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Hepatitis B virus serology : eight years of laboratory data in retrospect

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HEPATITIS B VIRUS SEROLOGY: EIGHT YEARS OF LABORATORY DATA IN RETROSPECT

John Gray Malcolm Sim

A dissertation submitted to the Faculty of Medicine,
University of the Witwatersrand, in partial fulfilment
of the requirements for the degree
Master of Medicine (Virology)

ABSTRACT

An analysis of 39,774 specimen records of diagnostic screening for hepatitis B virus (HBV), consisting of HBV surface antigen (HBsAg), antibody to surface antigen (anti-HBs) and antibody to HBV core antigen (anti-HBc) was performed. Specimens representative of all possible result combinations for these primary markers were found in the dataset and were investigated further by studying the distribution of extended marker frequencies within the groups defined by the primary marker combinations in an attempt to reconcile the patterns observed with standard profiles of HBV serology. The extended markers consisted of HBV e antigen (HBeAg), antibody to HBeAg (anti-HBe) and anti-HBc IgM. Unusual patterns of serological results which were identified were investigated further for the presence of HBsAg/anti-HBs immune complexing by acid dissociation techniques, and for the low level HBV chronic carrier state by polymerase chain reaction (PCR) amplification of HBV DNA. Results obtained indicated that immune complexing was not a common occurrence in the groups investigated, while preliminary findings in patients with isolated anti-HBc positivity demonstrated a 6.5% low level HBV carrier rate in this group.

DECLARATION

I declare that this dissertation is my own, unaided work and has not been submitted for any degree or examination at any other university.



John Gray Malcolm Sim

October 26, 1993

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1 INTRODUCTION

Hepatitis B virus (HBV) is an important universal viral pathogen responsible for considerable morbidity and mortality despite the existence of an effective and safe vaccine. There are thought to be more than 300 million HBV carriers worldwide (Zuckerman and Harrison, 1990), associated with 1 million deaths annually. It is estimated that there are more than 1.4 million chronically infected people in South Africa (Schoub, 1992). Furthermore, it has been suggested that about 1.2% of perinatally-infected individuals (the major route of transmission in this country), will die from the chronic sequelae of infection (Maynard *et al*, 1987). This would translate into 14 000 vaccine-preventable deaths per year (Schoub, 1992). While considerable progress has been made in HBV research in the relatively short time since its discovery at the end of the 1960's (Almeida *et al*, 1969; Boyer *et al*, 1968), there are many gaps in our understanding in areas such as pathogenesis, chronic infection, and the role of the virus in the aetiology of hepatocellular carcinoma. Even in the routine laboratory diagnosis of HBV infection, there are unresolved issues such as the basis for simultaneous specific antigen/antibody detection, antibody longevity, and the significance of isolated antibody to HBV core antigen (anti-HBc) in specimens. As in most areas of virology, molecular techniques have added a new dimension to HBV research and diagnosis. The polymerase chain reaction has revolutionized research into viruses such as HBV where cell monolayer isolation and culture is not an option at the present time.

1.1 THE VIRUS

1.1.1 Classification, Morphology and Antigenic Structure

1.1.1.1 Classification

Hepatitis B virus (HBV) is a typical member of the *hepadnaviridae*, a family of partially double-stranded DNA viruses with a remarkably small and efficient genome, which infect specific hosts across the full spectrum of the animal kingdom. The group displays close similarities in genome organization and they are all responsible for acute and persistent liver infections. Infection is not, however, confined to hepatic tissue. In human HBV, infection of a variety of extrahepatic tissues has been described, but the commonest site is peripheral blood leukocytes (Harrison, 1990). Extrahepatic infection has been documented for some of the animal viruses including duck hepatitis B virus (DHBV) (Halpern *et al*, 1986) and woodchuck hepatitis virus (Korba *et al*, 1986).

1.1.1.2 Morphology and Antigenic Structure

HBV consists of a number of morphologically defined and serologically important antigens. These are the surface antigen (HBsAg), the core antigen (HBcAg), the soluble and conformational variant of the core antigen referred to as the *e* antigen (HBeAg).

Both viral and subviral particles of HBV are found in the serum of infected individuals. These are illustrated in Figure 1, together with some of the antigenic and physical characteristics of the virus. For reasons which are not clear, viruses belonging to the *hepadnaviridae* are known to produce large

quantities of envelope particles during natural infection (Robinson, 1990). The 42 nm particle, otherwise referred to as the *Dane* particle, in deference to one of the researchers involved in the original description of the particle, represents the mature infectious virion. The virus is enveloped, with the envelope derived from the host cell cytoplasmic membrane. Densely packed into this lipid bilayer are three protein peplomers, which exist in glycosylated and unglycosylated forms. These are known as the S, M and L proteins. The S protein is the most abundant and represents the site of the principal neutralizing determinant, referred to as the *a* determinant. The function of the other two proteins, coded for by the pre-S part of the genome are not known, but the S and L, and probably the M are required for correct Dane particle morphogenesis (Ueda *et al*, 1991). The distribution of the proteins in the Dane and subviral particles also differs, which is probably a reflection of differences in protein targeting. Both the S protein and the pre-S2 product (L) share the same translocation signal, while the pre-S1 product

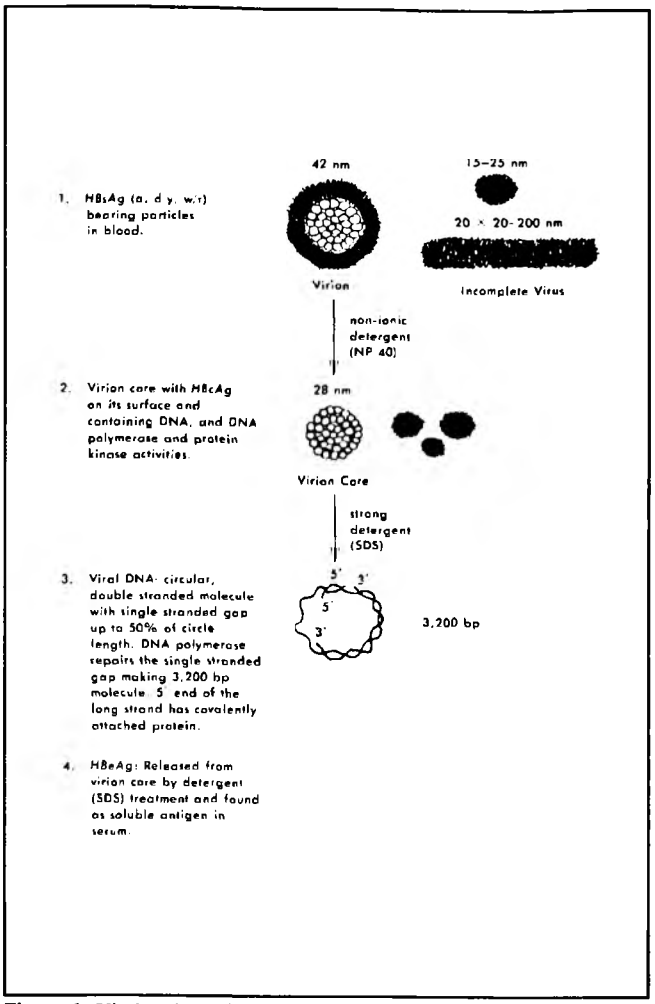


Figure 1 Viral and subviral particles of HBV (from Robinson, 1990).

(M) is handled differently (Slagle *et al*, 1992). The pre-S1 product is preferentially incorporated into virions rather than subviral particles. It has been stressed (Stirk *et al*, 1992), that HBsAg and pre-S together form a functional unit. It has been shown that pre-S binds to a receptor on HepG2 cells and it is suggested that this forms the first step in the internalization of the virus, although the HBV receptor remains unknown.

1.1.1.2.1 HBsAg

The HBsAg is associated with the S protein and has a number of antigenic specificities are associated with it. As mentioned above it is the seat of the group-specific determinant *a*, which is also the principle neutralizing determinant and is shared by all HBsAg preparations. In addition there are two pairs of subtype determinants (*d, y* and *w, r*) which, for the most part are mutually exclusive and behave as alleles (Robinson, 1990). Serological identification formed the basis for HBV subtype differentiation and geographically distinct patterns of subtype distribution have been identified. While the molecular basis for the subtype variations has been clarified (Okamoto *et al* , 1987; Norder *et al*; 1992), HBV subtyping based on these distinctions has recently been challenged (Naumann *et al*, 1993), and genome sequencing is likely to supervene.

1.1.1.2.2 HBcAg

The 28nm HBV core which can be released from virions by detergent disruption of the lipid envelope, is the site of the HBcAg, as well as HBeAg

(in a cryptic form), the viral DNA, and DNA polymerase and protein kinase activity (Robinson, 1990). HBcAg remains localized to hepatocytes during HBV infection, but the soluble variant HBeAg is secreted and invokes reaction from the host immune system.

1.1.1.2.3 HBeAg

HBeAg is coded for by the pre-C/C open reading frame (ORF) of the HBV genome. The pre-C differs from the C transcript by a secretory piece, which targets the polypeptide into the microsomal system for secretion. However, HBeAg expression may be elicited from both the pre-C and C proteins upon denaturation or limited protease treatment (Slagle *et al*, 1992). The pre-C protein is unusual in that it is both secreted and expressed in the cytoplasmic membrane of infected cells - a feature apparently only shared by the *gag* protein of murine leukaemia viruses (Schlicht, 1989). The presence of HBeAg in the serum of infected patients has been equated with HBV replication and is regarded as a marker of viral activity, while the emergence of anti-HBe is equated with recovery. However, HBV mutants, associated with the presence of a stop codon between the pre-C and C areas of the genome, and which are replication competent, have been described (Carmen *et al*, 1989). It has been suggested that this phenomenon may represent naturally-occurring escape mutants (Carman *et al*, 1991). The involvement of HBeAg in the host immune response is an interesting one, as it crosses the placenta of infected mothers

during gestation and acts as a tolerogen in the foetus and neonate (Milich *et al*, 1990), suggesting that the virus is particularly adapted to mother-child transmission.

1.1.2 Genome organization

The layout of the HBV genome is shown in Figure 2. With a partially double-stranded genome of 3.2 kbp, HBV represents the smallest genome of any virus known to infect man (Slagle *et al*, 1992). It is organized into four overlapping open reading frames coding for the envelope, core, polymerase and X gene product. The smallest of these the X ORF, codes for a product that is not essential for viral replication, but which is thought to play an as yet undefined role in pathogenesis *in vivo*. It has recently been shown to use a tumour promoter signalling pathway (Kekulé *et al*, 1993), which accounts for its promiscuous transcriptional transactivator behaviour (Rossner, 1992). HBV replication makes use of an RNA intermediate, and the polymerase possesses reverse transcriptional and RNase-H domains.

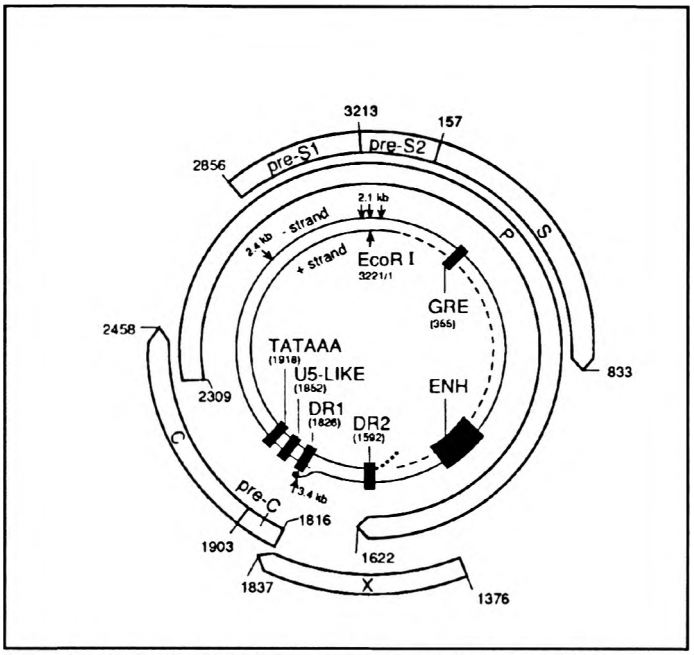


Figure 2 The layout of the HBV genome (From Slagle *et al*, 1992)

1.1.3 Pathogenesis of HBV-related disease

The pathogenesis of HBV infection is intrinsically interwoven with the immune response of the host. While the complexities of this interrelationship are not fully understood, the virus is not thought to mediate liver injury by a direct cytolytic route, but rather indirectly as a result of the effects of the cellular immune response directed against HBcAg and HBeAg on hepatocyte membranes. There is an inverse relationship between the status of the host immune system, and the acute manifestations of hepatic infection. Furthermore, immunosuppression or immunological immaturity are important risk factors for viral persistence and chronic infection. The humoral immune system is important in protection against infection, via neutralization of HBsAg, but also plays an important role in the extrahepatic manifestations of HBV infection, largely mediated by immune complex formation occurring in 10-20% of patients (Hollinger, 1990). The extrahepatic manifestations include (1) transient serum-sickness-like syndrome; (2) polyarteritis nodosa; (3) glomerulonephritis; (4) mixed cryoglobulinaemia; and (5) acrodermatitis of childhood - so-called Gianotti's disease (Hollinger, 1990). Immune complex formation is thus a well established component of HBV infection and pathogenesis.

1.1.4 Laboratory Diagnosis of HBV infection

The routine laboratory diagnosis of HBV infection is dependent on serological marker detection by radioimmunoassay (RIA) and enzyme immunoassay (EIA), with some variations and adaptations of these principles. The markers in routine use at the present time include HBsAg; antibody to HBsAg (anti-HBs); antibody to HBcAg (anti-HBc), including a capture assay for anti-HBc IgM; HBeAg; and antibody to

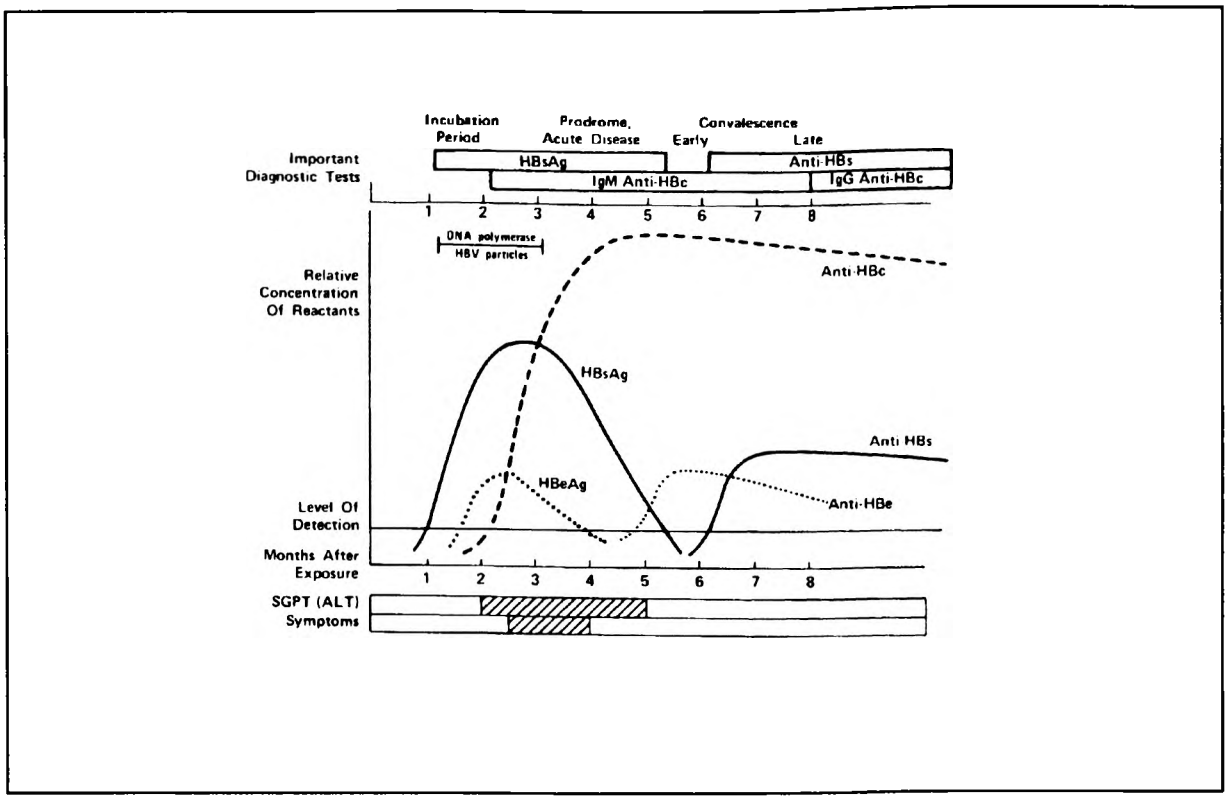


Figure 3 Serological and clinical patterns associated with HBV infection (from Hollinger, 1990).

HBeAg (anti-HBe). The stage of HBV infection is judged according to the profile of these markers detected at any one time against the typical patterns shown in Figure 3.

2 Aims and Organization of this Study

The stimulus to undertake this study was provided by a need to improve interpretative comment generation for routine hepatitis laboratory reporting at the National Institute for Virology (NIV). In the case of HBV infection, different combinations of serological results have specific clinical implications. Clear interpretative comment generation for HBV requires that all possible combinations of serological results be considered. To this end, the extensive computer database of laboratory results at the NIV was used to establish the frequency of patterns of HBV serology. This forms the basis for the first part of this research report. During the course of the search, unusual HBV serological patterns became

apparent, and the second and third sections of this study are devoted to aspects of unusual HBV serological profiles.

The research report is thus divided into the following three studies:

STUDY 1. Distribution of HBV serology results over an 8 year period at the National Institute for Virology (NIV)

AIM: to develop computer search routines for organizing, collating and analyzing the HBV serology results at the NIV, and then to relate the observed patterns to stages of infection.

STUDY 2. Investigation into techniques for the dissociation of immune complexes containing HBV surface antigen (HBsAg) and anti-HBs.

AIM: to titrate antigen/antibody combinations *in vitro* and determine whether acid dissociation techniques applied to these as well as patient serum is associated with release of antigen.

STUDY 3. Preliminary investigation of isolated HBV core antibody (anti-HBc) positivity on HBV screening by DNA amplification.

AIM: identify low level HBV carriage as a possible cause of isolated anti-HBc positivity on a random sample of such serological profiles identified on the NIV database.

3 STUDY 1. Distribution of HBV serology results over an 8 year period at the NIV

3.1 Introduction

The laboratory diagnosis of acute and chronic HBV infection depends on the detection of combinations of specific serological markers. The pattern of markers at any time is considered to represent different stages of infection as discussed in 1.1.4. It is commonplace (and perhaps appropriate), for many standard texts on clinical virology (Zuckerman *et al*, 1990; White and Fenner, 1986) to consider only a few of the possible combinations of markers and serological patterns typical of specific stages of infection when presenting HBV serology. However, in a busy diagnostic laboratory such as that at the NIV, unusual patterns are not uncommon and interpretive comments cannot be confined to the typical patterns only.

Table I All possible combinations of primary HBV screen markers.

Group	Anti-HBc	Anti-HBs	HBsAg
(a)	-	-	-
(b)	+	-	-
(c)	+	+	-
(d)	+	+	+
(e)	-	+	+
(f)	-	-	+
(g)	+	-	+
(h)	-	+	-

So in approaching the problem of interpretative comment generation, it was deemed necessary to consider all possible combinations of results and then to scan the HBV database to determine the distribution of these results. This database has accumulated all routine NIV result data over the past eight years and has proved a valuable source of information to the Institute. It was anticipated that the large number of records on the database would promote the recovery of some of the more unusual combinations.

At the NIV, as at many other laboratories, the screening of serum referred for HBV diagnosis is performed in a stepwise manner with specimens initially screened for HBsAg, anti-HBs and anti-HBc (referred to in this report as the "primary screen"), while those specimens positive for HBsAg are subsequently assessed for HBeAg, anti-HBe and anti-HBc IgM (the "extended markers"). The extended markers allow further diagnostic classification of results, especially with respect to the stage of HBV infection. If only positive and negative outcomes of tests are considered, then the primary screen generates 8 possible combinations, while a full extended screen (6 markers) generates 64 (2^6). Table I shows all the combinations of results possible for a primary HBV screen, and these are superimposed in Figure 4 on the background of the typical HBV serological profile discussed in 1.1.4. The groups in this diagram are arbitrarily alphabetically labelled and will henceforth be referred to according to this designation.

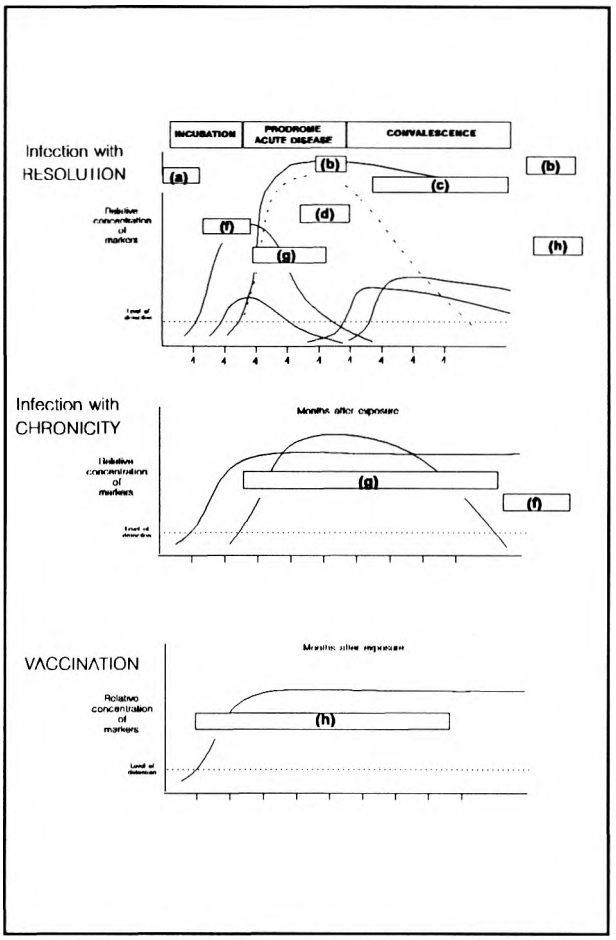


Figure 4 Standard HBV serological profile (modified from Hollinger, 1990) with primary screen groups superimposed.

3.2 Materials and Methods

The data used in this study were derived from the NIV database on which all laboratory results obtained over the time period 1985-1992 were recorded.

3.2.1 Data Structure and storage

The database used was dBASE III Plus (Ashton-Tate, 1985). Personal patient details and basic demographic information was held in a separate file to that containing the laboratory results, and information related via a specimen number (referred to as the NIV number). While some personal patient detail was transferred in the initial extraction procedure, all data was analyzed anonymously using this number and a further laboratory-specific number which is assigned to each specimen aliquot in the destination laboratory.

3.2.2 Data Extraction

Data extraction was performed on an IBM-compatible microcomputer. The routines were written and debugged in the interpretative dBASE command language and then compiled under Clipper ver 2.0 (Nantucket Corporation, Herts England).

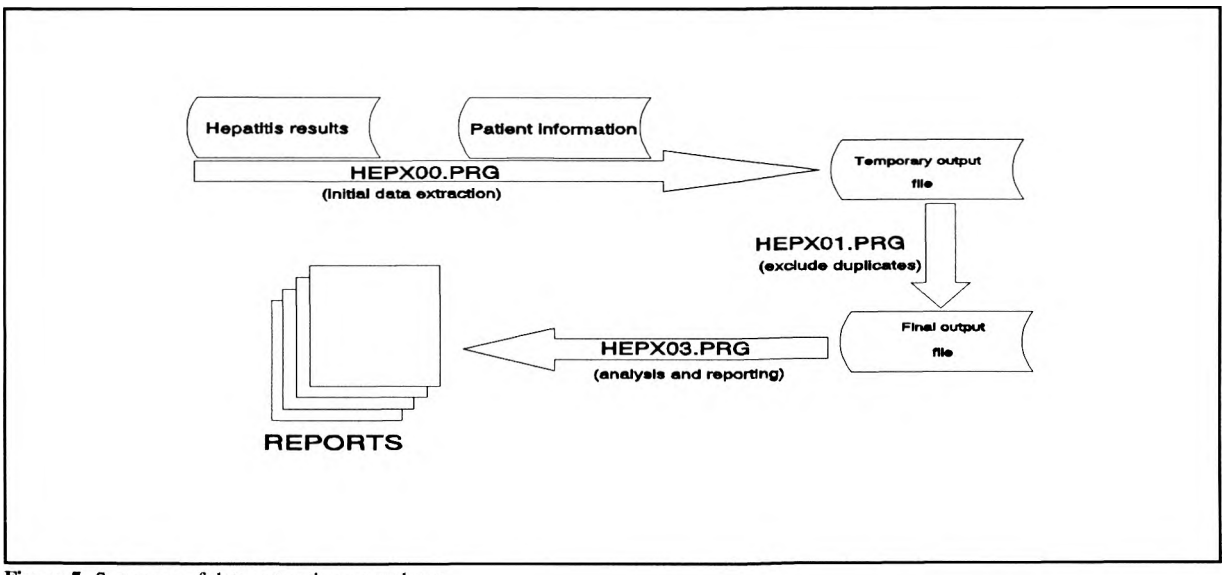


Figure 5 Summary of data extraction procedures

The source code for these extractions is included in the appendix to this section (see 3.3.4), but for convenience, the overall plan of the extraction is summarized in Figure 5.

3.2.3 Statistics

The Chi-square test was used to compare frequencies of marker patterns between groups. Where the expected count in one or more cells was less than 5, Fisher's exact test was applied.

3.3 Results

A total of 111,143 routine diagnostic specimens were received by the hepatitis laboratory for all hepatitis investigations between 1985 and December 1992. Of these, 39,774 (35.8%) were subjected to the standard primary HBV screen consisting of HBsAg, anti-HBs, and anti-HBc detection. Over the same time period, an abbreviated primary HBV screen consisting of HBsAg and anti-HBc only, was performed on 24,464 (22.0%) specimens. The results of the latter screen are not reported on further.

3.3.1 Distribution of HBV serological patterns obtained on Primary HBV screening

The results obtained from the breakdown of HBV primary screen serology are summarized in Table II.

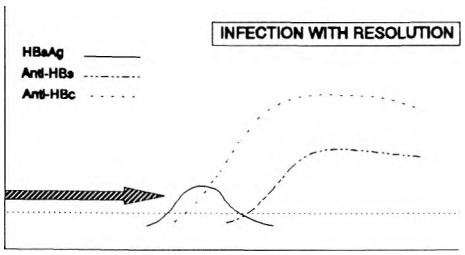
Table II Distribution of HBV serology results (1985-1992) according to patterns of possible outcomes.

Group	Anti-HBc	Anti-HBs	HBsAg	Total	%
a	-	-	-	20935	52.6
b	+	-	-	1581	4.0
c	+	+	-	9588	24.1
d	+	+	+	481	1.2
e	-	+	+	11	0.03
f	-	-	+	104	0.3
g	+	-	+	3554	8.9
h	-	+	-	3520	8.9
TOTAL				39774	

3.3.2 Distribution of Extended marker serological patterns within Primary screen groups

Figure 6 to Figure 9 summarize the distribution of extended HBV markers within each primary screen result group. The diagrams represent the probable stage of HBV infection for each primary screen group according to idealized patterns of serology developed from Figure 5. Group (e) was not included in the breakdown of extended marker results. This was because of its exceptionally low frequency in the database (0.03%), and the paucity of extended marker information. In Figure 8, the results for group (d) have intentionally been presented with those of group (g), because of their closely similar distributions. A comparison of the actual patterns of combined extended markers for these groups on the database revealed an even closer identity as shown in Figure 10. Figure 10 represents the frequencies of extended marker patterns within groups (d) and (g) as they actually occur on the database, so that for example, 76/481 (15.8%) of group (d) and 584/3554 (16.4%) of group (g) have the profile HBeAg negative, anti-HBe positive (the first data point

on the abscissa). The frequencies of these extended marker patterns were compared with the Chi-square test or Fisher's exact test (where the expected frequency within any cell was below 5) to detect differences. This data is presented in Table III.

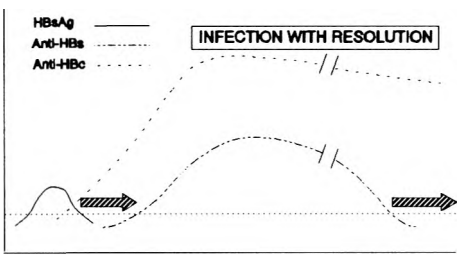


MARKER	POSITIVE	NEGATIVE	TOTAL
Anti-HBc (IgM)	0 (0.0)	77 (100)	77
HBeAg	1 (0.3)	331 (99.7)	332
Anti-HBe	7 (1.4)	511 (98.6)	518

Primary Screen Group

(a)

HBsAg -
Anti-HBs -
Anti-HBc -



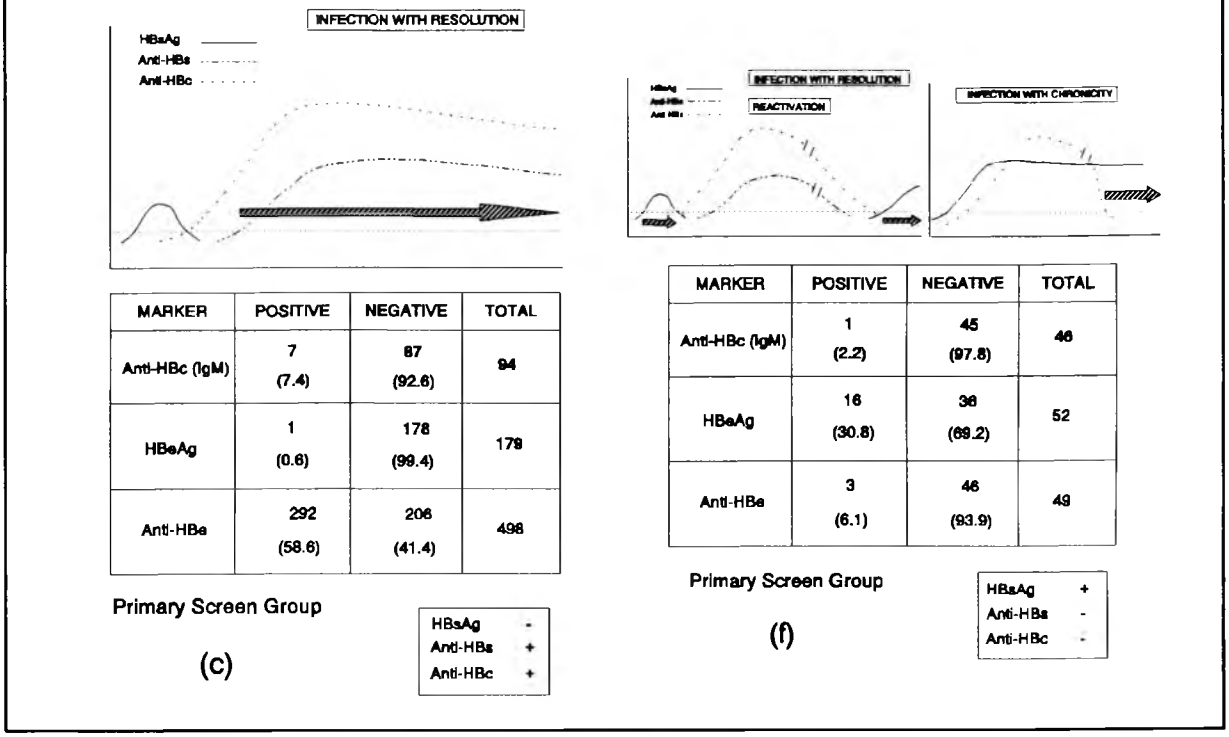
MARKER	POSITIVE	NEGATIVE	TOTAL
Anti-HBc (IgM)	34 (11.6)	259 (88.4)	293
HBeAg	1 (1.4)	73 (98.6)	74
Anti-HBe	79 (48.2)	85 (51.8)	164

Primary Screen Group

(b)

HBsAg -
Anti-HBs -
Anti-HBc +

Figure 6 Distribution of extended markers for primary screen groups (a) and (b). Figures in parentheses represent percentages.



MARKER	POSITIVE	NEGATIVE	TOTAL
Anti-HBc (IgM)	7 (7.4)	87 (92.6)	94
HBeAg	1 (0.6)	178 (99.4)	179
Anti-HBe	292 (58.6)	206 (41.4)	498

Primary Screen Group

(c)

HBsAg -
Anti-HBs +
Anti-HBc +

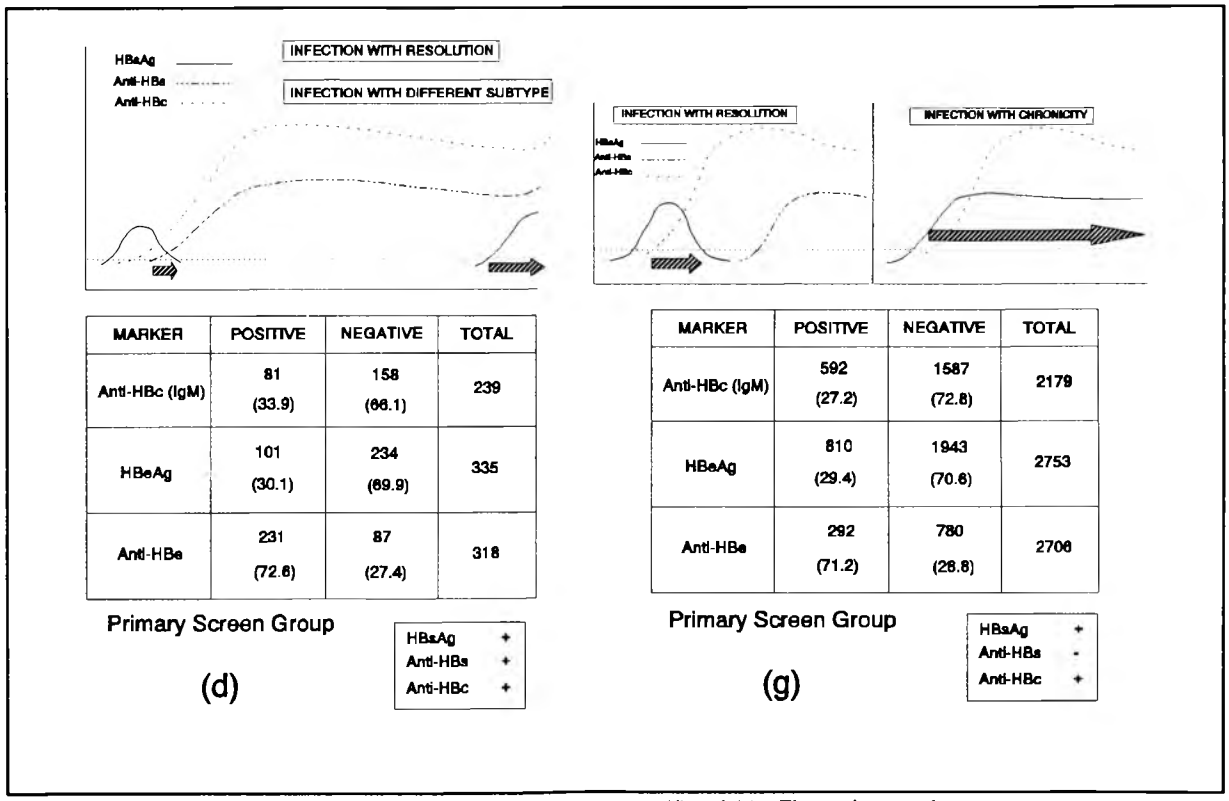
MARKER	POSITIVE	NEGATIVE	TOTAL
Anti-HBc (IgM)	1 (2.2)	45 (97.8)	46
HBeAg	16 (30.8)	36 (69.2)	52
Anti-HBe	3 (6.1)	46 (93.9)	49

Primary Screen Group

(f)

HBsAg +
Anti-HBs -
Anti-HBc -

Figure 7 Distribution of extended markers within primary screen groups (c) and (f). Figures in parentheses represent percentages.



MARKER	POSITIVE	NEGATIVE	TOTAL
Anti-HBc (IgM)	81 (33.9)	158 (66.1)	239
HBeAg	101 (30.1)	234 (69.9)	335
Anti-HBe	231 (72.6)	87 (27.4)	318

Primary Screen Group

(d)

HBsAg +
Anti-HBs +
Anti-HBc +

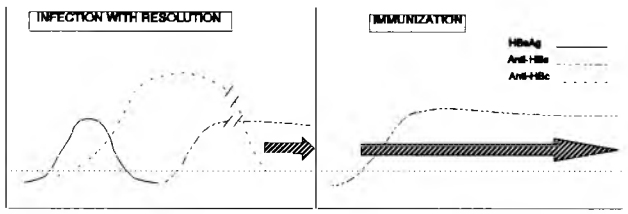
MARKER	POSITIVE	NEGATIVE	TOTAL
Anti-HBc (IgM)	592 (27.2)	1587 (72.8)	2179
HBeAg	810 (29.4)	1943 (70.6)	2753
Anti-HBe	292 (71.2)	780 (28.8)	2706

Primary Screen Group

(g)

HBsAg +
Anti-HBs -
Anti-HBc +

Figure 8 Distribution of extended markers within primary screen groups (d) and (g). Figures in parentheses represent percentages.



MARKER	POSITIVE	NEGATIVE	TOTAL
Anti-HBc (IgM)	0 (0.0)	14 (100.0)	14
HBeAg	0 (0.0)	51 (100.0)	51
Anti-HBe	5 (4.2)	114 (95.8)	119

Primary Screen Group

(h)

HBsAg -
Anti-HBs +
Anti-HBc -

Figure 9 Distribution of extended markers within primary screen group (h). Figures in parentheses represent percentages.

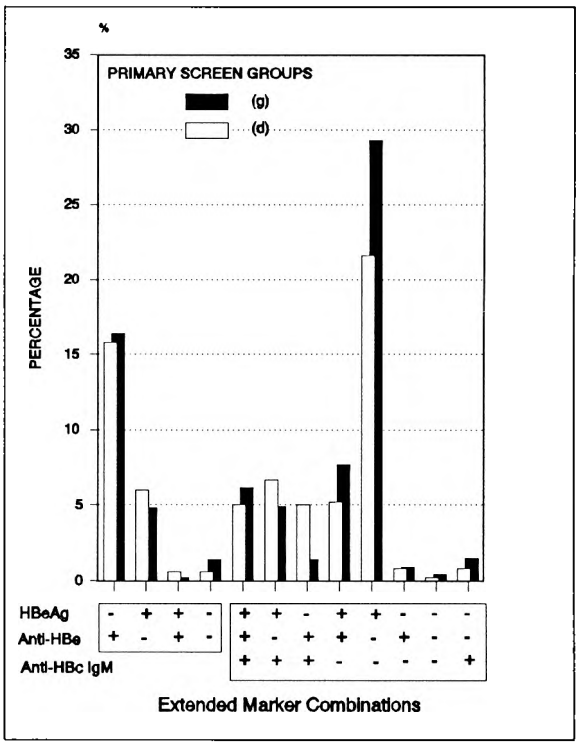


Figure 10 Comparison of all possible extended marker combination frequencies for primary screen groups (d) and (g). See text for details.

Table III Distribution of extended marker combinations for groups (d) and (g). Comparisons by Chi-square (*Fisher's exact test).

Extended Markers			Primary screen groups		P
HBeAg	Anti-HBe	Anti-HBc IgM	(d)	(g)	
-	+		76 (15.8)	584 (16.4)	ns
+	-		29 (6.0)	171 (4.8)	ns*
+	+		3 (0.6)	51 (1.4)	ns
-	-		4 (0.8)	52 (1.5)	ns
+	+	+	3 (0.6)	51 (1.4)	ns
+	-	+	24 (5.0)	220 (6.2)	ns
-	+	+	32 (6.7)	173 (4.9)	ns
+	+	-	5 (1.0)	49 (1.4)	ns
+	-	-	25 (5.2)	275 (7.7)	ns
-	+	-	104 (21.6)	1043 (29.3)	<0.001
-	-	-	4 (0.8)	33 (0.9)	ns*
-	-	+	1 (0.2)	15 (0.4)	ns*

3.3.3 Discussion

The shortcomings of looking at data in this way include the retrospective nature of the study, the question of extrapolating the findings beyond an academic laboratory situation, and the absence of background clinical information. Specimens referred to this laboratory are derived in the main from teaching hospitals with specialist referral clinics where patients with known liver disease are studied. Nevertheless, the considerable number of results obtained over an extended time period during which the procedures for detecting HBV markers have remained unchanged strengthens the credibility of these results. The identification of unusual marker combinations on our database, while not unique (Prozesky *et al*, 1984; Byskov *et al*, 1989) has also proved fruitful with respect to planning interpretative comments for routine laboratory reports.

Specimens on which primary HBV screening are performed at the NIV are largely those in which a diagnostic or management question is being asked. A full primary screen is not performed when requests are made for an assessment of immune status. Undoubtedly such specimens are included in the results presented, where the investigation requested or the diagnosis given did not clearly indicate a requirement for immune status assessment. Primary screen group (h) contains those specimens indicating immunity to HBV infection. The means of acquiring this immunity is not obvious from the result, and it could equally be due to vaccination or natural infection with loss of anti-HBc. Generally, anti-HBs is regarded as the first antibody to disappear, leaving the patient with an isolated anti-HBc result (Hoofnagle *et al*, 1973; Hollinger, 1990). While anti-HBe is not routinely assessed in these specimens, 4.2% of those in which it was were found to be positive (Figure 9). Anti-HBe levels usually decline in 30-50% of patients within 6 months of acute infection (Hollinger, 1990), so it is difficult to reconcile this with loss of anti-HBc. Although it is probably an unusual phenomenon in the population represented on this database, loss of anti-HBc antibodies following HBV infection in immunized children has been reported (Leboulleux *et al*, 1993). Nevertheless, it would seem reasonable to conclude from these results, that not all patients who recover from natural infection by HBV lose anti-HBs before anti-HBc.

One of the more striking results obtained in this study was the joint identity of primary screen groups (d) and (g) with respect to their congruent distribution of extended marker frequencies. Even when the frequencies of extended marker combinations within each of these groups was compared, the only difference found was a significantly higher frequency of isolated HBeAg positivity in primary screen group (g) see Table III. On the

face of it, it seems reasonable to conclude that they are really part of the same group. However, group (g) contains all specimens derived from typical HBV carriers - a chronic presentation of infection, and a primary screen pattern which is predictably relatively common on the NIV database (Table II). Group (d), on the other hand, would fit with either an early stage of convalescence (at the time of anti-HBs seroconversion), or a situation in which a subsequent infection of an immune individual developed, either as a result of no cross-protection to a different HBV subtype, or as a transient phenomenon after immunization. In the context of the latter suggestion, there is still no clear answer as to whether HBV vaccination protects against infection or disease - HBV infection has been documented after immunizing vaccination (Hadler *et al*, 1986; Wang *et al*, 1991; Hall, 1993). In either of these instances, it is difficult to reconcile the close extended marker pattern identity of (d) with the group containing chronic carriers, unless (d) represents a subgroup of chronic carriers in which anti-HBs is detectable. This is found in approximately 3-5% of chronic carriers in whom anti-HBs can be detected in low concentration, and many of these patients have more serious liver disease (Heijtink *et al*, 1982; Hollinger, 1990). The reciprocal suggestion that group (g) could represent a very early stage of HBV infection, prior to the development of the host immune response, is also very unlikely with respect to the frequency of this pattern on the database, as patients would be unlikely to present in numbers at this stage with signs and symptoms referrable to liver infection. Furthermore, the high frequency (71.2%) of anti-HBe positivity mitigates against this. On the other hand, 27.2% of specimens in group (g) were positive for anti-HBc IgM, suggestive of early infection. However, low concentrations of anti-HBc IgM have been shown to persist for up to 2 years in patients who have successfully resolved their infection as well as in many chronic carriers (Hollinger, 1990). The

antibody involved is usually a low molecular weight IgM in the case of chronic carriers (Sjogren *et al*, 1983; Tsuda *et al*, 1984), where it may have diagnostic and prognostic significance. Nevertheless, the significantly higher HBeAg frequency within group (g) would be compatible with an early stage of infection, or a higher frequency of HBeAg-positive chronic carriage. It is also unlikely that all the primary screen groups are homogenous with respect to the stage of infection they represent, especially groups (d) and (g) where the highest proportion of unusual HBV extended marker serological profiles on the NIV database reside.

The co-occurrence of HBsAg and anti-HBs is a serological pattern which has received a lot of attention in the literature (Nydegger and Miescher, 1980; Heijtink *et al*, 1982; Toti *et al*, 1983; Palla, Rizzi *et al*, 1983; Careoda *et al*, 1982; Grangeot-Keros *et al*, 1988; Carella *et al*, 1977; Ljunggren *et al*, 1991). This pattern is considered further in the second part of this report, and will not be discussed exhaustively here, suffice to note that the debate has concerned the basis for the co-occurrence of antigen and antibody, and whether such a finding has prognostic significance for the patient. Early case reports documenting this occurrence (Brandt *et al*, 1980; Couroucé-Pauty *et al*, 1979; Tabor *et al*, 1977) stressed the "two infection" hypothesis, whereby co-occurrence of HBsAg and anti-HBs may be explained by two subsequent infections with different subtypes, one of which gives rise to heterotypic antibodies (Le Bouvier *et al*, 1976). More recent investigations have challenged this, suggesting instead that anti-HBs positivity is associated with chronic liver disease, and represents a perturbation of the host immune response (Heijtink *et al*, 1982). Furthermore, it has been shown that immune complexes of HBsAg and anti-HBs IgM detectable during acute hepatitis have prognostic significance

in predicting the development of chronicity (Careoda *et al*, 1982; Toti *et al*, 1983; Ljunggren *et al*; 1991). Referring back to the results of the extended marker serological patterns, in the face of the above suggestions, it again makes more sense to regard group (d) as a subgroup of the larger chronic carrier group (g) in which anti-HBs is detectable. Unfortunately, no background clinical information was obtained to suggest an association with chronic liver disease in these patients. Certainly there are some important implications for future study in this area.

Primary HBV screen group (f), in which HBsAg alone is positive, is an uncommon finding on the database representing some 0.3% of the overall findings (Table II). This would seem appropriate with respect to the stages of infection it could represent (Figure 7). These include a very early stage of infection, prior to the development of the host immune response, and the onset of illness; reactivation of infection in a previously immune individual as a result of immunodeficiency; and finally a pattern associated with HBV chronicity in which anti-HBc is lost. Reactivation of HBV from extra-hepatic sites of latency, probably peripheral blood leukocytes (Harrison, 1990) has been described (Davis *et al*, 1987; Perrillo *et al*, 1984; Yoffe and Noonan, 1993), including in patients infected with HIV (Lazizi *et al*, 1988), an interaction which may assume greater importance in the future. This pattern has also been associated with the contentious HBV-2 virus (Coursaget *et al*, 1987). In general, HBV carriers are positive for anti-HBc, but a number of instances have been reported where this is not the case (WF Carman, personal communication). The distribution of extended markers in group (f) reveal the presence of anti-HBe in 6.1% (Figure 7). The presence of this marker is incompatible

with the very early stages of infection, indicating the possible presence of anti-HBc-negative carriers in group (f).

Isolated core antibody positivity on HBV screening - falling into group (b) in Table II, constitutes 4% of the results obtained in this laboratory. A number of reasons have been propounded for its occurrence. These include the following - false positivity, especially on enzyme immuno-assay; the so-called *core window* in early convalescence; naturally-acquired immunity with loss of anti-HBs; low level HBsAg carrier state where HBsAg is below levels of detection. These are discussed further as part of the third section of this report in which results of preliminary investigations into the low level carrier state are presented (see section 2, 5). It is, however, pertinent at this stage to briefly discuss the extended marker distribution associated with group (b) identified in the database search. 11.6% of specimens assessed were positive for anti-HBc IgM, suggestive of the core window period. 48.2% of those specimens in which it was assayed, were positive for anti-HBe, a marker which may be positive during the core window period, as well as in later convalescence. It is difficult, however, to reconcile the presence of anti-HBe with the late stage of naturally-acquired immunity where there is loss of anti-HBs. As mentioned above, anti-HBe levels become undetectable in 30-50% of individuals within 6 months of infection, while anti-HBs persists for a lifetime in over 80% of patients (Hollinger, 1990).

Primary screen group (e) was ommitted from extended marker analysis in the results because of its low frequency on the database. It has to be regarded as a very unusual combination, and one could speculate, in those instances where the results are carefully

checked, that it may represent reactivation of latent infectionin a situation where an individual with long-term immunity from primary infection (and loss of anti-HBc), becomes immunocompromised. It could also represent infection with a second viral subtype, or mutant virus, in a host possessing heterotypic antibody derived from previous vaccination or wild-type infection. Finally, it could represent an effect of treatment with interferon. Clearly, in dealing with such patterns of serology, additional clinical information and cautious interpretation become appropriate.

3.3.4 Appendix

3.3.4.1 Initial data Extraction (HEPX00.PRG)

```
*HEPX00.PRG  HEPATITIS EXTRACTION PROGRAM - this is a basic but useful programme which extracts data from
the patient file and the results file
CLEAR ALL
SET TALK OFF
SET BELL OFF
SET SAFETY OFF
SET DELETED ON
SET CONFIRM ON
KHEADING="HEPX00 - Basic Hepatitis Result/Patient Extraction"
KHEAD2=" "
DO WHILE .T.
 K_HEPIN=SPACE(15)
 K_PATIN=SPACE(15)
 K_HEPOUT=SPACE(15)
 CLEAR
 @ 1,0 TO 3,79
 @ 2,INT((70-LEN(KHEADING+" "+KHEAD2))/2) SAY KHEADING+" "+KHEAD2
 @ 2,70 SAY DATE()
 @ 08,05 SAY "Enter name of Hepatitis file ..... " GET K_HEPIN PICTURE "!!!!!!!!!!!!!!!"
 @ 09,05 SAY "              Patient file ....... " GET K_PATIN PICTURE "!!!!!!!!!!!!!!!"
 @ 10,05 SAY "              Output file ........ " GET K_HEPOUT PICTURE "!!!!!!!!!!!!!!!"
 READ
 IF K_HEPIN=SPACE(15)
  QUIT
 ENDIF
 SELECT 2
 USE &K_PATIN ALIAS BB
 SELECT 1
 USE HEPEXT
 ZAP
 COPY STRU TO &K_HEPOUT
 USE &K_HEPOUT
 APPEND FROM &K_HEPIN
 INDEX ON NIV_NO TO HEPX1
 UPDATE ON NIV_NO FROM B REPLACE HOSP_NAME WITH B->HOSP_NAME,HOSP_NO WITH B->HOSP_NO,DATE_TAKN WITH
B->DATE_TAKN,PATI_RACE WITH B->PATI_RACE,PATI_SEX WITH B->PATI_SEX,PATI_AGE WITH B->PATI_AGE,INVESTIG WITH
B->INVESTIG,DIAGNOSE WITH B->DIAGNOSE RANDOM
ENDDO
RETURN
```

3.3.4.2 Programme to exclude duplicate numbers (HEPX01.PRG)

```
*HEPX01.PRG  Hepatitis extraction programme - combines hep results
*from the same niv_no using hepatitis files only.
CLEAR ALL
SET TALK OFF
SET BELL OFF
SET SAFETY OFF
SET DELETED ON
SET CONFIRM ON
KHEADING="HEPX01 - combines results by NIV no from hepatitis files"
KHEAD2=" "
DO WHILE .T.
 K_HEPIN=SPACE(15)
 K_HEPOUT=SPACE(15)
 CLEAR
 @ 1,0 TO 3,79
 @ 2,INT((70-LEN(KHEADING+" "+KHEAD2))/2) SAY KHEADING+" "+KHEAD2
 @ 2,70 SAY DATE()
 @ 08,05 SAY "Enter name of Hepatitis file ..... " GET K_HEPIN PICTURE "!!!!!!!!!!!!!!!"
 @ 10,05 SAY "              Output file ........ " GET K_HEPOUT PICTURE "!!!!!!!!!!!!!!!"
```

```
READ
IF K_HEPIN=SPACE(15)
 QUIT
ENDIF
SELECT 1
USE HEPEXT
ZAP
COPY STRU TO &K_HEPOUT
APPEND FROM &K_HEPIN
INDEX ON NIV_NO TO HEPX1
SELECT 3
USE &K_HEPOUT ALIAS HEPOUT
SELECT 1
GO TOP
DO WHILE .NOT. EOF()
 SELECT 2
 USE HEXTEMP
 ZAP
 K_LAST=HEPEXT->NIV_NO
 SELECT HEXTEMP
 APPEND BLANK
 REPLACE HEXTEMP->PATI_NAME WITH HEPEXT->PATI_NAME
 REPLACE HEXTEMP->NIV_NO WITH HEPEXT->NIV_NO
 REPLACE HEXTEMP->TEST_NO WITH HEPEXT->TEST_NO
 REPLACE HEXTEMP->SPEC_NO WITH HEPEXT->SPEC_NO
 REPLACE HEXTEMP->TEST_CNTR WITH HEPEXT->TEST_CNTR
 REPLACE HEXTEMP->TEST_RESU WITH HEPEXT->TEST_RESU
 REPLACE HEXTEMP->TEST_COMM WITH HEPEXT->TEST_COMM
 REPLACE HEXTEMP->HOSP_NAME WITH HEPEXT->HOSP_NAME
 REPLACE HEXTEMP->HOSP_NO WITH HEPEXT->HOSP_NO
 REPLACE HEXTEMP->DATE_TAKN WITH HEPEXT->DATE_TAKN
 REPLACE HEXTEMP->DATE_RCVD WITH HEPEXT->DATE_RCVD
 REPLACE HEXTEMP->PATI_RACE WITH HEPEXT->PATI_RACE
 REPLACE HEXTEMP->PATI_SEX WITH HEPEXT->PATI_SEX
 REPLACE HEXTEMP->PATI_AGE WITH HEPEXT->PATI_AGE
 REPLACE HEXTEMP->LANGUAGE WITH HEPEXT->LANGUAGE
 SELECT HEPEXT
 SKIP
 DO WHILE HEPEXT->NIV_NO=K_LAST .AND. .NOT. EOF()
  L_IND=.T.
  SELECT HEXTEMP
  DO WHILE .NOT. EOF()
   IF K_LAST<>"          "
    IF HEXTEMP->niv_no=HEPEXT->niv_no
      L_IND=.F.
      K_RES1=SUBSTR(HEXTEMP->TEST_RESU,1,2)
      K_RES2=SUBSTR(HEXTEMP->TEST_RESU,3,2)
      K_RES3=SUBSTR(HEXTEMP->TEST_RESU,5,2)
      K_RES4=SUBSTR(HEXTEMP->TEST_RESU,7,2)
      K_RES5=SUBSTR(HEXTEMP->TEST_RESU,9,2)
      K_RES6=SUBSTR(HEXTEMP->TEST_RESU,11,2)
      K_RES7=SUBSTR(HEXTEMP->TEST_RESU,13,2)
      K_RES8=SUBSTR(HEXTEMP->TEST_RESU,15,2)
      IF SUBSTR(HEPEXT->TEST_RESU,1,2)<>"  "
       K_RES1=SUBSTR(HEPEXT->TEST_RESU,1,2)
      ENDIF
      IF SUBSTR(HEPEXT->TEST_RESU,3,2)<>"  "
       K_RES2=SUBSTR(HEPEXT->TEST_RESU,3,2)
      ENDIF
      IF SUBSTR(HEPEXT->TEST_RESU,5,2)<>"  "
       K_RES3=SUBSTR(HEPEXT->TEST_RESU,5,2)
      ENDIF
      IF SUBSTR(HEPEXT->TEST_RESU,7,2)<>"  "
       K_RES4=SUBSTR(HEPEXT->TEST_RESU,7,2)
      ENDIF
      IF SUBSTR(HEPEXT->TEST_RESU,9,2)<>"  "
       K_RES5=SUBSTR(HEPEXT->TEST_RESU,9,2)
      ENDIF
      IF SUBSTR(HEPEXT->TEST_RESU,11,2)<>"  "
       K_RES6=SUBSTR(HEPEXT->TEST_RESU,11,2)
      ENDIF
      IF SUBSTR(HEPEXT->TEST_RESU,13,2)<>"  "
       K_RES7=SUBSTR(HEPEXT->TEST_RESU,13,2)
      ENDIF
```

```
       IF SUBSTR(HEPEXT->TEST_RESU,15,2)<>"  "
        K_RES8=SUBSTR(HEPEXT->TEST_RESU,15,2)
       ENDIF
       SELECT HEXTEMP
       REPLACE HEXTEMP->TEST_RESU WITH
 K_RES1+K_RES2+K_RES3+K_RES4+K_RES5+K_RES6+K_RES7+K_RES8
       EXIT
      ENDIF
      SKIP
     ELSE
      EXIT
     ENDIF
    ENDDO
    IF L_IND
     SELECT HEXTEMP
     APPEND BLANK
     REPLACE HEXTEMP->PATI_NAME WITH HEPEXT->PATI_NAME
     REPLACE HEXTEMP->NIV_NO WITH HEPEXT->NIV_NO
     REPLACE HEXTEMP->TEST_NO WITH HEPEXT->TEST_NO
     REPLACE HEXTEMP->SPEC_NO WITH HEPEXT->SPEC_NO
     REPLACE HEXTEMP->TEST_CNTR WITH HEPEXT->TEST_CNTR
     REPLACE HEXTEMP->TEST_RESU WITH HEPEXT->TEST_RESU
     REPLACE HEXTEMP->TEST_COMM WITH HEPEXT->TEST_COMM
     REPLACE HEXTEMP->HOSP_NAME WITH HEPEXT->HOSP_NAME
     REPLACE HEXTEMP->HOSP_NO WITH HEPEXT->HOSP_NO
     REPLACE HEXTEMP->DATE_TAKN WITH HEPEXT->DATE_TAKN
     REPLACE HEXTEMP->DATE_RCVD WITH HEPEXT->DATE_RCVD
     REPLACE HEXTEMP->PATI_RACE WITH HEPEXT->PATI_RACE
     REPLACE HEXTEMP->PATI_SEX WITH HEPEXT->PATI_SEX
     REPLACE HEXTEMP->PATI_AGE WITH HEPEXT->PATI_AGE
     REPLACE HEXTEMP->LANGUAGE WITH HEPEXT->LANGUAGE
    ENDIF
    SELECT HEPEXT
    SKIP
   ENDDO
   SELECT HEXTEMP
   GO TOP
   IF .NOT. EOF()
    USE
    SELECT HEPOUT
    APPEND FROM HEXTEMP
   ENDIF
   SELECT HEPEXT
  ENDDO
  SELECT HEPEXT
ENDDO
RETURN
```

3.3.5 Programme to analyze Primary and Extended HBV screen results

```
* HEPX03.PRG  Hepatitis extraction programme - counts and prints
* different hepatitis result combinations according to the type of screen done.
CLEAR ALL
SET TALK OFF
SET BELL OFF
SET SAFETY OFF
SET DELETED ON
SET CONFIRM ON
KHEADING="HEPX02 - Counts Hepatitis screen results to print"
khead2=" "
k_hepin=space(15)
clear
@ 1,0 to 3,79
@ 2,int((70-len(kheading+" "+khead2))/2) say kheading+" "+khead2
@ 2,70 say date()
@ 08,05 say "Enter name of Hepatitis file....." get k_hepin picture "!!!!!!!!!!!!!!!"
read
if k_hepin=space(15)
 quit
endif
```

```
select 2
use &k_hepin alias HEPIN
go top
*The following procedure will only look at those specimens where a current
*routine screen has been performed i.e HBcAb, HBsAb, HBsAg
set filter to substr(test_resu,3,2)<>"  ".and.substr(test_resu,9,2)<>"  ".and.substr(test_resu,13,2)<>"  "
count all to k_total
count all for
 substr(test_resu,3,1)="-".and.substr(test_resu,9,1)="-".and.substr(test_resu,13,1)="-" TO K_TOTAL1
count all for
 substr(test_resu,3,1)="+".and.substr(test_resu,9,1)="-".and.substr(test_resu,13,1)="-" TO K_TOTAL2
count all for
 substr(test_resu,3,1)="+".and.substr(test_resu,9,1)="+".and.substr(test_resu,13,1)="-" TO K_TOTAL3
count all for
 substr(test_resu,3,1)="+".and.substr(test_resu,9,1)="+".and.substr(test_resu,13,1)="+" TO K_TOTAL4
count all for
 substr(test_resu,3,1)="-".and.substr(test_resu,9,1)="+".and.substr(test_resu,13,1)="+" TO K_TOTAL5
count all for
 substr(test_resu,3,1)="-".and.substr(test_resu,9,1)="-".and.substr(test_resu,13,1)="+" TO K_TOTAL6
count all for
 substr(test_resu,3,1)="+".and.substr(test_resu,9,1)="-".and.substr(test_resu,13,1)="+" TO K_TOTAL7
count all for
 substr(test_resu,3,1)="-".and.substr(test_resu,9,1)="+".and.substr(test_resu,13,1)="-" TO K_TOTAL8
SET DEVICE TO PRINT
@ PROW()+1,03 SAY "Breakdown of hepatitis serology results where routine screen done"
@ PROW()+2,03 SAY "Database used = "
@ PROW(),20 SAY K_HEPIN
@ PROW()+2,01 SAY "HBcAb HBsAb HBsAg"
@ PROW()+1,03 SAY "-      -      -"
@ PROW(),20 SAY K_TOTAL1 PICTURE "99999"
@ PROW()+1,03 SAY "+      -      -"
@ PROW(),20 SAY K_TOTAL2 PICTURE "99999"
@ PROW()+1,03 SAY "+      +      -"
@ PROW(),20 SAY K_TOTAL3 PICTURE "99999"
@ PROW()+1,03 SAY "+      +      +"
@ PROW(),20 SAY K_TOTAL4 PICTURE "99999"
@ PROW()+1,03 SAY "-      +      +"
@ PROW(),20 SAY K_TOTAL5 PICTURE "99999"
@ PROW()+1,03 SAY "-      -      +"
@ PROW(),20 SAY K_TOTAL6 PICTURE "99999"
@ PROW()+1,03 SAY "+      -      +"
@ PROW(),20 SAY K_TOTAL7 PICTURE "99999"
@ PROW()+1,03 SAY "-      +      -"
@ PROW(),20 SAY K_TOTAL8 PICTURE "99999"
@ PROW()+2,01 SAY "Total number = "
@ PROW(),15 SAY K_TOTAL
SELECT 1
USE HSTEMP
ZAP
APPEND   FROM   &K_HEPIN   for   substr(test_resu,3,2)<>"      ".and.substr(test_resu,9,2)<>"      ".and.
substr(test_resu,13,2)<>"  "
REPLACE ALL HSTEMP->RESU_KEY WITH
 SUBSTR(TEST_RESU,13,1)+SUBSTR(TEST_RESU,9,1)+SUBSTR(TEST_RESU,3,1)+SUBSTR(TEST_RESU,5,1)+;
SUBSTR(TEST_RESU,11,1)+SUBSTR(TEST_RESU,7,1)
REPLACE ALL RESU_NUM WITH 1
INDEX ON RESU_KEY TO HSTEMP
TOTAL ON RESU_KEY FIELDS RESU_NUM TO TEMP
USE TEMP
GO TOP
DO WHILE .NOT. EOF()
 DO CASE
  CASE SUBSTR(RESU_KEY,1,1)="+"
   K_WKA="P"
  CASE SUBSTR(RESU_KEY,1,1)="-"
   K_WKA="N"
  OTHERWISE
   K_WKA=" "
 ENDCASE
 DO CASE
  CASE SUBSTR(RESU_KEY,2,1)="+"
   K_WKA=K_WKA+"P"
  CASE SUBSTR(RESU_KEY,2,1)="-"
   K_WKA=K_WKA+"N"
  OTHERWISE
```

```
   K_WKA=K_WKA+" "
 ENDCASE
 DO CASE
  CASE SUBSTR(RESU_KEY,3,1)="+"
   K_WKA=K_WKA+"P"
  CASE SUBSTR(RESU_KEY,3,1)="-"
   K_WKA=K_WKA+"N"
  OTHERWISE
   K_WKA=K_WKA+" "
 ENDCASE
 DO CASE
  CASE SUBSTR(RESU_KEY,4,1)="+"
   K_WKA=K_WKA+"P"
  CASE SUBSTR(RESU_KEY,4,1)="-"
   K_WKA=K_WKA+"N"
  OTHERWISE
   K_WKA=K_WKA+" "
 ENDCASE
 DO CASE
  CASE SUBSTR(RESU_KEY,5,1)="+"
   K_WKA=K_WKA+"P"
  CASE SUBSTR(RESU_KEY,5,1)="-"
   K_WKA=K_WKA+"N"
  OTHERWISE
   K_WKA=K_WKA+" "
 ENDCASE
 DO CASE
  CASE SUBSTR(RESU_KEY,6,1)="+"
   K_WKA=K_WKA+"P"
  CASE SUBSTR(RESU_KEY,6,1)="-"
   K_WKA=K_WKA+"N"
  OTHERWISE
   K_WKA=K_WKA+" "
 ENDCASE
 REPLACE TEMP->RESU_KEY WITH K_WKA
 SKIP
ENDDO
P_TOT=0
GO TOP
SET DEVICE TO PRINT
L_FIRST=.T.
DO WHILE .NOT. EOF()
 IF PROW()>57 .OR. L_FIRST
  @ 01,10 SAY ".H.H.H.H.H.H.            . H H H H H H . ."
  @ 02,10 SAY ".B.B.B.B.B.B.            . B B B B B B . ."
  @ 03,10 SAY ".s.s.c.c.e.e.            . c c e s e s . ."
  @ 04,10 SAY ".A.A.A.A.A.A.            . A A A A A A . ."
  @ 05,10 SAY ".g.b.b.b.g.b.            . b b b b g g . ."
  @ 06,10 SAY ". . . .m. . . Number     .   m        . ."
  @ 07,10 SAY "----------------------------------------"
  L_FIRST=.F.
 ENDIF
 @ PROW()+1,10 SAY ". . . . . . ..      ....."
 @ PROW(),11 SAY SUBSTR(TEMP->RESU_KEY,1,1)
 @ PROW(),13 SAY SUBSTR(TEMP->RESU_KEY,2,1)
 @ PROW(),15 SAY SUBSTR(TEMP->RESU_KEY,3,1)
 @ PROW(),17 SAY SUBSTR(TEMP->RESU_KEY,4,1)
 @ PROW(),19 SAY SUBSTR(TEMP->RESU_KEY,5,1)
 @ PROW(),21 SAY SUBSTR(TEMP->RESU_KEY,6,1)
 @ PROW(),25 SAY TEMP->RESU_NUM
 P_TOT=P_TOT+TEMP->RESU_NUM
 @ PROW(),35 SAY TEMP->TEST_RESU
 SKIP
ENDDO
@ PROW()+1,25 SAY "----"
@ PROW()+1,25 SAY P_TOT PICTURE '99999'
@ PROW()+1,25 SAY "===="
EJECT
SET DEVICE TO SCREEN
*The following procedure will only look at those specimens where HBsAg and HBcAb have been done ("previous
screen")
SELE 2
go top
set filter to
```

```
set filter to substr(test_resu,3,2)<>"  ".and.substr(test_resu,13,2)<>"  ".and.substr(test_resu,9,2)="  "
count all to k_tot2
count all for substr(test_resu,3,1)="+".and.substr(test_resu,13,1)="+" TO K_TOT9
count all for substr(test_resu,3,1)="+".and.substr(test_resu,13,1)="-" TO K_TOT10
count all for substr(test_resu,3,1)="-".and.substr(test_resu,13,1)="+" TO K_TOT11
count all for substr(test_resu,3,1)="-".and.substr(test_resu,13,1)="-" TO K_TOT12
SET DEVICE TO PRINT
@ PROW()+5,03 SAY "Breakdown of hepatitis serology results where PREVIOUS routine screen done"
@ PROW()+2,03 SAY "Database used = "
@ PROW(),20 SAY K_HEPIN
@ PROW()+2,01 SAY "HBcAb HBsAg"
@ PROW()+1,03 SAY "+     +"
@ PROW(),20 SAY K_TOT9 PICTURE "99999"
@ PROW()+1,03 SAY "+     -"
@ PROW(),20 SAY K_TOT10 PICTURE "99999"
@ PROW()+1,03 SAY "-     +"
@ PROW(),20 SAY K_TOT11 PICTURE "99999"
@ PROW()+1,03 SAY "-     -"
@ PROW(),20 SAY K_TOT12 PICTURE "99999"
@ PROW()+2,01 SAY "Total number = "
@ PROW(),15 SAY K_TOT2 PICTURE "99999"
SELECT 1
USE HSTEMP
ZAP
APPEND    FROM    &K_HEPIN    for    substr(test_resu,3,2)<>"        ".and.substr(test_resu,13,2)<>"
".and.substr(test_resu,9,2)="  "
R  E  P  L  A  C  E      A  L  L      H  S  T  E  M  P  -  >  R  E  S  U  _  K  E  Y      W  I  T  H
SUBSTR(TEST_RESU,13,1)+SUBSTR(TEST_RESU,9,1)+SUBSTR(TEST_RESU,3,1)+SUBSTR(TEST_RESU,5,1)+;
SUBSTR(TEST_RESU,11,1)+SUBSTR(TEST_RESU,7,1)
REPLACE ALL RESU_NUM WITH 1
INDEX ON RESU_KEY TO HSTEMP
TOTAL ON RESU_KEY FIELDS RESU_NUM TO TEMP
USE TEMP
GO TOP
DO WHILE .NOT. EOF()
 DO CASE
  CASE SUBSTR(RESU_KEY,1,1)="+"
   K_WKA="P"
  CASE SUBSTR(RESU_KEY,1,1)="-"
   K_WKA="N"
  OTHERWISE
   K_WKA=" "
 ENDCASE
 DO CASE
  CASE SUBSTR(RESU_KEY,2,1)="+"
   K_WKA=K_WKA+"P"
  CASE SUBSTR(RESU_KEY,2,1)="-"
   K_WKA=K_WKA+"N"
  OTHERWISE
   K_WKA=K_WKA+" "
 ENDCASE
 DO CASE
  CASE SUBSTR(RESU_KEY,3,1)="+"
   K_WKA=K_WKA+"P"
  CASE SUBSTR(RESU_KEY,3,1)="-"
   K_WKA=K_WKA+"N"
  OTHERWISE
   K_WKA=K_WKA+" "
 ENDCASE
 DO CASE
  CASE SUBSTR(RESU_KEY,4,1)="+"
   K_WKA=K_WKA+"P"
  CASE SUBSTR(RESU_KEY,4,1)="-"
   K_WKA=K_WKA+"N"
  OTHERWISE
   K_WKA=K_WKA+" "
 ENDCASE
 DO CASE
  CASE SUBSTR(RESU_KEY,5,1)="+"
   K_WKA=K_WKA+"P"
  CASE SUBSTR(RESU_KEY,5,1)="-"
   K_WKA=K_WKA+"N"
  OTHERWISE
   K_WKA=K_WKA+" "
```

```
 ENDCASE
 DO CASE
  CASE SUBSTR(RESU_KEY,6,1)="+"
   K_WKA=K_WKA+"P"
  CASE SUBSTR(RESU_KEY,6,1)="-"
   K_WKA=K_WKA+"N"
  OTHERWISE
   K_WKA=K_WKA+" "
 ENDCASE
 REPLACE TEMP->RESU_KEY WITH K_WKA
 SKIP
ENDDO
P_TOT=0
GO TOP
SET DEVICE TO PRINT
L_FIRST=.T.
DO WHILE .NOT. EOF()
 IF PROW()>57 .OR. L_FIRST
  @ 01,10 SAY ".H.H.H.H.H.H.              . H H H H H H . ."
  @ 02,10 SAY ".B.B.B.B.B.B.              . B B B B B B . ."
  @ 03,10 SAY ".s.s.c.c.e.e.              . c c e s e s . ."
  @ 04,10 SAY ".A.A.A.A.A.A.              . A A A A A A . ."
  @ 05,10 SAY ".g.b.b.b.g.b.              . b b b b g g . ."
  @ 06,10 SAY ". . . .m. . . Number       .   m          . ."
  @ 07,10 SAY "-----------------------------------------"
  L_FIRST=.F.
 ENDIF
 @ PROW()+1,10 SAY ". . . . . . ..       ....."
 @ PROW(),11 SAY SUBSTR(TEMP->RESU_KEY,1,1)
 @ PROW(),13 SAY SUBSTR(TEMP->RESU_KEY,2,1)
 @ PROW(),15 SAY SUBSTR(TEMP->RESU_KEY,3,1)
 @ PROW(),17 SAY SUBSTR(TEMP->RESU_KEY,4,1)
 @ PROW(),19 SAY SUBSTR(TEMP->RESU_KEY,5,1)
 @ PROW(),21 SAY SUBSTR(TEMP->RESU_KEY,6,1)
 @ PROW(),25 SAY TEMP->RESU_NUM
 P_TOT=P_TOT+TEMP->RESU_NUM
 @ PROW(),35 SAY TEMP->TEST_RESU
 SKIP
ENDDO
@ PROW()+1,25 SAY "----"
@ PROW()+1,25 SAY P_TOT PICTURE '99999'
@ PROW()+1,25 SAY "===="
EJECT
SET DEVICE TO SCREEN
EJECT
RETURN
```

4. STUDY 2. Investigation into techniques for the dissociation of immune complexes containing HBV surface antigen (HBsAg) and anti-HBs.

4.1 Introduction

The simultaneous detection of HBsAg and anti-HBs in clinical specimens at the NIV is an uncommon but regular occurrence constituting 1.2% of the results obtained from primary HBV screening (Table II). The basis for its occurrence, and significance is disputed (Heijtink *et al*, 1982; Grangeot-Keros *et al*, 1988). There is evidence that the finding is associated with the circulation of immune complexes (Heijtink *et al*, 1982) and that immune complex detection, even in acute hepatitis, may have important prognostic significance (Careoda *et al*, 1982; Palla *et al*, 1983; Toti *et al*, 1983; Ljunggren *et al*, 1991). Immune complexes are also thought to play an important role in some of the extrahepatic manifestations of acute and chronic HBV infection, such as arthropathy, skin rash and glomerulonephritis (Hollinger, 1990; Appel, 1993).

In this study, it was decided to work from the hypothesis that specific antigen/antibody co-positivity on HBV screening, is associated with the presence of immune complexes and to investigate those combinations where complexes could be expected to be present - specifically primary screen group (d). Furthermore, the close relationship (in terms of extended HBV marker distribution) documented between primary screen combinations (d), (featuring HBsAg/anti-HBs co-positivity), and group (g) (see 3.3.2), (in which HBsAg but not anti-HBs was detected), made it logical to explore the existence of immune complexes in the latter group as well. As an initial step, to establish an experimental model of immune complexing

with which to assess immune complex dissociation methods, varying titrations of HBsAg- and anti-HBs-positive pooled sera were combined in a checkerboard configuration to create immune complexes *in vitro*. Following which, acid dissociation procedures to disrupt antigen/antibody interactions, which have been successfully applied to human immunodeficiency virus (HIV) core protein detection (Nishanian *et al*, 1990; Fauvel *et al*, 1993) were used.

4.2 Materials and Methods

4.2.1 Selection of Sera

Two groups of serum were selected from the NIV serum banks for this part of the study.

4.2.1.1 Specimens for *in vitro* immune complex creation : 20 anti-HBs-positive specimens, in which anti-HBc was also detected (suggestive of naturally-acquired immunity), were anonymously selected by laboratory number from the database according to their origin from Baragwanath or Hillbrow hospitals. Both of these hospitals belong to the University of the Witwatersrand teaching complex and serve, at this time, mainly Black patients. This choice was made so as to decrease the risk of encountering sera with unusual HBsAg subtype specificity. A similar number of HBsAg-positive simple carriers were selected from the same hospitals. Specimens were subjected to retesting (by radioimmunoassay) in the hepatitis laboratory at the NIV and only those with scintillation counts greater than 8,000 cpm were used.

Aliquots of anti-HBs- and HBsAg-positive sera were removed and separately pooled.

4.2.1.2 Specimens for immune complex detection by Acid Dissociation: Specimens with primary HBV screen result combinations placing them into groups (d) and (g) were also selected for study. These two groups were each further subdivided into "early" and "late" on the basis of extended marker patterns. Specimens from these two groups were regarded as *early* if they were positive for HBV e antigen (HBeAg) as well as antibody of the IgM class against HBV core antigen (HBcAg). Similarly, they were regarded as *late* if they were negative for the above two markers, but positive for antibody to HBeAg (anti-HBe).

The numbers of specimens selected were as follows:

d-early 19 specimens (all available from total of 24)

d-late 20 specimens

g-early 20 specimens

g-late 20 specimens

4.2.2 Detection of HBsAg and Anti-HBs by Radioimmunoassay

HBsAg and anti-HBs were detected by radioimmunoassay using standard kits and procedures, (*AUSRIA*™ and *AUSAB*™ respectively) supplied by Abbott Laboratories Diagnostics Division, North Chicago. The method as described by the manufacturer was followed in both these procedures apart from the different cutoff calculation (refer to 4.2.2.2 below), and the fact that in the acid dissociation techniques used in this experiment, kit controls were used at the final dilution (in saline) of the treated specimens.

4.2.2.1 Radioimmunoassay for the Detection of HBsAg

The principle of this "sandwich" RIA procedure is that polystyrene beads coated with anti-HBs derived from guinea pigs are incubated with serum and controls, during which any HBsAg becomes attached to the specific antibody on the solid phase. After the bead has been washed, the presence of bound HBsAg is detected by adding ^{125}I-anti-HBs, and then detecting the radioactive signal in a gamma scintillation counter in counts per minute (cpm). The amount of HBsAg in the specimen is thus proportional to the radioactivity measured. A specimen is regarded as positive by relating its reading to the cutoff calculated by multiplying the negative control mean cpm by a factor of 2.1.

4.2.2.2 Radioimmunoassay for the Detection of Anti-HBs

A similar RIA "sandwich" principle to that in 4.2.2.1 is used in this test. Polystyrene beads coated with human HBsAg (subtypes *ad* and *ay*) are incubated with serum and controls, during which any anti-HBs present becomes attached to the specific antigen on the solid phase. The presence of bound anti-HBs is detected by adding ^{125}I-labelled HBsAg, and then detecting the radioactive signal in a gamma scintillation counter in cpm. The amount of anti-HBs present in the specimen is thus proportional to the radioactivity measured. A specimen is regarded as positive by relating its reading to the cutoff calculated by multiplying the negative control mean by a factor of 2.7.

Note that the latter factor is higher than the 2.1 recommended by the kit manufacturer (Abbott Diagnostics Division) and is based on specific experience with this test at the NIV.

4.2.3 Checkerboard titration of HBV Immune Complexes

4.2.3.1 Establishing range of dilutions for Checkerboard titration

Serial 1:4 dilutions using normal saline of each of the pooled sera (see 4.2.1.1) were prepared in Wasserman tubes and measured by the RIA procedures described above (4.2.2).

300μl of each dilution of HBsAg-positive and anti-HBs-positive pooled serum was mixed in Wasserman tubes. In a modification of the method described by Schoub *et al* (1986), for poliovirus neutralization, these were incubated at 37°C for 4 hours, mixed on a vortex mixer every hour, and then placed at 4°C overnight. Controls consisting of serial dilutions of each pool and made up to volume with normal saline were included.

RIA's to detect HBsAg and anti-HBs were performed on each specimen according to the method described in 4.2.2.

4.2.4 Acid Dissociation of HBV Immune Complexes

Two different methods for the acid dissociation of immune complexes were used in this study. Both of these have been used in the detection of HIV p24 antigen detection (Fauvel *et al*, 1993; Nishanian *et al*, 1990).

4.2.4.1 Acid Dissociation of Immune Complexes - Method according to Fauvel *et al* (1993)

Reagents:

0.15M Glycine HCl (pH 2.0)

3.5M TRIS base (pH 10.8)

Procedure:

To 200μl of each serum specimen, 380μl of glycine HCl was added and incubated at 70°C in a water bath for 10 minutes. Following this, 20μl of 3.5M TRIS base was added to neutralize the acid. All controls in which acid dissociation was not performed, were made up to a 1:3 dilution with normal saline in order to remain consistent with the dilution resulting from acid treatment.

Specimens were then immediately put up on the respective RIA procedures described in 4.2.2.

4.2.4.2 Method 2 - Acid Dissociation of Immune Complexes - method according to Nishanian *et al* (1990)

Reagents:

0.5N HCl

0.5N NaOH

Procedure:

To 100μl of each serum specimen was added 50μl of 0.5N HCl. This was vortex-mixed, and placed at 37° in a water bath for 60 minutes. On

removal from the water bath, 50μl of 0.5N NaOH were added while checking the pH with pH indicator strips (Neutralit pH 5-10, Merck, Darmstadt) to achieve a pH of approximately 7.0. The resulting 1:2 dilution was applied to all controls using normal saline.

4.2.5 Statistics

The Wilcoxon signed ranks test was used to compare the medians of acid-treated experimental groups with non-treated controls for each of the dissociation methods used.

4.3 Results

4.3.1 Artificial Immune Complex generation - Checkerboard Titration

The results of combining serial dilutions of pooled HBsAg-positive and anti-HBs-

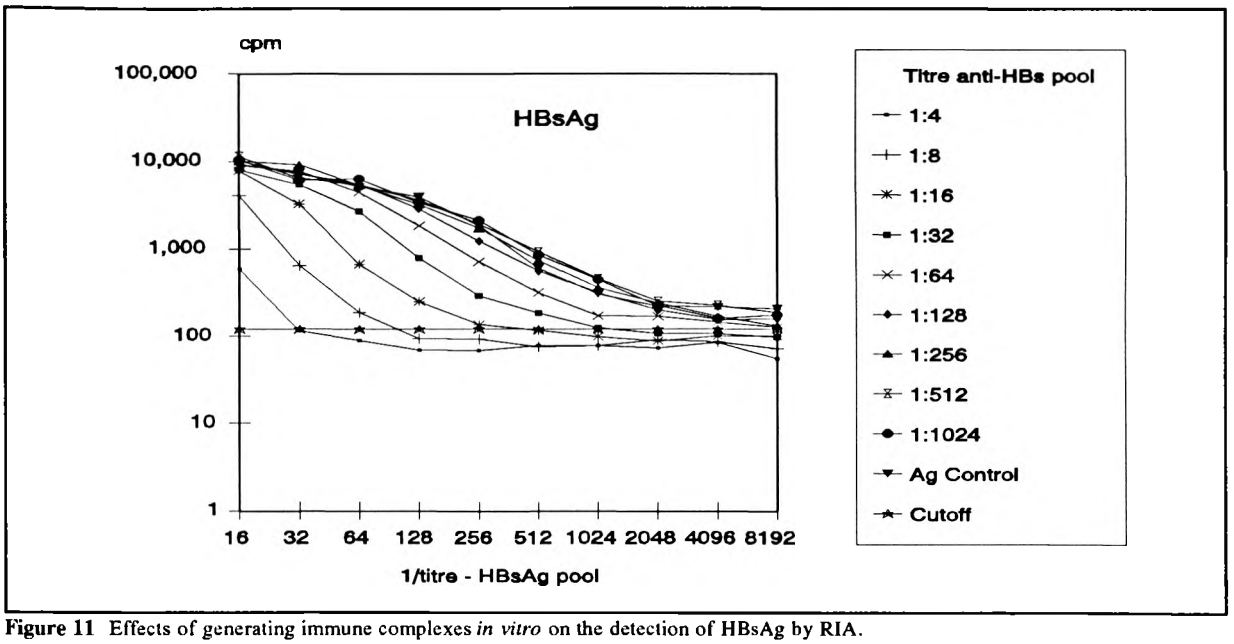


Figure 11 Effects of generating immune complexes *in vitro* on the detection of HBsAg by RIA.

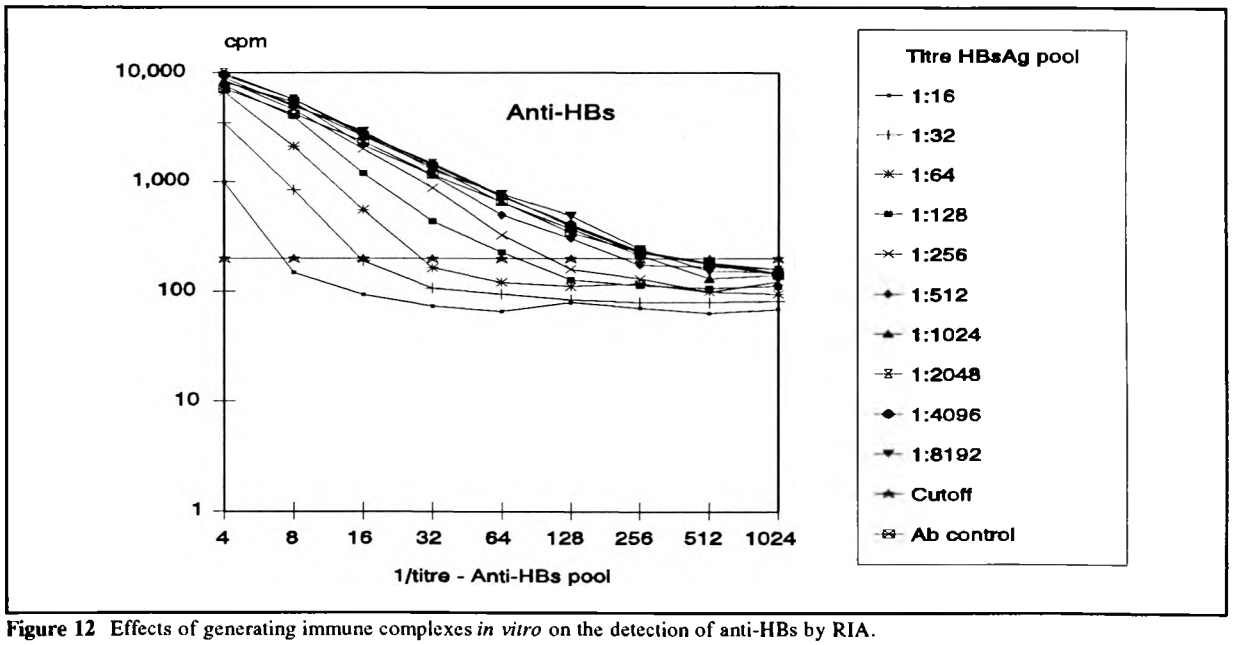


Figure 12 Effects of generating immune complexes *in vitro* on the detection of anti-HBs by RIA.

positive sera are shown in Figure 11 and Figure 12 for HBsAg and anti-HBs respectively. The effect of immune complex formation on the result of the RIA for HBsAg and anti-HBs can clearly be seen. In Figure 11, all dilutions of the anti-

HBs serum pool below 1:128, reduced the scintillation count for each dilution of the HBsAg pool below the HBsAg control. The stronger antibody dilutions were capable of reducing the count below the cutoff value for the test (i.e. converting a positive to a negative result), for even the lower HBsAg pool dilutions. A similar effect on anti-HBs detection by RIA was observed (Figure 12) in the same specimens. It can also be seen that a number of the specimens were simultaneously positive for both anti-HBs and HBsAg.

4.3.2 Acid Dissociation of HBV Immune Complexes

4.3.2.1 Effect of Acid Dissociation on HBsAg/anti-HBs immune complexes created *in vitro*

It is apparent in Figure 13 and Figure 14 that both acid dissociation methods successfully released HBsAg from immune complexes. The figures represent the results obtained for the methods of Fauvel *et al* (1993) and Nishanian *et al* (1990) respectively. Only at titres of anti-HBs below 1:16 in the method of Fauvel *et al*, the dissociation can be seen to have been incomplete (Figure 13). However, different sets of immune complex pools were used in testing the two acid dissociation methods, and it can be seen by studying the control plots, that there was greater immune interaction in the pools used in the latter method.

Acid dissociation completely removed all traces of anti-HBs reactivity from specimens, suggesting that these techniques were responsible for denaturing antibody. A comparison of the acid dissociation techniques with respect to the

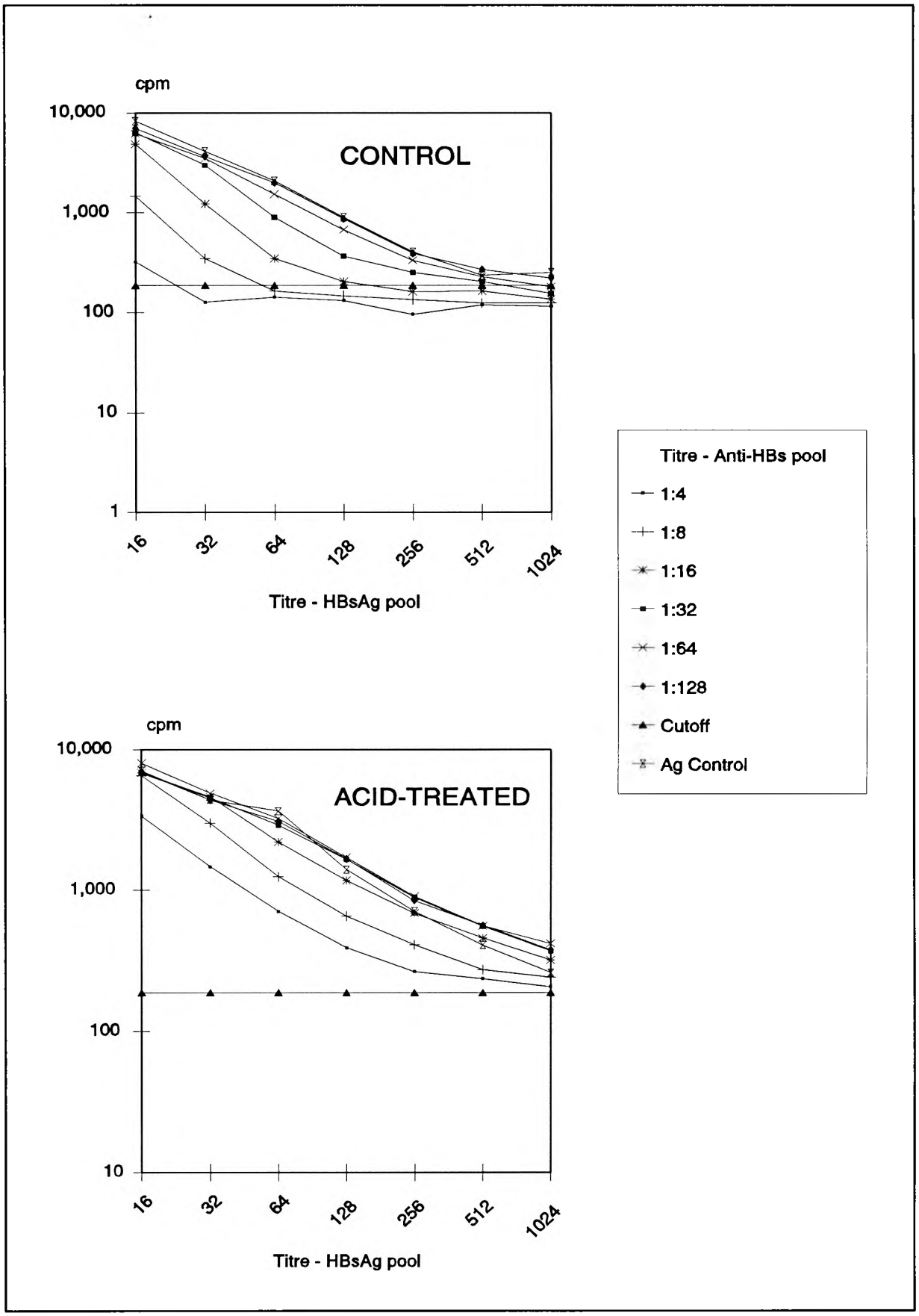


Figure 13 The effect of acid dissociation (method of Fauvel *et al*, 1993) on HBsAg/anti-HBc immune complexes created *in vitro* .

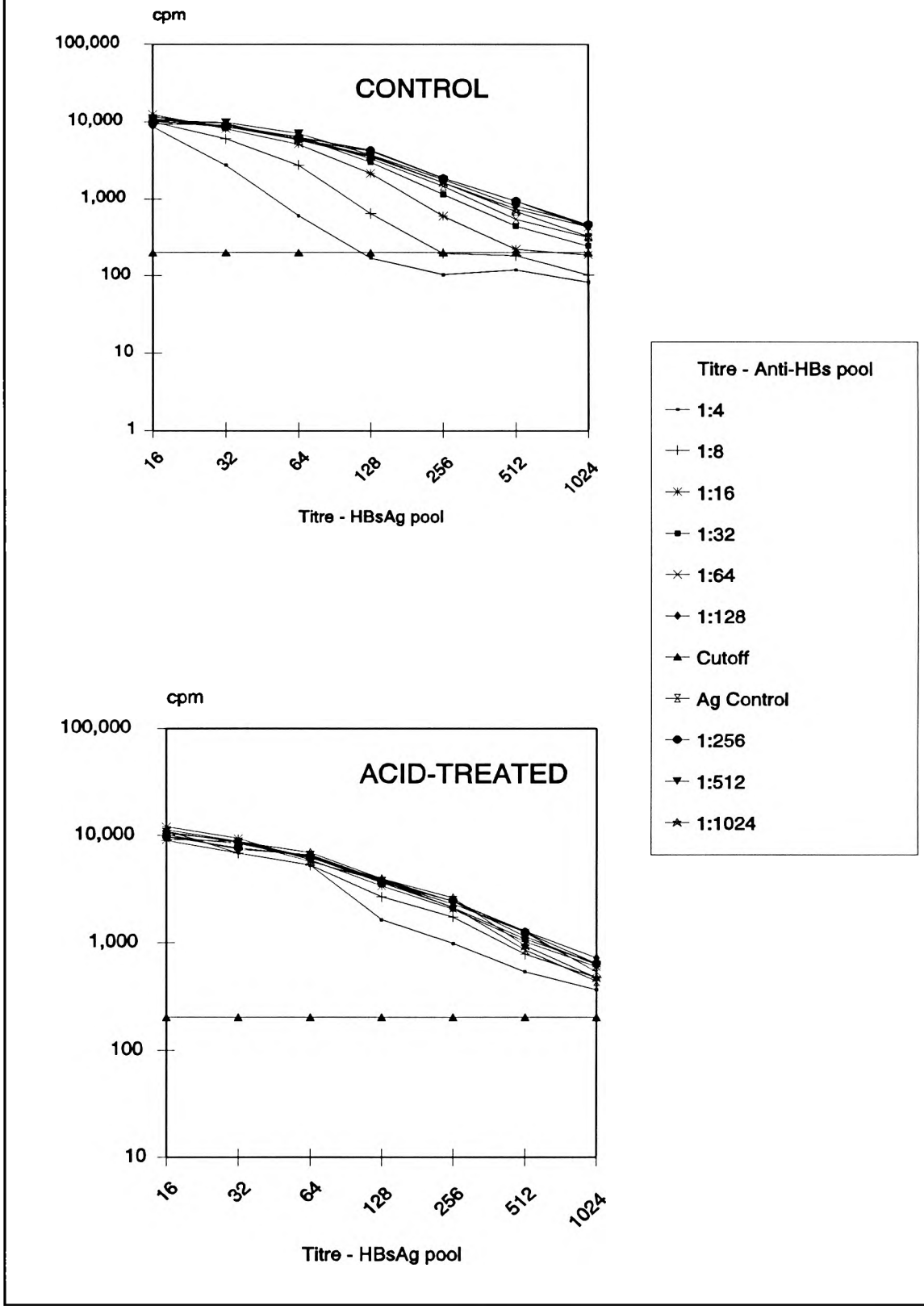


Figure 14 Effect of acid dissociation (method of Nishanian *et al*, 1990) on HBsAg/anti-HBs immune complexes created *in vitro*.

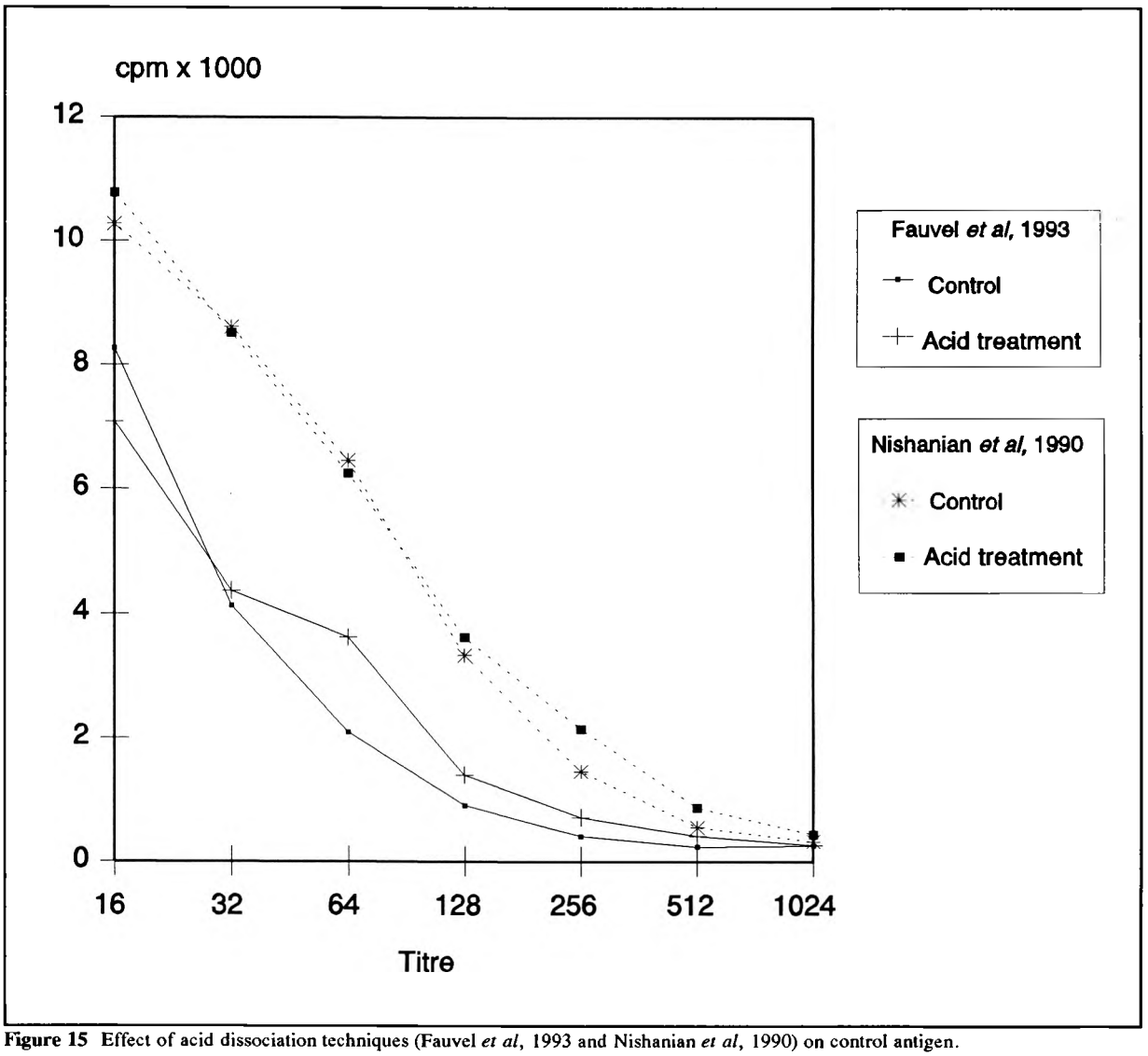


Figure 15 Effect of acid dissociation techniques (Fauvel *et al*, 1993 and Nishanian *et al*, 1990) on control antigen.

effect on the control dilutions of antigen is shown in Figure 15. It did not appear from this comparison, within the range of dilutions used, that the acidification had any effect on HBsAg in the specimens. Note that the difference in overall count between the two methods apparent in Figure 15 is a reflection of the final dilution factor in the tests. However, as is demonstrated in the next section, when neat serum specimens were used in the tests, the addition of the second neutralizing reagent according to the method of Fauvel *et al* (1993) resulted in sudden precipitation, which could well have had a confounding and unpredictable effect on the results.

4.3.2.2 Effect of Acid Dissociation procedures on HBsAg detection in patient sera considered to be possible sources of HBsAg/anti-HBs immune complexes

Serum from specimens selected according to the criteria given in 4.2.1.2 were subjected to the same two methods of acid dissociation. Unfortunately, due to the number of procedures performed on the original specimens selected, there was a certain amount of sample attrition. The results reported in this section relate only to those specimens for which there was a sufficient quantity of serum to complete both of the acid dissociation procedures.

While using the first of these two methods (Fauvel *et al*, 1993), with neat serum specimens, a sudden and profound precipitation was noted caused by the addition of the second reagent (3.5M TRIS base). This occurred to some extent in all specimens, but in the majority it was severe enough to cause problems with pipetting in subsequent steps. This phenomenon was not observed when investigating the pooled specimens in which immune complexing was established artificially, but these specimens were never used at dilutions below 1:4. In an attempt to troubleshoot the procedure, a number of specimens were repeated at varying dilutions and it was noted that dilution had a significant effect on the amount of precipitate formed.

Two specimens (laboratory numbers S7175 and U3771) within the groups selected for the acid dissociation, demonstrated the presence of immune complexes by responding with a markedly increased scintillation count on the RIA for HBsAg after acid treatment. In the case of S7175, the increase was

ten fold and occurred in both methods of immune dissociation attempted. U3771 showed a three fold increase in count after acid treatment in the second method used (Nishanian *et al*, 1990), but not in the first (Fauvel *et al*, 1993). Both these specimens had had repeatedly low positive HBsAg reactions, and both belonged, in terms of their primary HBV screen results, to group (d), being positive for all three primary screen markers. In addition, both were positive for anti-HBe, and negative for the other extended markers placing them into the *late* (d) category.

Table IV and Table V contain the results recorded for the primary screen groups as a whole. When the primary screen groups were compared together, for each dissociation technique, they showed a significant decline in median scintillation count after acid treatment. The same tendency, in some instances significant, was noted in the various subgroups defined within each primary screen category.

Table IV Effect of acid dissociation (Fauvel *et al*, 1993) on HBsAg detection by RIA.
See text for details. cpm = counts per minute

Primary HBV screen subgroup		n	HBsAg RIA mean (cpm)		p
			Acid Dis	Control	
d	early	5	9962	12051	0.18
	late	15	9803	10106	0.09
g	early	9	8511	10569	< 0.05
	late	12	9068	11821	< 0.05
All subgroups		41	9341	11651	< 0.001

Table V Effect of acid dissociation (Nishanian *et al*, 1990) on HBsAg detection by RIA.
cpm=counts per minute

Primary HBV screen subgroup		n	HBsAg RIA mean (cpm)		p
			Acid Dis	Control	
d	early	5	13704	18002	0.28
	late	15	15459	16016	0.75
g	early	9	14276	17086	0.19
	late	12	15091	16880	< 0.05
All subgroups		41	15130	16367	< 0.05

4.4 Discussion

The results of this study show that immune complexes consisting of HBsAg and anti-HBs can be formed *in vitro* and dissociated with acid treatment procedures in a manner analagous to HIV p24 core antigen detection. Furthermore, over a range of different dilutions of pooled anti-HBs- and HBsAg-positive combinations, both markers can be detectable in the same specimen (Figure 11 and Figure 12) - at least demonstrating that homotypic antigen and antibody are detectable under these circumstances and mirroring the finding in patient sera.

The results do not support the conclusion that immune complexing plays an important role in explaining the results of the primary screen groups selected for study. However, within the primary screen group (d), evidence supporting the presence of acid-dissociable imune complexes was obtained in at least one specimen, a result which was duplicated using two different methods. There are a number of considerations to be

made in reviewing these results. Firstly, the sample numbers were small. Secondly, the procedures used for immune complex dissociation may require further adaptation and development. Specifically the technique of Fauvel *et al* (1993), seems to reduce the HBsAg detection more than that of Nishanian *et al* (1990). This could be at least partly due to the significant amount of precipitation associated with this procedure. It is perhaps of interest, that a similar technique has recently been used in HIV p24 detection (Pokriefka *et al*, 1993) using less concentrated TRIS base, the reagent responsible for inducing precipitation in the neat specimens particularly . The dissociation method of Nishanian *et al* (1990), while it did not produce any precipitation had the drawback of requiring that the pH of specimens after the acid treatment step be returned to a reading of 7 by the addition of sodium hydroxide, a difficult and time-consuming operation. A third factor for consideration is the choice of specimens. The result profile most likely to be associated with circulating immune complexes is that where HBsAg is detectable in the marginal or low positive range. Future work in this area will consider other dissociation techniques, the choice of specimens, or patients where prospective study design possibilities exist. The association between immune complex circulation and chronic liver disease (Heijtink *et al*, 1982) is also not resolved, while the recent finding, in acute HBV infection, that immune complexing between HBsAg and anti-HBs IgM is predictive of HBV chronicity (Ljunggren *et al*, 1991) deserves further consideration in our population. The method used in the latter study is especially attractive as it represents a simple adaptation of the routine RIA procedures for HBV detection to achieve "immune complex capture". Unfortunately it was deemed beyond the scope of the present study to include this procedure.

5. STUDY 3. Preliminary investigation of isolated HBV core antibody (anti-HBc) positivity on HBV screening by DNA amplification.

5.1 Introduction

The interpretation of isolated HBV core antibody positivity has been recognized as a problem for some time and it may be associated with either (1) a chronic low level HBV carriage, (2) naturally acquired immunity with loss of anti-HBs over time, (3) passive transfer of anti-HBc (perinatally and after transfusion or immunoglobulin administration) or (4) nonspecific cross-reacting antibody (Draelos *et al*, 1987). To this list could be added the *core window*, but this alternative can be readily excluded by testing for anti-HBc IgM. Some of these alternatives are dichotomously opposed and have implications for the interpretation of HBV serology. Isolated core antibody positivity is relatively uncommon finding in our own laboratory, representing only about 4% of HBV screening results, and only 11.6% of these have an extended marker profile representing the *core window* (see Figure 6).

This section of the report is a preliminary investigation into the frequency with which we encounter low level HBV carriage in our population of isolated anti-HBc positives using DNA amplification by the polymerase chain reaction (PCR) method.

5.2 Materials and Methods

5.2.1 Selection of Sera

A random sample of 50 sera identified on the NIV database as belonging to the primary HBV screen group (b) were anonymously selected by laboratory number.

5.2.2 Retesting of Sera (Radioimmunoassay)

All specimens were subjected to repeat testing by RIA in the hepatitis laboratory of the NIV to confirm the finding of isolated anti-HBc positivity. They were also tested for anti-HBc IgM, to identify those specimens representing the core window.

5.2.3 Nucleic Acid Extraction from Serum

The method used for DNA extraction from serum was based on that described by Patou and Ayliffe (1991), a simple and rapid method of extraction.

5.2.3.1 Method

50 μl of sera were added to 100 μl of a solution containing 50 mmol potassium chloride, 10 mmol TRIS-HCl (pH 8.3), 2.5 mmol magnesium chloride, 0.1 mg/ml gelatin, 0.45% Nonidet-P40 (BDH Chemicals, Poole England), and 0.006 mg proteinase K (Boehringer Mannheim GmbH, W Germany), the latter being separately aliquotted and added just prior to use.

The mixture was then incubated at 65°C for 1 hour, followed by 95°C for 10 minutes to inactivate the proteinase K. The precipitate formed was pelletted by centrifuging at 14,000 rpm for 15 minutes and the supernatant removed for use in the PCR assay.

5.2.4 Polymerase Chain Reaction (PCR)

As this was a preliminary investigation, only a single primer pair, and a single round of amplification was used. The PCR protocol and PCR buffer used was that described by Kaneko *et al* (1989a and 1989b). However, in addition to this protocol, a rapid PCR method was also tried, which has been reported to yield a sensitivity equivalent to that of nested protocols (Vandenvelde *et al*, 1993).

Amplified bands were visualized under ultra-violet light on agarose gels stained with ethidium bromide.

5.2.4.1 Primers

The oligonucleotide primers used in the amplification were derived from the S ORF as follows:

Sense (21 mer): 5'- gACAAgAAgAATCCTCACAATACC - 3' (220-240 from *EcoR1* site)

Antisense (24 mer): 5'- AgCCCTACgAACCACTgAACAAAT - 3' (688-711 from *EcoR1* site)

These primers were designed in our laboratory (SM Bowyer, unpublished results)

5.2.4.2 PCR Controls

5.2.4.2.1 Positive controls

Serum: Serum derived from a patient with high levels of HBV DNA (> 1000 pg/ml) was diluted 1:10 and aliquotted for use as a positive control.

Plasmid: Plasmid dilutions were also used as positive controls and to establish the sensitivity of the assay. The plasmid used was pAM 12, a gift from the laboratory of J Gerin (Rockville MD, USA), which consists of the entire HBV genome (subtype *adw*) cloned into pBR 325 at the *EcoR1* site.

5.2.4.2.2 Negative controls: Distilled water was used as a negative control, interposed regularly between specimens in each PCR run. In addition, serum derived from patients immunized against HBV and known to be negative for HBV DNA, was included with each round of serum DNA extraction, and then used as a negative control in each PCR run.

5.2.4.3 PCR Buffer and Reaction Mixture (Kaneko *et al*, 1989a and 1989b)

Extracted serum (10 μl) was added to a 90 μl reaction volume (layered with two drops of liquid paraffin) containing 10mmol TRIS-HCl (pH 8.3), 50 mmol potassium chloride, 1.5 mmol magnesium chloride, 0.01% (weight/volume) bovine serum albumin, 2.5 units Taq DNA polymerase (Promega Corporation, Madison USA), 200 μmol of each dNTP, and 1 μmol of each primer. Immediately prior to amplification, specimens were mixed on a vortex mixer, and then briefly centrifuged.

5.2.4.4 35 cycle technique: The reaction was performed on a programmable heat cycling block (TRIO-Thermoblock, Biometra Göttingen, FRG). Samples were heated to 94°C for 1 minute (denaturation of DNA), cooled to 45°C for 1 minute (primer hybridization), and incubated at 72°C for 2 minutes (primer extension). 35 such cycles were used, following which specimens were kept at 72°C for 3 minutes (final extension), and then stored at 4°C until fractionated on an agarose gel.

5.2.4.5 99 cycle technique: The reaction was performed on a programmable heat cycling block in which tube temperature was monitored (Hybaid, Hybaid Ltd Teddington UK). 99 cycles of the following sequence were performed: samples were heated to 95°C for 1 second (denaturation of DNA), then cooled to 50°C for 1 second (primer hybridization), follwing which they were incubated at 72°C for 1 second (primer extension). After the 99th cycle, the samples were incubated at 72°C for final extension, then stored at 4°C until fractionated on an agarose gel.

5.2.5 Agarose Gel Electrophoresis: A 15 μl aliquot of PCR reaction mixture was fractionated by 2.5% agarose gel electrophoresis containing ethidium bromide, and then visualized by UV flourescence.

5.3 Results

5.3.1 Sensitivity of PCR assay

The sensitivity of the PCR assay was established using dilutions of plasmid containing known copy number. It proved possible, on repeated occasions to amplify HBV DNA at a starting level of 10fg/ml with both PCR protocols. Figure 16 shows the results obtained with both protocols using varying quantities of plasmid. Note the "misfire" positives in the 99 cycle protocol. This is a term which has been coined to describe the smear appearance of some positive PCR results probably induced by unfavourable storage conditions (Quan *et al*, 1992)

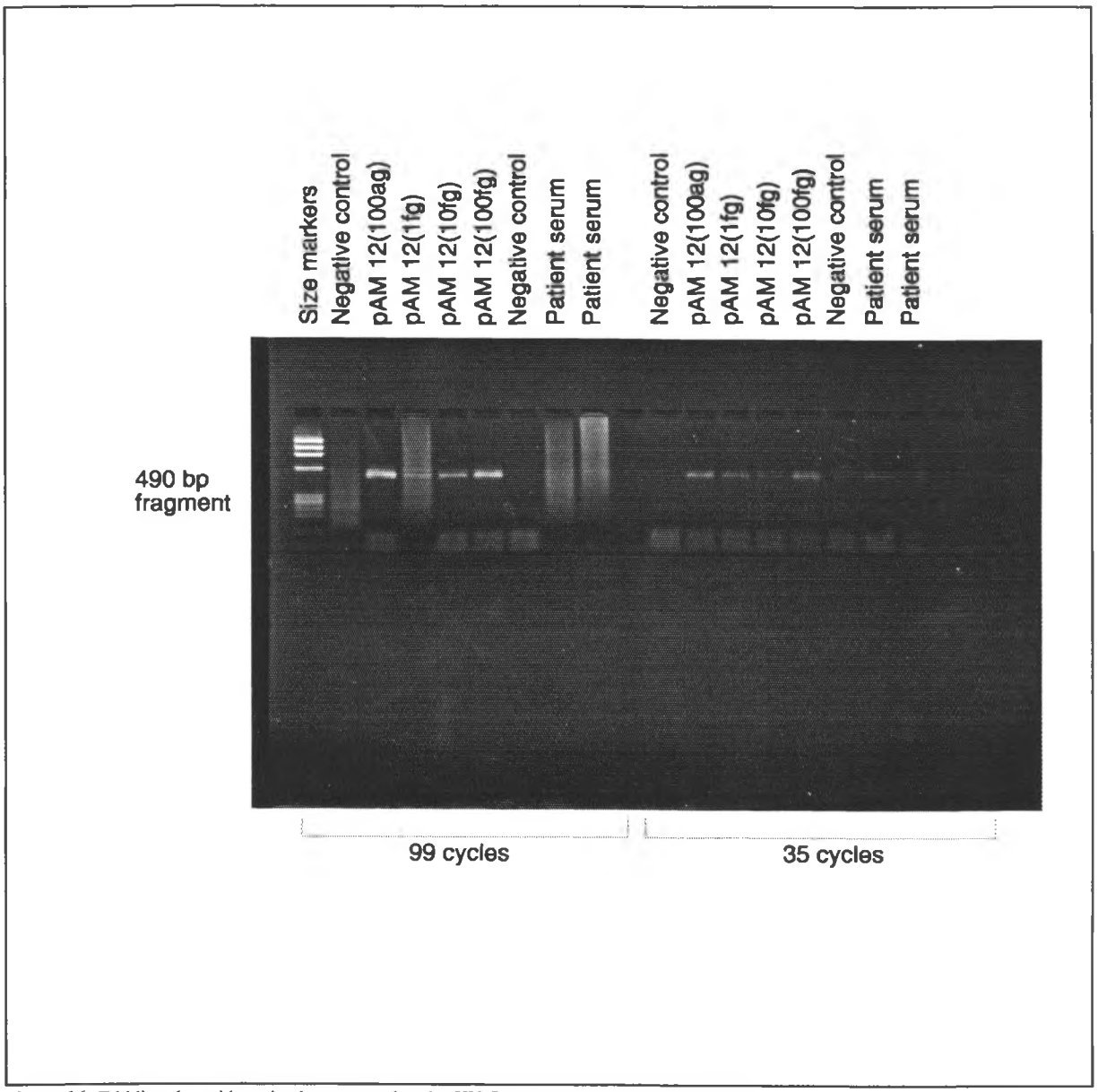


Figure 16 Ethidium bromide-stained agarose gel under UV flouresecnce. See text for details.

5.3.2 Results

A total of 35 specimens with repeatedly positive isolated anti-HBc were successfully subjected to both PCR protocols in which all controls performed appropriately. 2/35 (5.7%) reacted with weakly positive bands on the 35 cycle protocol. These two specimens were also positive on the 99 cycle technique. However, in 7/35 specimens, on the latter protocol, "misfire" results (see Figure 16) were obtained, 6 of these occurred in specimens which were negative

by the 35 cycle method. The 99 cycle protocol produced this type of result in a number of amplifications, including in positive controls. Nevertheless, as can be seen in Figure 16, the level of amplification achieved with this protocol when amplified bands were clearly visible, (i.e. not "misfire positives") was significantly greater than that achieved in the 35 cycle technique.

Of the 35 specimens selected, 4 (11.4%) were found to be positive for anti-HBc IgM by the hepatitis laboratory at the NIV.

5.4 Discussion

The results of this section of the study must be regarded as preliminary. Only one primer set was used, and hybridizational confirmation of specific amplification was not performed. Nevertheless the results are of interest, and form the kernel of future investigations into HBV.

The 35 cycle PCR, which demonstrated adequate sensitivity, revealed that 5.7% of the isolated core positive specimens may contain HBV DNA, and so be compatible with the diagnosis of low level HBV carriage. These specimens included 4 (11.4%) which were found to be anti-HBc IgM positive, compatible with the diagnosis of the core window. This figure is remarkably similar to that obtained in the first part of this report, for the database as a whole, where 11.6% of specimens belonging to primary screen group (b) were also anti-HBc IgM positive (Figure 6). None of the specimens with this profile on which PCR was performed were found to be positive. If these specimens are then excluded from the analysis, the frequency of possible low level carriers in the isolated anti-HBc positive group is 6.5%. While no attempt was made to select isolated anti-

HBc positive specimens according to the scintillation count obtained in their initial assessment in the hepatitis laboratory, it is of great interest to note that the two positives on the 35 cycle PCR protocol both had low counts in the anti-HBc RIA. As this test is a competitive assay, this suggests high levels of anti-HBc in these specimens.

While some difficulties were experienced in obtaining results with the 99 cycle protocol, mainly a problem of interpreting the cause and significance of the so-called "misfire" results, there was a definite difference in sensitivity between this method and the more usual 35 cycle approach. If it is possible to achieve sensitivities approximating those for nested protocols, then this unconventional PCR cycling would be of great value in avoiding the contamination risk associated with nesting.

Isolated anti-HBc has been reported as an uncommon finding (0.1-2.0%) in low-risk populations (Hanson and Polesky, 1984; Hadler *et al*, 1984), but the prevalence has been reported as high as 6.0% in high-risk populations (Hadler *et al*, 1984; Tedder *et al*, 1980). An intermediate rate of 4.0% on the NIV database (Table II) is probably an appropriate reflection of the high- and low-risk elements of the population served by this laboratory.

The key issue in isolated anti-HBc-positivity is what it means in terms of diagnosis, and possible infectivity of the patient. There is a spectrum of possibilities, ranging from false positivity, through established immunity following natural infection, to low level HBV carriage and even active liver disease. Isolated anti-HBc positivity has been reported in patients with chronic liver disease, hitherto unsuspected of being HBV-

related, and in whom HBV DNA was detected in liver biopsies (Bréchot *et al*, 1985; Fagan *et al*, 1991).

One of the problems of isolated HBV core antibody positivity been the frequency of false positive anti-HBc results, especially with enzyme immunoassays (Caspari *et al*, 1989; Blackburn *et al*, 1990; Schmidt *et al*, 1989). False-positive rates of 72.9% have been reported (Lai *et al*, 1992). Treatment of anti-HBc-positive sera with a reducing agent (dithiothreitol) has been reported to reduce the false positivity rate, and a reduction-sensitive factor (probably a nonspecific IgM anti-HBc species) has been isolated from sera with this profile (Spronk *et al*, 1991; Robertson *et al*, 1991). An interesting caveat to consider in relation to this discovery, is whether the anti-HBc IgM assay is similarly afflicted by false-positivity.

Low level HBV carriage and infectivity has been a concern for some time (Katchaki *et al*, 1980; Katchaki *et al*, 1982; Cossart *et al*, 1982; Sugg *et al*, 1982) and it is still a fact that 10-15% of cases of posttransfusion hepatitis are caused by HBV (Vandenvelde *et al*, 1993). If the preliminary results presented in the current study stand up to further scrutiny, a low carrier rate of 6.5% in the group of isolated anti-HBc-positive patients will be of importance to this laboratory. The issue obviously also has great implications for blood transfusion services. Indeed, the whole subject of isolated anti-HBc has bedevilled transfusion services, not only from this point-of-view, but also because anti-HBc was regarded as a contentious surrogate marker for non-A, non-B hepatitis prior to the discovery of hepatitis C virus (HCV). A considerable wastage of blood for transfusion results from this profile (Aoki *et al*, 1993). One of the methods for

determining whether a patient is a low level carrier or not, is a trial of HBV vaccine, when carriers show up as non-responders, while those who possess anti-HBc as a vestige of previous HBV infection (with loss of anti-HBs) will develop an easily detectable secondary or anamnestic response, and those who are HBV-naive (false positive anti-HBc) will reveal a typical primary vaccine response (Draelos *et al*, 1987: Hollinger, 1990). Aoki *et al* (1993), used this method in patients derived from a blood transfusion service to show that 53/58 (91%) of patients responded to vaccination, 44 with primary responses and the remainder with an anamnestic response. Similar results were reported by Draelos *et al* (1987). All nonresponders were tested by PCR for HBV DNA, but none was positive. Contrasting with these findings, Kroes *et al* (1991), in a high-risk population in the Netherlands reported a 10% rate of HBV DNA positivity in a group of isolated anti-HBc reactive subjects. Of interest in this study was an anti-HBe-positive rate of 30.8% in the same group - which compares with the findings in the first part of this report (Figure 6), where anti-HBe was reactive in 48.2% of primary screen group (b). Much higher rates of low level HBV carriage (28.6-35.2%) in isolated core antibody positive individuals have been reported from high HBV prevalence regions of the Far East using PCR amplification of HBV DNA (Luo *et al*, 1991). While these results may be corroborated, one has to remain ever vigilant of the problem of PCR contamination. It is our experience at the NIV that the PCR procedure is exquisitely sensitive to contamination, despite prevalent awareness and precaution.

6 Concluding Discussion

A total of 39,774 serological results, in which an HBV primary screen consisting of RIA testing for HBsAg, anti-HBs and anti-HBc were derived from eight years of routine hepatitis testing at the NIV. All possible result combinations of these three markers were represented on the database.

The most unusual of these was that associated with co-positivity of HBsAg and anti-HBs in the absence of anti-HBc. This combination is difficult to reconcile with the usual course of HBV serological changes, but could be representative of situations such as reactivation of infection in immunosuppressed states, and as such may become a more frequent finding with the development of the HIV epidemic.

The co-occurrence of anti-HBs and HBsAg associated with anti-HBc, referred to as primary screen group (d), was also an uncommon finding on the database (1.2%). A very close similarity in the distribution of extended marker patterns, not seen between any other two primary screen groups, and difficult to reconcile with chance in this extended dataset, was observed between this group and one of the more common serological profiles, primary screen group (g), in which HBsAg and anti-HBc were detected together with negative anti-HBs. The extended marker pattern identity between these two groups was sufficiently strong to conclude that group (d) represented subset of group (g), in which virtually all chronic carriers on this database were located. This has implications for interpreting the basis for the co-occurrence of HBsAg/anti-HBs. The finding is probably associated with chronic carriers in whom anti-HBs appears, rather than previously immune patients becoming reinfected with

a second viral subtype, and may be associated more frequently with those who have chronic hepatitis, with possible immunological perturbations. Further investigations in these groups, utilizing two acid dissociation techniques, did not show that immune complexing, which is a well recognized manifestation of HBV infection, was a significant cause of antibody/antigen co-positivity. Nevertheless, at least one specimen with this profile demonstrated clear dissociation in both techniques attempted. An experimental model of HBV immune complexing was successfully used to assess these dissociation techniques and to demonstrate the effects of immune complex formation on HBsAg and anti-HBs detection by RIA. Further development of these techniques could prove valuable in the identification of patients at risk of chronic outcome.

Isolated anti-HBc positivity as a finding in HBV serology was relatively uncommon on the database at 4% of findings. The extended marker patterns suggested that 11.4% of these could be a manifestation of the so-called *core window*. PCR amplification of HBV DNA was used to detect low level HBV carriers in this group. Preliminary results obtained on a sample of sera with this profile suggested that 6.5% of the true isolated anti-HBc positives could be low level carriers, a finding of importance in the diagnostic service at the NIV but which requires further confirmation.

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