesting an elimination of the viral antigens with time[27].

In some geographical areas the incidence rates of HBV infection and HCC do not coincide [34,35,36]. This suggests that some HCC cases are not HBV related. Other hepatotropic viruses like Non A Non B hepatitis viruses may act as initiators. Exposure to aflatoxin in early life may also initiate carcinogenesis.

Aflatoxin is a metabolic product of the mould Aspergilus
flavus a common food spoilage fungus in hot humid
environments. Several studies have examined the correlation
between aflatoxin and HCC [31-41]. In all a definite
relationship between aflatoxin intake and the incidence of
HCC was noted. Aflatoxin B1 has been found in the livers of
a high proportion of HCC patients and not in controls
from the same locality. The metabolites of aflatoxin are
carcinogenic and are normally detoxified in the liver by
glutathion conjugation. There is genetic variation in
the degree of aflatoxin metabolite binding and inactivation.
Eighty per cent of Africans are slow inactivators of
Isoniazid and debrisoquin and may also be slow inactivators
of aflatoxin metabolites. They would therefore be exposed
to the carcinogenic effects of epoxides for prolonged
periods of time. This may partly explain the high
incidence of HCC in Africa.

Certain chemicals such as N-Nitroso compounds used
in laundries and dry cleaners and organochlorine pesticides
have been found to cause hepatocellular carcinoma in
animals but their role in human HCC is not yet defined[33].

5.4   PREVENTION OF HBV ASSOCIATED HCC AND LIVER CIRRHOSIS

No effort should be spared in the study and evaluation
of factors involved in the causation of cirrhosis and HCC.
Infection with HBV at an early age may lead to a persistent
carrier state which greatly increases the risk of HCC and
cirrhosis as the body is unable to eliminate the virus.
Active immunisation of infants in high incidence areas
and high risk groups in low incidence areas would be
quite effective in controlling the infection. In view
of the current high costs of the vaccine it may be necessary
to select only the infants of HBV positive mothers for
immunization. However facilities to screen all mothers
for HBV nationwide may prove to be expensive too. Careful
planning is called for before any HBV immunization strategy
is implemented.

Screening of donated blood is now routine and prevents
transmission of HBV through blood and its products.

Improved farming and food storage methods would reduce
contamination with aflatoxin. Efforts may be concentrated
on regions with particularly high levels of aflatoxin
in food[33]. High aflatoxin levels may be related to
climate and altitude. People in high incidence areas should be
adviced to avoid alcohol and tobacco which enhance the
carcinogenic effects of HBV and aflatoxin.

6.   <u>CONCLUSIONS</u>

The significantly high rates of HBSAg in HCC and cirrhosis in contrast to low rates in normal liver support the hypothesis that HBV is involved in the causation of HCC and liver cirrhosis.

Hepatitis B core antigen was not detected as frequently as HBSAg in HCC and cirrhosis but was totally absent from normal liver samples. About half the cases of HCC had associated cirrhosis while the other half had no detectable cirrhosis. The rate of HBV components positivity was similar in both groups suggesting that HBV was equally responsible for the aetiology of HCC whether associated with cirrhosis or not. Some infected persons may proceed directly to HCC without an intervening stage of cirrhosis. This is thought to occur most often when HBV is acquired at birth or in infancy.

The importance of biopsy size in a study of this nature is clearly brought out. Open biopsies provide more tissue and thus improve the chances of detection of the randomly distributed antigens. Since no antigen is detectable in the tumour itself all samples examined should include some non-neoplastic tissue.

7.   RECOMMENDATIONS

Hepatocellular carcinoma and liver cirrhosis are common
diseases in Kenya for which no cure is available. Morbidity
and mortality from these two conditions are very high.
Every effort should therefore be expended towards their
prevention.

Vaccines for immunization against HBV have recently
been developed[35]. Immunization of persons at high risk
such as infants, health personnel and dialysis patients
should be advocated.

Future research should be directed towards the production
of a cheaper vaccine suitable for the developing countries
where the incidence of HBV is highest. Factors that may be
invloved in the aetiology of HCC and cirrhosis either
independently or in association with HBV should be evaluated
to determine their contribution to these diseases in Kenya
and other high incidence countries.

8.1   <u>APPENDIX 1</u>

<u>HAEMATOXYLIN AND EOSIN STAINING METHOD</u> (Standard)

1.   De-wax the sections in two changes of xylene.
2.   Remove xylene by dipping sections in two changes
      of absolute alcohol. One minute in each bath is
      sufficient. Pass sections through 90% and 70%
      alcohol to avoid the possibility of diffusion
      currents causing damage and perhaps detachment
      of the sections.
3.   Wash the sections in tap water to dehydrate
4.   Stain in Cole's haematoxylin for 10 minutes.
5.   Wash well in running tap water for 2-3 minutes.
6.   Differentiate in 1% hydrochloric acid in 70%
      alcohol for a few seconds. (three dips).
7.   Wash in tap water.
8.   Blue in Scots tap water for 30 seconds.
9.   Stain in 1 per cent eosin for 5 minutes.
10.   Wash off surplus stain in water.
11.   Dehydrate in alcohol, clear in xylene and mount
      in Distrin Phthalate xylene.

8.2   APPENDIX 2

ORCEIN STAIN FOR HBSAg

Oxidation before staining reduces the stainablity of
the cytoplasm and nucleus thus giving a better contrast
for the orcein positive areas.

  A: Oxidation

1.   Sections of about 5 um thickness are placed in 0.3%
      potassium permanganate solution with 0.015 ml
      sulfuric acid for 5 minutes.
2.   The sections are then placed in 1.5% oxalic acid
      until colourless.
3.   Place in 70% alcohol for 5 minutes.

  B: Staining

1.   1 g orcein is dissolved in 100 ml of 70% alcohol,
      pH is adjusted to 1-2 with hydrochloric acid.
      The sections are placed in this solution for 4-6 hours.
2.   Differentiate in absolute alcohol.
3.   Clear in xylene then mount using Distrin Phthalate
      xylene (DPx).

8.3   APPENDIX 3

IMMUNOPEROXIDASE STAINING TECHNIQUE [31]

A.   Removal of paraffin and rehydration of tissues

a) Place sections in an oven 56°-60°C for 30 minutes.
b) Transfer slide immediately into a xylene bath and
    incubate for 3 minutes. Repeat once.
c) Shake off excess liquid and place slides in fresh
    absolute ethanol for 3 minutes. Repeat once.
d) Shake off excess liquid and place slides in 95%
    ethanol for 3 minutes. Repeat once.
    Rinse slides in gently running tap water for 30
    seconds then commence staining procedure.

B. Staining procedure

i)   Wipe excess liquid from slides, place on a flat
        surface. Apply 3% hydrogen peroxide solution.
        This suppresses endogenous peroxidase activity.
        Incubate for 5 minutes, at room temperature.
        Rinse with distilled water and place the slides
        in 0.05m Tris buffer (pH.6) bath for 5 minutes.
ii)   Wipe off excess liquid. Cover slide with blocking
        serum and incubate for 20 minutes at room temperature.
        This serum quenches non-specific protein binding
        to certain tissue elements. Tap off excess serum.
iii)   Divide slides marked for different antigens into
        groups and apply antibody to target antigen (HBSAg
        of HBCAg) to test slides. Negative control reagent
        (lacks specific antibody) is added to negative
        control slides. Incubate for 20 minutes.
        Rinse gently with Tris buffer then place in Tris
        buffer bath for 5-20 minutes.
iv)   Wipe off excess liquid cover slides with link
        antibody (antibody to primary antibody). Incubate
        for 20 minutes. Rinse gently with Tris buffer
        then place in a Tris buffer water bath for 5-10 minutes.

v)   Wipe off excess liquid. Cover slides with
Peroxidase anti-peroxidase (PAP) and incubate for
20 minutes. Because the antibody of the PAP reagent
is from the same species of animal as the primary
antibody, they will be united by the link antibody
at the site of the tissue antigen.
Rinse in buffer, place in buffer bath for 5-20 minutes.

vi)   Cover slides with freshly prepared Diaminobenzene
(DAB).

vii) Incubate at room temperature for 1.2 hours.
Rinse gently with distilled water.

C.   <u>Counterstaining</u>

i)   Place slides in Meyers haematoxylin x 3 min
wash in running tap water.

ii)   Differentiate in 1% acid alcohol
wash in running tap water

iii) Blue in Scots tap water
Wash in running tap water

iv) Dehydrate in graded alcohol
clear in xylol
mount using Distrin Phthalate xylene (DPX).

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