

Diagnosis and Monitoring of Hepatic Injury. I. Performance Characteristics of Laboratory Tests

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Purpose: To review information on performance characteristics for tests that are commonly used to identify acute and chronic hepatic injury.

Data Sources and Study Selection: A MEDLINE search was performed for key words related to hepatic tests, including quality specifications, aminotransferases, alkaline phosphatase, γ -glutamyltransferase, bilirubin, albumin, ammonia, and viral markers. Abstracts were reviewed, and articles discussing performance of laboratory tests were selected for review. Additional articles were selected from the references.

Guideline Preparation and Review: Drafts of the guidelines were posted on the Internet, presented at the AACC Annual Meeting in 1999, and reviewed by experts. Areas requiring further amplification or literature review were identified for further analysis. Specific recommendations were made based on analysis of published data and evaluated for strength of evidence and

clinical impact. The drafts were also reviewed by the Practice Guidelines Committee of the American Association for the Study of Liver Diseases and approved by the committee and the Association's Council.

Recommendations: Although many specific recommendations are made in the guidelines, some summary recommendations are discussed here. Alanine aminotransferase is the most important test for recognition of acute and chronic hepatic injury. Performance goals should aim for total error of <10% at the upper reference limit to meet clinical needs in monitoring patients with chronic hepatic injury. Laboratories should have age-adjusted reference limits for enzymes in children, and gender-adjusted reference limits for aminotransferases, γ -glutamyltransferase, and total bilirubin in adults. The international normalized ratio should not be the sole method for reporting results of prothrombin time in liver disease; additional research is needed to determine the reporting mechanism that best correlates with functional impairment. Harmonization is needed for alanine aminotransferase activity, and improved standardization for hepatitis C viral RNA measurements.

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Hepatocyte injury is encountered frequently in the practice of medicine. The incidence of acute viral hepatitis has decreased markedly in the past decade, following the introduction of vaccines for hepatitis A and B and testing of the blood supply for hepatitis C. Other forms of acute hepatic injury have not changed appreciably in incidence, and recognition of chronic hepatic injury has increased. Worldwide, an estimated 300–350 million individuals (5–6% of the world population) are chronically infected with hepatitis B virus (HBV),⁷ with an estimated 1–1.25

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An Approved Guideline of the National Academy of Clinical Biochemistry "Laboratory Medicine Practice Guidelines" and the American Association for the Study of Liver Diseases.

Presented in part at the American Association for Clinical Chemistry Annual Meeting, July 25–26, 1999, New Orleans, LA.

A complete monograph of these guidelines will be published by the National Academy of Clinical Biochemistry. Reprints are not available from the authors.

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Received March 24, 2000; accepted October 6, 2000.

⁷ Nonstandard abbreviations: HBV, hepatitis B virus; HCV, hepatitis C virus; NACB, National Academy of Clinical Biochemistry; AASLD, American Association for the Study of Liver Diseases; HAV, hepatitis A virus; HDV, hepatitis D virus; HEV, hepatitis E virus; ALT, alanine aminotransferase (EC 2.6.1.2); AST, aspartate aminotransferase (EC 2.6.1.1); ALP, alkaline phosphatase (EC 3.1.3.1); GGT, γ -glutamyltransferase (EC 2.3.2.2); PT, prothrombin

million of these in the United States. An estimated 170 million individuals (3% of the world population) are chronically infected with hepatitis C virus (HCV), with 2.1–2.8 million of these in the United States (1). Cirrhosis is currently the ninth leading cause of death in the United States (2); deaths from cirrhosis are predicted to increase 223% by 2008 and 360% by 2028 as a result of cases developing from chronic HCV infection (3). Hepatocellular carcinoma is the fifth leading cause of cancer death worldwide, with most deaths occurring in Asia and Africa. The incidence of hepatocellular carcinoma is rising worldwide; in the United States, it has doubled in the past 20 years (4) and is expected to increase another 68% over the next decade from cancers developing in HCV-infected individuals (3).

Knowledge in the field of hepatocyte injury has increased rapidly, expanding the number of tests available for diagnosing and monitoring viral, metabolic, and immunologic etiologies of hepatocyte injury. At the same time, changes in the healthcare environment and Medicare reimbursement policies have made it important to have useful guidelines to follow in diagnosis and monitoring of patients with liver disease. The National Academy of Clinical Biochemistry (NACB) has, for several years, developed evidence-based laboratory medicine practice guidelines for the diagnosis and monitoring of various disorders. The American Association for the Study of Liver Diseases (AASLD) also publishes clinical practice guidelines for treatment of patients with liver disease. The present guidelines represent a consensus of both guideline committees. They have been reviewed and approved by the AASLD Council.

These guidelines, intended for use by physicians and laboratories, suggest preferable approaches to the diagnostic aspects of care. These guidelines are intended to be flexible, in contrast with “standards of care”, which are inflexible policies to be followed in almost every case. The guidelines presented have been developed in a manner consistent with the AASLD Policy Statement on Development and Use of Practice Guidelines. Specific recommendations are based on relevant published information. In an attempt to standardize recommendations, the Practice Guidelines Committee of AASLD has adopted modified categories of the Quality Standards of the Infectious Diseases Society of America. These categories (Table 1) are reported with each recommendation, using the Roman numerals I–IV to determine quality of evidence on which recommendations are based and the letters A–E to determine the strength of recommendation. Because of

Table 1. AASLD categories reflecting evidence supporting guidelines (A–E) and quality of evidence on which recommendation is based (I–IV).

Category	Explanation
I	Evidence from multiple well-designed randomized controlled clinical trials, each involving a number of patients to be of sufficient statistical power
II	Evidence from at least one large well-designed clinical trial with or without randomization, from cohort or case-controlled analytical studies, or well-designed metaanalysis
III	Evidence based on clinical experience, descriptive studies, or reports of expert committees
IV	Not rated
A	Survival benefit
B	Improved diagnosis
C	Improvement in quality of life
D	Relevant pathophysiologic parameters improved
E	Impacts cost of healthcare

the nature of these guidelines, only categories B and E are used in the recommendations.

Methods

The NACB developed a committee in March 1998, composed of two biochemists (D.D., J.L.), two virologists (D.G., F.N.), and two hepatologists (R.K., L.S.), to develop guidelines for diagnosis and monitoring of hepatic injury. After an initial meeting to determine areas that should be addressed by the guidelines, a comprehensive literature search was conducted of English-language articles in Index Medicus from 1966 to 1998, with “Knowledge Finder” as a search engine. Key search words included hepatitis A (HAV), hepatitis B (HBV), hepatitis C (HCV), hepatitis D (HDV), hepatitis E (HEV), hepatitis G, TT virus, alcoholic hepatitis, α_1 -antitrypsin, Wilson’s disease, hemochromatosis, autoimmune hepatitis, primary biliary cirrhosis, sclerosing cholangitis, cirrhosis, hepatocellular carcinoma, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), γ -glutamyltransferase (GGT), bilirubin, ammonia, and laboratory analytical performance goals. Compound searches were performed using the terms “fibrosis markers and chronic hepatitis”, “HCV and genotype”, and “prothrombin time (PT) and liver disease”. For several of the key words, the search was repeated in August 1999 for articles from 1998 and 1999. The filter was set for fuzzy logic for word matching and to select the top 1000 matches in order of relevance. All titles with high or moderate relevance values were reviewed, and if they appeared to address the topics selected, the abstracts were reviewed to select articles for further study. A total of >750 articles were selected for review; additional references were selected from the bibliographies of the articles selected. Assessment of strength of the evidence for each recommendation was based on the criteria of AASLD (Table 1).

time; P-5'-P, pyridoxal-5'-phosphate; CAP, College of American Pathologists; CLIA, Clinical Laboratory Improvement Act of 1988 amendments; ISI, international sensitivity index; INR, international normalized ratio; HBsAg, hepatitis B virus surface antigen; HBcAg, hepatitis B virus core antigen; HBeAg, hepatitis B virus e antigen; RIBA, recombinant immunoblot assay; SOD, superoxide dismutase (EC 1.15.1.1); EIA, enzyme immunoassay; and FDA, Food and Drug Administration.

The guidelines were reviewed in a multistep process. An initial draft was prepared by the committee and reviewed by 11 experts: 8 in the field of hepatology, and 3 in laboratory medicine. On the basis of the comments received, a second draft of the guidelines was prepared and presented to the AASLD Practice Guidelines Committee and NACB board of directors. A third draft was then prepared and posted on the NACB website for open comments, and presented in a 2-day, NACB-sponsored symposium at the AACC Annual Meeting in July 1999. A transcript of the comments made at the symposium was reviewed by the committee, and modifications to the guidelines were again presented to the AASLD Practice Guidelines Committee and the NACB board of directors for final comments. The guidelines were then reviewed and approved by the AASLD Council. These guidelines represent the product of the final modifications based on those comments.

Recommended Guidelines

The following serum tests should be used to evaluate patients with known or suspected liver disease: AST, ALT, ALP, total bilirubin, direct bilirubin, total protein, and albumin (IIIB, E). A panel with all of these tests is currently approved by the Health Care Financing Administration for Medicare reimbursement. Total protein is not discussed extensively in this guideline because of its limited utility in evaluation of liver status; its primary utility as a liver-related test is in allowing recognition of increased γ -globulins, to aid in recognition of patients at increased likelihood of having autoimmune chronic hepatitis.

PERFORMANCE SPECIFICATIONS FOR LIVER TESTS

A recent conference, sponsored by the IFCC, WHO, and the International Union for Pure and Applied Chemistry, addressed strategies for establishing performance specifications for laboratory tests (5, 6). Performance specifica-

tions for laboratory tests can be established by different methods, including (in decreasing order of importance) medical outcome studies, data on biological variation, opinions of clinicians or professional societies, or data from proficiency testing or government directives (7). Goals should specify acceptable imprecision (degree of reproducibility of measurement), bias (difference between measured results and the true value), and total error (defined as bias + 1.65 \times imprecision). When goals are derived from biological data, the target for imprecision is less than one-half of the intraindividual variation (difference between measurements in a single person; CV_i) for the test, whereas the target for bias is less than one-fourth of the average intraindividual and interindividual (difference in values within a population of individuals; CV_g) variation, calculated as $\frac{1}{4} (CV_i^2 + CV_g^2)^{1/2}$ (8). Table 2 summarizes published data on performance specifications and within-laboratory precision for liver-related tests. Throughout this report, goals are defined based on the upper reference limit for all tests except albumin; there is no clinical significance to most occurrences of low concentrations of enzymes, bilirubin, or ammonia.

REFERENCE INTERVALS

Reference intervals refer to the range of values for a laboratory test seen in a specific population, typically described by upper and lower reference limits. Reference intervals serve as a means for physicians to compare results in their patients to expected values in particular settings. Most commonly, reference intervals are derived from samples of presumably healthy individuals and defined as the central 95% of results from healthy volunteers, tested under defined conditions (for example, fasting, drawn in the morning, with the patient seated for 10–15 min). Reference intervals may be established for different groups by partitioning the values to account for differences between groups of individuals, such as between men and women or between children and adults.

Table 2. Performance specifications and precision for liver tests (%).

Source	Type	ALT	AST	ALP	GGT	Albumin	Bilirubin
		Performance specifications					
CLIA	Mandate	TE ^a = 20	TE = 20	TE = 30		TE = 10	TE = 20 or 0.4 mg/dL
European (5)	Biological variation	I = 13.6	I = 7.2	I = 3.4	NS	I = 1.4	I = 11.3
		B = 13.6	B = 6.2	B = 6.4		B = 1.1	B = 9.8
		TE = 36	TE = 18	TE = 12		TE = 3.4	TE = 28
Ricos et al. (215)	Biological variation	I = 12.2	I = 6.0	I = 3.2	I = 6.9	I = 1.6	I = 12.8
		B = 12.2	B = 5.4	B = 6.4	B = 10.8	B = 1.3	B = 10
		TE = 32	TE = 15	TE = 12	TE = 22	TE = 3.9	TE = 31
Skendzel et al. (35)	Clinician opinion	NS	TE = 26	NS	NS	NS	TE = 23
Within-laboratory imprecision, %							
Lott et al. (13)	Proficiency tests	8	9	5	6	NS	NS
Ross et al. (216)	Proficiency tests	NS	NS	NS	NS	4.4	8.9

^a TE, total error; I, imprecision or degree of reproducibility; B, bias or difference from correct result; NS, not specified.

Partitioning is necessary when data show that results are significantly different between particular subgroups. Although individual laboratories seldom perform such extensive studies to establish reference limits, the validity of the limits used must be verified by testing a small number of healthy individuals to assure that the reference limits suggested in studies performed by the manufacturers of methods or reagents or published in the literature are acceptable for the population tested by the laboratory.

For a few tests, reference limits are based on data associated with risk of developing particular disease manifestations or complications. For example, upper reference limits for cholesterol have been established based on risk of development of atherosclerosis in prospectively followed individuals. Similarly, upper reference limits for fasting glucose have been defined by risk of developing diabetic complications in several cross-sectional surveys of apparently healthy individuals. For such health-derived reference limits to be used, there must be a high degree of comparability of results between laboratories, a process termed harmonization. In the case of cholesterol, this was accomplished by use of fresh serum samples, with results determined by a reference method, to prepare calibration curves for methods used in individual laboratories.

For most of the liver-associated tests discussed here, there are few data to suggest a risk-based reference limit. In the case of ALT, however, there are data to suggest that a value of 45 U/L in men is a clinically useful upper reference limit. Several studies, discussed in Part II of the Guidelines (9), have shown that treatment of chronic HCV infection is not indicated if ALT is within the reference interval, although this is controversial. The outcomes for treated patients with only slight increases in ALT (1–1.3 times the upper reference limit of 45 U/L) are similar to those for patients with larger increases in ALT activity (10). Furthermore, studies of blood donors (before availability of HCV tests) found that risk of transmission of hepatitis increased markedly in donors with ALT even slightly above the reference limit of 45 U/L, whereas there was no difference in likelihood in those with activities in the top one-third of the reference interval compared with those with lower ALT activities (11, 12). To use a single reference limit, harmonization of ALT methods among laboratories would be required. A pilot project using ALT reference material RM8430 showed an ability to reduce the range of ALT activities obtained by laboratories using a single analytical method (13).

AMINOTRANSFERASES

AST (EC 2.6.1.1) and ALT (EC 2.6.1.2) are widely distributed throughout the body. AST is found primarily in heart, liver, skeletal muscle, and kidney, whereas ALT is found primarily in liver and kidney, with lesser amounts in heart and skeletal muscle (14–16). The AST and ALT activities in liver are ~7000- and 3000-fold higher than serum activities, respectively (17). ALT is exclusively

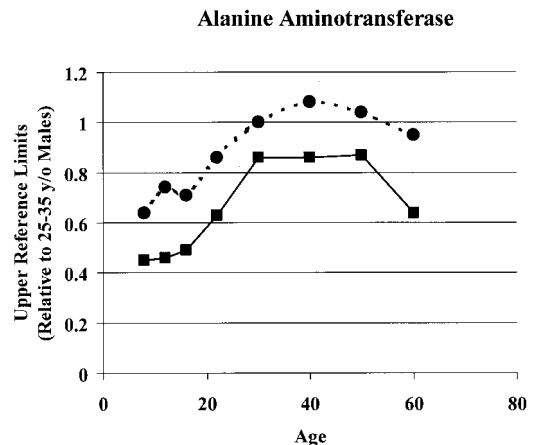


Fig. 1. Age and gender effects on upper reference limits for ALT.

The upper reference limit for 25- to 35-year-old males was set at 1.0 relative value units. ALT upper reference limits increase from childhood to approximately age 40, with greater increases seen in males (●) than in females (■); upper reference limits are ~30% higher in males 40 years of age than in males 25 years of age. After age 40, ALT upper reference limits again decline, with the decline more pronounced in males than in females. Data from Siest et al. (22).

cytoplasmic; both mitochondrial and cytoplasmic forms of AST are found in all cells (18). The half-life of total AST in the circulation is 17 ± 5 h, whereas that of ALT is 47 ± 10 h (19). The half-life of mitochondrial AST averages 87 h (20). In adults, AST and ALT activities are substantially higher in males than in females and vary with age (Figs. 1 and 2) (21, 22). Until approximately age 15, AST activity is slightly higher than that of ALT, with the pattern reversing by age 15 in males but persisting until age 20 in females (21). In adults, AST activity tends to be lower than that of ALT until approximately age 60, when the activities become roughly equal. An informal survey of attendees at a recent national meeting indicated that one-half of laboratories have gender-based reference intervals for AST and ALT, and fewer have age-adjusted

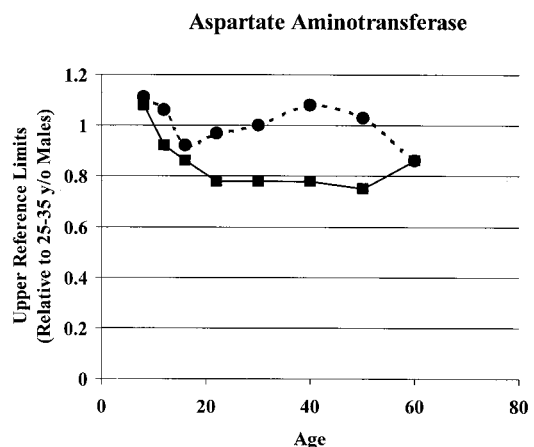


Fig. 2. Age and gender effects on upper reference limits for AST.

The upper reference limit for 25- to 35-year-old males was set at 1.0 relative value units. AST upper reference limits increase from childhood to young adulthood but change relatively little with increasing age in adults until after age 60. At all ages, except childhood and old age, AST upper reference limits are ~25–30% higher in males (●) than in females (■). Data from Siest et al. (22).

reference intervals. Because upper reference limits vary little between the ages of 25 and 60, age-adjusted reference limits need not be used for this population, which comprises most persons with chronic liver injury. Separate reference limits are needed for children and older adults; these may require national efforts to obtain enough samples from healthy individuals to accurately determine reference limits.

Liver disease is the most important cause of increased ALT activity and a common cause of increased AST activity. Several factors other than liver disease must be considered in interpreting AST and ALT activities; these are summarized in Table 3. In most types of liver disease, ALT activity is higher than that of AST; exceptions include alcoholic hepatitis and Reye syndrome. The reasons for the higher AST activity in alcoholic hepatitis appear to be multiple. Alcohol increases mitochondrial AST activity in plasma, whereas other causes of hepatitis typically do not (23). Most forms of liver injury decrease hepatocyte activity of both cytosolic and mitochondrial AST, but alcohol produces a decrease only in cytosolic AST activity (24). Pyridoxine deficiency, common in alcoholics, decreases hepatic ALT activity (25), and alcohol induces release of mitochondrial AST from cells without visible cell damage (26).

AST and ALT typically are measured by catalytic activity (27); both require pyridoxal-5'-phosphate (P-5'-P)

for maximum activity, although the effect of deficient P-5'-P on ALT is greater than the effect on AST (28). In renal failure, AST and ALT are significantly lower than in healthy individuals, perhaps because of serum binders of P-5'-P, as total P-5'-P is increased; decreased free P-5'-P reduces enzymatic activity (29). In a recent College of American Pathologists (CAP) proficiency survey, the average variation in values between laboratories using the same methods was 4–9% at aminotransferase values in the reference interval. When average results between laboratories using different assays were compared, the range was 39–85 U/L for the same specimen (30). Unexpectedly abnormal results are often normal on repeat testing (31, 32). Because of marked differences between laboratories, harmonization of methods is a priority. Alternative methods to minimize differences between laboratories, such as expressing results as multiples of the reference limit (33), have been shown to minimize between-laboratory variation (34).

Current target values for performance goals for total error in ALT activity measurements are 20% [Clinical Laboratory Improvement Act of 1988 amendments (CLIA)] and 32% (based on biological variation in healthy individuals). Data from outcome studies are not available for most laboratory tests for liver evaluation, with the exception of ALT. Few data exist on the biological variation of ALT in chronic hepatitis, particularly hepatitis C,

Table 3. Factors affecting AST and ALT activity, other than liver injury.

Factor	AST	ALT	Reference(s)	Comments
Time of day		45% variation during day; highest in afternoon, lowest at night	217, 218	No significant difference between 0900 and 2100; similar in liver disease and health
Day-to-day	5–10% variation from one day to next	10–30% variation from one day to next	219–221	Similar in liver disease and health, and in elderly and young
Race/gender	15% higher in African-American men		222	No significant difference between African-American, other women
BMI ^a	40–50% higher with high BMI	40–50% higher with high BMI	21, 223–225	Direct relationship between weight and AST, ALT
Meals	No effect	No effect	21	
Exercise	Threefold increase with strenuous exercise	20% lower in those who exercise at usual levels than in those who do not exercise or exercise more strenuously than usual	225–228	Effect of exercise seen predominantly in men; minimal difference in women (<10%). Enzymes increase more with strength training
Specimen storage	Stable at room temp for 3 days, in refrigerator for 3 weeks (<10% decrease); stable for years frozen (10–15% decrease)	Stable at room temperature for 3 days, in refrigerator for 3 weeks (10–15% decrease); marked decrease with freezing/thawing	229–233	Stability based on serum separated from cells; stable for 24 h in whole blood, marked increase after 24 h
Hemolysis, hemolytic anemia	Significant increase	Moderate increase attributable to release from red cell		Dependent on degree of hemolysis; usually severalfold lower than increases in LDH
Muscle injury	Significant increase	Moderate increase	228	Related to amount of increase in CK
Other	Macroenzymes	Macroenzymes	23, 235	Typically stable increase, affects only AST or ALT

^a BMI, body mass index; LDH, lactate dehydrogenase; CK, creatine kinase.

although it is commonly stated that ALT results are highly variable. In a study of 186 patients with confirmed chronic HCV infection, the average intraindividual CV was 39%. Although the majority of patients had fluctuations in enzyme activities over time, approximately one-third had ALT that varied little over time, with an average CV of 23% (D. Dufour, unpublished observations). As discussed earlier, there is evidence that distinguishing mildly increased ALT activity from normal ALT activity has clinical relevance (10–12). Thus, accurate determination of ALT at the reference limit is critical for correct treatment of patients with HCV infection.

The consensus of the authors and the AASLD Practice Guidelines committee is that performance criteria for ALT should be defined at the upper reference limits and that current performance goals are inadequate for clinical use. The data in patients with stable ALT suggest that a total error of <10% is required at the upper reference limits for accurate detection of patients who may benefit from treatment for HCV. Current data on within-laboratory precision (Table 2) suggest that this target cannot be met by current methods. It will likely be necessary to develop a standardization program for ALT measurements, similar to that used for creatine kinase MB. This may require use of other methods, such as immunoassay, to achieve the necessary total error target for management of patients with chronic hepatitis.

Performance goals for total error in AST activity measurement are 15–20%, by both CLIA requirements and based on biological variation. These meet the perceived needs of clinicians for diagnosis and management of liver disease (35). Performance goals are not as critical for AST as for ALT; a lower percentage of AST results are abnormal in chronic HCV compared with ALT (33% vs 71%). AST seldom is abnormal (6%) when ALT is normal, except in cirrhosis (D. Dufour, unpublished observations). Performance goals based on biological variation (Table 2) are adequate for clinical use, and precision goals for AST seem capable of meeting clinically relevant performance needs (35).

Recommendations:

- Assays for ALT activity should have total analytical error of $\leq 10\%$ at the upper reference limit (IIIB). Current published performance goals for AST, with total error of 15–20%, are adequate for clinical use (IIIB).
- Standardization of ALT values between methods and across laboratories is a priority need for patient care. Until standardization is accomplished, use of normalized results should be considered (IIIB).
- At a minimum, laboratories should have separate upper reference limits for adult males and females; reference limits should also be established for children and adults over age 60 by cooperative efforts (IIB).
- Unexpectedly increased ALT and/or AST should be evaluated by repeat testing; in individuals engaging in strenuous exercise, it should be repeated after a period

of abstinence from exercise. Research is needed to determine the appropriate time interval required (IIIB and IIIE).

ALP

ALP (EC 3.1.3.1), which is involved in metabolite transport across cell membranes, is found (in decreasing order of abundance) in placenta, ileal mucosa, kidney, bone, and liver (36–41). The bone, liver, and kidney ALP isoenzymes share a common protein structure coded for by the same gene (42, 43), but they differ in carbohydrate content. The half-life of the liver isoenzyme is 3 days (44–46). Age- and gender-related changes in ALP upper reference limits are illustrated in Fig. 3. Interpretation of ALP results using appropriate reference populations is particularly important in children; reference limits differ little in adult males and females between the ages of 25 and 60. After age 60, reference limits increase in women, although studies have not consistently evaluated subjects for the presence of osteoporosis, which can increase ALP activity in serum (47). Separate reference intervals are required for children and for pregnant women.

Cholestasis stimulates synthesis of ALP by hepatocytes, and bile salts facilitate release of ALP from cell membranes (48–50). Other factors affecting ALP are summarized in Table 4.

The method for total ALP in widest use is the *p*-nitrophenylphosphate method of Bowers and co-workers (51, 52). CAP surveys typically show variations of 5–10% between laboratories using the same manufacturer's assay; assays using different manufacturers' reagents vary widely (53). Complexing agents such as citrate, oxalate, or EDTA bind cations such as zinc and magnesium, necessary cofactors for ALP activity measurement, causing falsely decreased values as low as zero in plasma samples

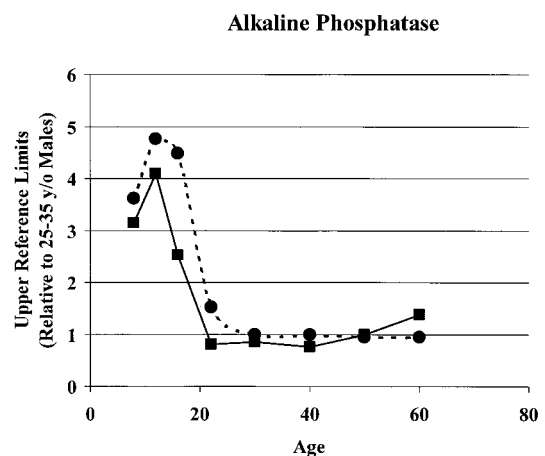


Fig. 3. Age and gender effects on upper reference limits for ALP.

The upper reference limit for 25- to 35-year-old males was set at 1.0 relative value units. ALP is manyfold higher in children and adolescents, reaching adult activities by approximately age 25. Values are slightly higher in males (●) than in females (■) until late in life. In adult males, upper reference limits do not change with age, whereas in females upper reference limits increase after menopause. Data from Siest et al. (22).

Table 4. Factors affecting ALP activity, other than liver injury.

Factor	Change	Reference(s)	Comments
Day-to-day	5–10%	219–221	Similar in liver disease and health, and in elderly and young
Food ingestion	Increases as much as 30 U/L	236–239	In patients of blood groups B and O; remains increased up to 12 h; attributable to intestinal isoenzyme
Race/gender	15% higher in African-American men; 10% higher in African-American women	222	
BMI ^a	25% higher with increased BMI	239	
Exercise	No significant effect	225, 227	
Specimen storage	Stable for up to 7 days in refrigerator, months in freezer	231	
Hemolysis	Hemoglobin inhibits enzyme activity	240	
Pregnancy	Increases up to two- to three-fold in third trimester	241	Attributable to placental isoenzyme
Smoking	10% higher	222, 239	
Oral contraceptives	20% lower	242	
Other	High in bone disease, tumors producing ALP; low after severe enteritis (in children) and in hypophosphatasia	240	Can be separated from liver causes by ALP isoenzymes and/or normal GGT

^a BMI, body mass index.

collected with the agents. Blood transfusion (containing citrate) causes transient decrease in serum ALP through a similar mechanism.

Separation of tissue-nonspecific ALP forms (bone, liver, and kidney) is difficult because of structural similarity; high-resolution electrophoresis and isoelectric focusing are the most useful techniques. Bone-specific ALP can be measured by heat inactivation (a poor method), immunologically, and by electrophoretic methods. Immunoassays of bone ALP are now available from several sources (54–56) and can be used to monitor patients with bone disease. Because there is good agreement between increases in ALP of liver origin and increases in the activity of other canalicular enzymes such as GGT, measurement of GGT activity is a good indication of a liver source, but does not rule out coexisting bone disease (57).

In contrast to most enzymes, intraindividual variation in ALP is low, averaging slightly more than 3% (Table 2). The current average within-laboratory imprecision of 5% is close to recommended performance specifications; a total error of 10–15% would meet health-based target values of 12%. The CLIA-specified total error range of 30% appears too wide for clinical use and should be narrowed.

Recommendations:

- Assays for ALP activity should have total analytical error of ≤10–15% at the upper reference limit (IIIB).
- Separate reference limits should be provided for children, based on age and gender, and for pregnant women. A single reference interval is adequate for adults over age 25 (IIB).

- Specimens for ALP activity should be obtained in the fasting state; if not, mildly increased patient values should be reevaluated in the fasting state before further evaluation (IIB and IIE).
- Assays for ALP isoenzymes or measurement of other associated enzymes (such as GGT) are needed only when the source is not obvious from clinical and laboratory features (IIIB and IIIE)

GGT

GGT (EC 2.3.2.2), a membrane-bound enzyme, is present (in decreasing order of abundance) in proximal renal tubule, liver, pancreas (ductules and acinar cells), and intestine (58–60). GGT activity in serum comes primarily from liver. The half-life of GGT in humans is ~7 to 10 days; in alcohol-associated liver injury, the half-life increases to as much as 28 days, suggesting impaired clearance (61). Age- and gender-related differences in GGT are summarized in Fig. 4. In adult men, a single reference interval is adequate between the ages of 25 and 80. Although upper reference limits are approximately twofold higher in those of African ancestry, information on racial characteristics is not commonly provided to laboratories; it would thus be difficult for laboratories to report values with the appropriate race-based reference range. In women and children, GGT upper reference limits increase gradually with age and are considerably lower than those in adult men. Separate reference limits should be established for men and women and for different age ranges in women and children. In children, this will probably require a cooperative effort of laboratories

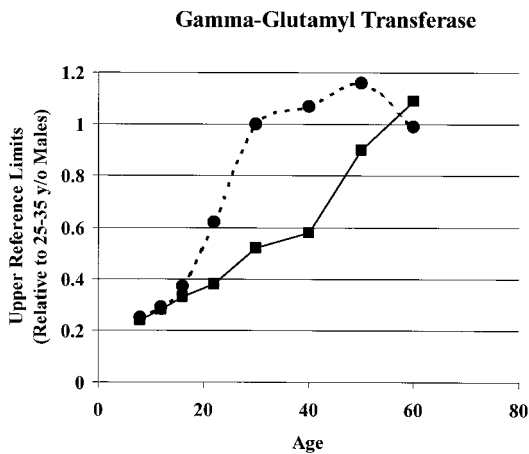


Fig. 4. Age and gender effects on upper reference limits for GGT.

The upper reference limit for 25- to 35-year-old males was set at 1.0 relative value units. GGT upper reference limits increase throughout life in females (■) but are relatively stable in males (●) over age 30. Before age 50, upper reference limits in males are ~25–40% higher than those in females, but the differences decrease with increasing age. Data from Siest et al. (22).

to obtain adequate numbers of specimens from healthy children.

GGT is slightly more sensitive than ALP in obstructive liver disease. GGT is increased an average of 12-fold above the upper reference limit in 93–100% of those with cholestasis, whereas ALP is increased an average of 3-fold above the upper reference limit in 91% of the same group (57, 62, 63). GGT appears to increase in cholestasis by the same mechanisms as does ALP (63, 64). GGT is increased in 80–95% of patients with any form of acute hepatitis

(65–67). Other factors that affect GGT activity are summarized in Table 5. Patients with diabetes, hyperthyroidism, rheumatoid arthritis, and obstructive pulmonary disease often have an increased GGT; the reasons for these findings are largely obscure. After acute myocardial infarction, GGT may remain abnormal for weeks (68, 69). These other factors cause a low predictive value of GGT (32%) for liver disease (70).

The IFCC method described by Shaw et al. (71) is used by most laboratories. Precision with activities less than one-half the upper reference limit is ~10%; at approximately twice the upper reference limit, it is closer to 5%. Mean values obtained in adults by different assays show dramatic differences, ranging from 37 to 90 U/L (72). Performance goals for GGT are based primarily on biological variation, with total error tolerance limits of ~20%. These are adequate for clinical purposes, given the limited clinical utility of GGT measurements.

Recommendations:

- Assays for glutamyltransferase activity should have total analytical error of $\leq 20\%$ at the upper reference limit (IIIB).
- Use of fasting morning specimens is recommended (IIB).
- Although a single upper reference limit is appropriate for adult men, separate reference limits (based on age) are needed for children and adult women (IIB).
- Because of lack of specificity, GGT should be reserved for specific indications such as determining the source of an increased alkaline phosphatase (IIIB and IIIE).

Table 5. Factors affecting GGT, other than liver injury.

Factor	Change	Reference(s)	Comments
Day-to-day	10–15%	219–221	Similar in liver disease and health, and in elderly and young
Race	Approximately double in African Americans	222	Similar differences in adult males, females
BMI ^a	25% higher with mild increase in BMI; 50% higher with BMI >30	223	Effect similar in adult males, females
Food ingestion	Decreases after meals; increases with increasing time after food ingestion	243	
Exercise	No significant effect	243	
Specimen storage	Stable for up to 7 days in refrigerator, for months in freezer	240	
Pregnancy	25% lower during early pregnancy	244, 245	
Drugs	Increased by carbamazepine, cimetidine, furosemide, heparin, isotretinoin, methotrexate, oral contraceptives, phenobarbital, phenytoin, valproic acid	246	Values up to 2 times reference limits are common, may be up to 5 times reference limits, especially with phenytoin
Smoking	10% higher with 1 pack/day; approximately double with heavier smoking	243	
Alcohol consumption	Direct relationship between alcohol intake and GGT	243, 247–249	May remain increased for weeks after cessation of chronic and alcohol intake

^a BMI, body mass index.

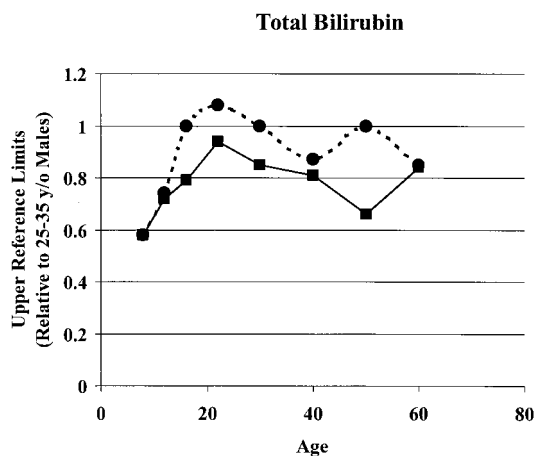


Fig. 5. Age and gender effects on upper reference limits for total bilirubin.

The upper reference limit for 25- to 35-year-old males was set at 1.0 relative value units. Upper reference limits increase throughout childhood and adolescence, reaching peak values at approximately age 20; after this, values gradually decrease with increasing age. At all ages, upper reference limits are higher in males (●) than in females (■), although the differences are minimal at the extremes of life. Data from Siest et al. (22).

BILIRUBIN

Daily production of unconjugated bilirubin is 250–350 mg, mainly from senescent erythrocytes (73). Clearance at normal values is $5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$, or $\sim 400 \text{ mg/day}$ in adults; the rate does not increase significantly with hemolysis (74). The half-life of unconjugated bilirubin is $< 5 \text{ min}$ (75). UDP-glucuronyltransferase catalyzes rapid conjugation of bilirubin in the liver; conjugated bilirubin is excreted into bile and is essentially absent from blood in healthy individuals. δ -Bilirubin, sometimes termed bili-protein, occurs when conjugated bilirubin covalently binds to albumin (76); it has a half-life of ~ 17 –20 days (the same as albumin), which accounts for the prolonged jaundice in patients recovering from hepatitis or obstruction (77). Age- and gender-related changes in bilirubin reference limits are illustrated in Fig. 5. Increases in conjugated bilirubin are highly specific for disease of the liver or bile ducts (78). Increased conjugated bilirubin

may also occur with impaired energy-dependent bilirubin excretion in sepsis and total parenteral nutrition and after surgery (79). With recovery from hepatitis or obstruction, conjugated bilirubin falls quickly, whereas δ -bilirubin declines more slowly (80). Gilbert syndrome, found in $\sim 5\%$ of the population, causes mild unconjugated hyperbilirubinemia because of impaired UDP-glucuronyltransferase (EC 2.4.1.17) activity (81). Total bilirubin rarely exceeds 68 – $85 \text{ } \mu\text{mol/L}$ (4 – 5 mg/dL), even during prolonged fasting, unless other factors that increase bilirubin are also present (82). Other factors affecting bilirubin are summarized in Table 6.

Bilirubin typically is measured using two assays for total and “direct reacting” or direct bilirubin; subtracting direct from total gives “indirect bilirubin”. The direct bilirubin assay measures the majority of δ -bilirubin and conjugated bilirubin and a variable but small percentage of unconjugated bilirubin (83–85). High pH or the presence of a wetting agent promotes reaction of unconjugated bilirubin in the direct assay; the reagent for direct bilirubin should have at least 50 mmol/L HCl to prevent measurement of unconjugated bilirubin (86). Light can convert unconjugated bilirubin to a photoisomer that reacts directly (87); it also causes total bilirubin to decrease by $0.34 \text{ } \mu\text{mol} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$ ($0.02 \text{ mg} \cdot \text{dL}^{-1} \cdot \text{h}^{-1}$). Direct spectrophotometry (dry film methods) measures conjugated and unconjugated bilirubin and calculates δ -bilirubin as the difference between the sum of these and total bilirubin. Some authors have suggested that conjugated bilirubin is better than direct bilirubin to measure recovery from liver disease (88).

In a recent CAP survey, at a total bilirubin concentration of $38 \text{ } \mu\text{mol/L}$ (2.5 mg/dL), the average variation in laboratories using the same method was 5%; however, the mean with different methods ranged from 34 to $46 \text{ } \mu\text{mol/L}$ (2.0 – 2.7 mg/dL). At a concentration of $27 \text{ } \mu\text{mol/L}$ (1.5 mg/dL), the average variation using the same method was 8% (89).

Performance goals for total bilirubin measurement allow 20% (CLIA) to 30% (biological variation) total error

Table 6. Factors affecting bilirubin, other than liver injury.

Factor	Change	Reference(s)	Comments
Day-to-day	15–30%	219	
Food ingestion	Bilirubin increases an average of one- to twofold with fasting up to 48 h	250, 251	Averages 20–25% higher after overnight fast than after meals
Race	33% lower in African-American men, 15% lower in African-American women	222, 252	Compared with values in other racial/ethnic groups
Exercise	30% higher in men	227	No significant effect in women
Light exposure	Up to 50% decrease in 1 h	87	Affects unconjugated bilirubin more than direct-reacting bilirubin
Pregnancy	Decreases 33% by second trimester	241	Similar in second, third trimester
Hemolysis	Cross-reacts in some assays	240	Hemoglobin absorbs light at the same wavelength as bilirubin, falsely increasing results
Oral contraceptives	15% lower	242	
Hemolytic anemia	Increases in unconjugated bilirubin	240	

(Table 2). Clinicians felt that a 23% change in bilirubin at the upper reference limits indicated a significant change in condition (Table 2) (35). Thus, CLIA performance goals appear to meet clinical performance needs. Few data exist on performance goals for direct bilirubin. There is poor reproducibility of direct bilirubin between laboratories as a result of a variety of factors (90, 91). Because conjugated bilirubin is essentially absent from normal serum (80), it is reasonable to expect that laboratories should report direct bilirubin of $\leq 1.7 \mu\text{mol/L}$ (0.1 mg/dL) in virtually all healthy individuals.

Recommendations:

- Assays for total bilirubin should have a total analytical error of $\leq 20\%$ [or $6.8 \mu\text{mol/L}$ (0.4 mg/dL)] at the upper reference limit (IIIB).
- Separate upper reference limits should be used for total bilirubin in men and women. Although total bilirubin upper reference limits decline with age in adults, there is little significance to slight increases in bilirubin, and separate adult age-adjusted upper reference limits are not needed. In children, separate reference intervals should be used (IIIB).
- There are no data on analytical performance goals for direct or conjugated bilirubin. Laboratories should assure that direct bilirubin measurements are $< 1.7 \mu\text{mol/L}$ (0.1 mg/dL) in most healthy individuals. Additional data are needed on performance goals in patients with increased conjugated bilirubin (IIIB).

ALBUMIN

Albumin is the most abundant plasma protein and is produced by hepatocytes. The rate of production is dependent on several factors, including the supply of amino acids, plasma oncotic pressure, concentrations of inhibitory cytokines (particularly interleukin-6), and the number of functioning hepatocytes (92). Albumin functions as the major determinant of plasma oncotic pressure and serves as a transport protein for drugs, hormones, and waste products such as bilirubin; it also serves as a source of amino acids for the synthesis of other proteins. The half-life of plasma albumin typically is ~ 19 – 21 days. Plasma albumin concentrations are low in neonates, typically 28 – 44 g/L (2.8 – 4.4 g/dL). By the first week of life, adult values of 37 – 50 g/L (3.7 – 5.0 g/dL) are reached, increasing to 45 – 54 g/L (4.5 – 5.4 g/dL) by age 6 and remaining at these concentrations through young adulthood before declining to typical adult values. There is no significant difference in reference limits between males and females (93). Increases of serum albumin typically are secondary to hemoconcentration caused by dehydration, prolonged tourniquet use during collection, or specimen evaporation (92). The main causes for decreased albumin include protein loss (nephrotic syndrome, burns, protein losing enteropathy), increased albumin turnover (catabolic states, glucocorticoids), decreased protein intake (malnutrition, very low protein diets), and liver disease (92).

Plasma albumin seldom is decreased in acute hepatitis because of its long half-life, but in chronic hepatitis albumin gradually falls with progression to cirrhosis (92). Albumin concentrations are a marker of decompensation and prognosis in cirrhosis (94).

Albumin is most commonly measured by dye-binding methods, particularly bromocresol green and bromocresol purple; currently, $\sim 50\%$ of laboratories use each method. Bromocresol green methods may overestimate albumin (95), although differences between the two methods are small (92). Bromocresol purple underestimates albumin in renal failure (96, 97) and in patients with increased δ -bilirubin (98, 99), making this method unsuitable for patients with jaundice. Protein electrophoresis and staining may overestimate albumin because of higher binding of the dye to albumin than to other proteins (92). Immunoassays for albumin are available but not widely used in plasma (100). Results from recent CAP surveys indicate that average variation in albumin among laboratories using the same method is low (2–3%), although there are significant differences between laboratories using different methods (101). Such differences appear less dramatic when fresh specimens rather than CAP survey materials are used.

Performance goals for albumin measurement based on biological variation are typically $\sim 4\%$, whereas CLIA allows an error of 10%. The clinical uses of albumin measurements for liver disease are primarily in recognition of cirrhosis and in determining its severity; these require significant changes from reference limits. Data from CAP surveys indicate that only 2% of laboratories can meet the error limits based on biological variation. The opinion of the committee is that the CLIA performance goals are adequate for clinical purposes.

Recommendations:

- Total error of $< 10\%$ at the lower reference limit is adequate for clinical purposes; performance goals based on biological variation, although an ideal goal for measurement, cannot be met by most laboratories (IIIB).
- Assays for albumin in patients with liver disease should use bromocresol green. Bromocresol purple and electrophoresis determinations of albumin may be inaccurate in patients with liver disease (IIB).

PT

PT measures time for clotting of plasma after addition of tissue factor and phospholipid; it is influenced by the activity of factors X, VII, V, II (prothrombin), and I (fibrinogen). All of these factors are made by the liver, and three (II, VII, and X) are activated by vitamin K by the addition of a second, γ -carboxyl group onto glutamic acid residues. Immunoassays are available to measure “proteins induced by vitamin K antagonists (PIVKA)”, the most common measuring des- γ -carboxy prothrombin (102). PT is relatively insensitive to deficiency of any single clotting factor; there is no significant increase until

the concentration of any one factor falls below 10% of normal (103).

PT is commonly reported in seconds and compared to patient reference values (104). To minimize variation between reagents, each is assigned an International Sensitivity Index (ISI), compared to a reference method. The lower the amount of tissue factor (which initiates clotting in the assay), the lower the ISI value and the longer the PT. To adjust for differences in the ISI of reagents, a derived term, the international normalized ratio (INR), is used; the value is calculated as:

$$\text{INR} = \left(\frac{\text{PT}_{\text{patient}}}{\text{PT}_{\text{control mean}}} \right)^{\text{ISI}}$$

(105). For example, a sample with PT of 20 s with high ISI reagents had a PT of 40 s when tested with low ISI reagents, but the INR was essentially identical with both reagents (103). INR thus normalizes results in a patient on warfarin, despite differences in the ISI of reagents used. The use of reagents with low ISI improves the reproducibility of INR measurements, making the use of low ISI reagents ideal for monitoring anticoagulant therapy (106).

The effect of ISI is much greater on PT in warfarin use than in liver disease, so that the INR does not accurately reflect inhibition of coagulation in liver disease (103, 107–109). In liver disease, a decrease in the ISI of the reagents used produces only a slight increase in PT. For example, a sample from a patient with liver disease, tested with three differing ISI reagents, had INR values that varied between 1.86 and 2.90 although the difference in PT was only 3.6 s (103). If reagents with a low ISI are used, the INR markedly overestimates the degree of coagulation impairment in liver disease. A possible cause for the discrepancy in INR utility between warfarin use and liver disease is the marked difference in the relative amounts of native prothrombin vs des- γ -carboxy prothrombin present in the two conditions. Patients on warfarin or with vitamin K deficiency have markedly increased des- γ -carboxy prothrombin and decreased native prothrombin, whereas patients with acute hepatitis or cirrhosis have decreased native prothrombin but only slightly increased des- γ -carboxy prothrombin (110). Some preparations of tissue factor are inhibited by des- γ -carboxy prothrombin (110).

PT is reproducibly increased, usually at least 3 s beyond the population mean, in acute ischemic (111, 112) and toxic (113) hepatitis, but it rarely is increased >3 s in acute viral (114) or alcoholic (115, 116) hepatitis. PT often is increased in obstructive jaundice and may respond to parenteral vitamin K administration. In chronic hepatitis, PT typically is within reference limits, but it increases as progression to cirrhosis occurs and is increased in cirrhotic patients (117). Other factors affecting PT are summarized in Table 7.

Abnormal PT values are highly dependent on the ISI of the reagents used, although reference values are similar

Table 7. Factors affecting PT.

Factor	Change	Reference(s)
Specimen storage	No change at room temperature up to 3 days; refrigeration falsely shortens PT	121, 253–255
Citrate concentration	3.2% citrate minimizes problems compared with 3.8% citrate	121
Inadequate tube filling	Falsely increases PT	256, 257
High hematocrit	Falsely increases PT	256, 257
Other factors	Warfarin, malabsorption, vitamin K deficiency, drugs that decrease vitamin K production (especially antibiotics, fibric acid derivatives), and consumptive coagulopathy increase PT	

(103). Reagents with the same ISI typically give different results on different instruments, even of the same model (118–120). In addition, when reagents with the same ISI are used, a specimen can yield different INRs (106, 121–123). The variability (CV) of PT results among laboratories using the same instrument and reagents is 3–8% when PTs are prolonged, and variation is greater for the INR than it is for the PT itself. Within a single laboratory, the average variation (CV) in INR is estimated to be 10% (124). The difference in PT between laboratories using different reagents may be marked; in one study, the average difference was 20% (122). Recently, use of calibrant plasmas to determine the ISI in each laboratory for its own reagents and instrument has been shown to significantly improve agreement of INR values between laboratories (122, 125, 126).

Recommendations:

- PT in seconds rather than the INR should be used to express results of PT in patients with liver disease (IIIB); however, this does not standardize results between laboratories (IIB).
- Additional research into standardization of reagents and use of derived indices (percentage of activity, INR) in liver disease is needed (IVB).

AMMONIA (NH₃)

NH₃ is a product of amino acid metabolism and is cleared primarily by urea synthesis in the liver. *Helicobacter pylori* in the stomach appears to be an important source of NH₃ in patients with cirrhosis (127, 128). In liver disease, increased NH₃ typically is a sign of hepatic failure. High concentrations are seen with deficiency of urea cycle enzymes (129), in Reye syndrome (130), and with acute or chronic hepatic encephalopathy (131, 132). Mild increases in plasma NH₃ are seen in patients with chronic hepatitis, in proportion to the extent of disease (133). The use of NH₃ for monitoring of patients with encephalopathy is controversial; some studies have shown good correlation

of NH_3 concentrations with degree of encephalopathy (130, 132), whereas others have not (134). NH_3 appears to enhance the effects of γ -aminobutyric acid (135) and to increase benzodiazepine receptors (136); both γ -aminobutyric acid and benzodiazepines have been implicated in the pathogenesis of hepatic encephalopathy. On the other hand, clinical features seen in persons with isolated hyperammonemia are not identical to those of hepatic encephalopathy (137). Factors affecting NH_3 are summarized in Table 8. Specimens should have plasma separated from cells within 1 h of collection to avoid artifactual increases in NH_3 ; in patients with liver disease, separation within 15 min is ideal (138, 139).

Several methods have been used to measure NH_3 (138), with enzymatic assays currently the most widely used. One manufacturer uses slide technology with alkaline pH to convert NH_4^+ to NH_3 and then measures NH_3 with bromphenol blue. Reproducibility within laboratories using the same method averages 10–20%, with mean values using different methods differing by <10% on average (140).

Recommendations:

- Measurement of plasma NH_3 for diagnosis or monitoring of hepatic encephalopathy is not routinely recommended in patients with acute or chronic liver disease;

it may be useful in patients with encephalopathy of uncertain etiology (IIIB).

- Ideally, arterial, rather than venous, specimens should be used (IIB).
- Plasma should be separated from cells within 15 min of collection to prevent artifactual increases in NH_3 (IIB).

HEPATITIS SEROLOGIC MARKERS AND NUCLEIC ACID TESTING

HAV. IgM antibodies to HAV (anti-HAV IgM) typically are present at onset of symptoms and remain detectable for 3–6 months after infection in most patients, but they persist for >200 days in 13.5% of cases and may remain up to 420 days (141, 142). Prevalence of HAV RNA in blood is highest when ALT is highest, and the mean duration of viremia is 18 days (143). Total anti-HAV antibodies persist for long periods after infection, perhaps for life (144); seroprevalence increases with increasing age, ranging from 11% in children <5 years to 74% in adults >50 years (145). HAV vaccine induces detectable anti-HAV antibodies within 2–4 weeks of the initial dose of vaccine (146, 147), and the antibodies remain detectable at 5 years in 99% of individuals completing vaccination (148). There are no commercially available antigen detection tests for HAV, but immune electron microscopy and immunoassay methods have been used to detect HAV antigen in stool filtrates and other specimens (149, 150).

Table 8. Factors affecting NH_3 , other than liver injury.

Factor	Change	Reference(s)	Comments
Age	Four- to eightfold higher in neonates; two- to threefold higher in children <3 years; reaches adult concentrations by adolescence	258	
Specimen source	Arterial higher than venous; differences greater in renal, hepatic disease; capillary blood falsely increased because of NH_3 in sweat if skin inadequately cleaned	131, 138, 259	Arterial NH_3 correlates better with change in liver function than venous NH_3 ; tourniquet use, clenching fist increase venous NH_3
Exercise	Increases up to threefold after exercise	260	Increase greater in males than in females
Smoking	Increases 10 $\mu\text{mol/L}$ after 1 cigarette	138	
Delay in analysis	NH_3 increases because of metabolism by red blood cells: 20% in 1 h and 100% by 2 h	139, 261, 262	Use of ice water, rapid centrifugation, and separation of plasma minimize increases; rate of increase higher in liver disease because of high GGT activity in specimens, releasing NH_3 from peptides
Other factors	Increased in acute leukemia, blood transfusion, bone marrow transplantation, portal-systemic shunts, gastrointestinal bleeding, or high protein intake	263, 264	
Medications	Valproic acid and glycine (in irrigation fluids used in prostate, endometrial resection) increase NH_3 production	265, 266	

Recommendations:

- Anti-HAV IgM should be used to diagnose acute HAV infection (IB); HAV RNA tests are needed only for research purposes (IIIB).
- Total antibody should be used for determining immune status for HAV (IB).

HBV. Hepatitis B is a DNA virus with several protein antigens that can induce antibody responses. The most abundant, HBV surface antigen (HBsAg) is produced in excess along with viral particles and during nonreplicative phases of infection. HBV core antigen and e antigen (HBcAg and HBeAg) are produced by the same region of the viral genome and are found in infectious particles. A typical serological and clinical course of acute HBV infection is shown in Fig. 6 (151). IgM antibody to HBcAg (anti-HBc) usually is considered the gold standard for diagnosis of acute hepatitis B (152, 153), but it may also be present at fluctuating, low titers in patients with chronic hepatitis B, particularly when patients also have positive plasma HBeAg and episodes of rising ALT, indicating reactivation of disease (153–155). Total anti-HBc typically persists for life (156). HBsAg is characteristically present and anti-HBs absent at presentation in patients with acute HBV infection, but both are occasionally absent (152, 153), leaving IgM anti-HBc the only marker of infection (“core window”). Isolated positive anti-HBc also may represent low-level viremia, loss of anti-HBs many years after recovery, or a false-positive result (153, 156–159). Two

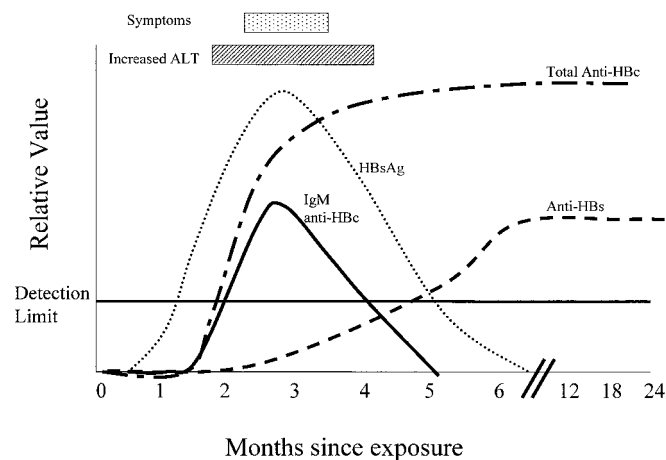


Fig. 6. Time course of serologic markers in acute hepatitis B infection with resolution.

After infection, the first marker of infection to appear is the HBsAg, at 1–3 months after exposure. Approximately 1–2 months later, the first antibody response is IgM anti-HBc, generally around the time of increases in AST and ALT activities in plasma. Total anti-HBc (measuring both IgM and IgG antibodies) also is positive at this time and subsequently. At the time of onset of jaundice, most patients have both HBsAg and IgM anti-HBc. With clearance of virus, anti-HBs will become detectable. In a small percentage of patients, there may be a transient period where neither HBsAg nor anti-HBs can be measured; the only commonly measured marker present at this time will be IgM anti-HBc, a pattern termed the core window. Although not illustrated here, such patients will usually be positive for HBeAg or anti-HBe if a second test is needed to confirm the anti-HBc result. With recovery from HBV infection, anti-HBc and anti-HBs persist for life in most individuals.

Table 9. Serologic diagnosis of HBV infections.^a

Marker	Incubation	Acute infection	Past infection	Chronic infection	Vaccination
HBsAg	+ ^b	+	–	+	–
HBeAg	+	+	–	±	–
HBV DNA	+	+	± ^c	+	–
Anti-HBc					
IgM	–	+	–	± ^d	–
Total	–	+	+	+	–
Anti-HBe	–	–	±	± ^e	–
Anti-HBs	–	–	+	–	+

^a Modified from Chernesky et al. (163).

^b +, detectable; –, not detectable; ±, may be detectable.

^c PCR methods.

^d May be positive in 10–15% patients with reactivation of infection.

^e Patients with chronic HBV infection usually have detectable HBeAg or anti-HBe.

factors are associated with likelihood of false-positive results: low anti-HBc reactivity and absence of anti-HBs in sensitive radioimmunoassays. In several studies, virtually none of those with low anti-HBc activity and negative anti-HBs showed an anamnestic response to a single injection of HBsAg vaccine, whereas 35–40% of those with weakly positive anti-HBs and 50–80% of those with high anti-HBc activity responded (157, 159–161). Convalescence from infection is indicated by loss of HBsAg and development of anti-HBs. Concomitant HBsAg and anti-HBs may be seen in a small number of patients with chronic HBV infection. This phenomenon appears to be particularly common in patients on maintenance hemodialysis (7%) compared with other HBsAg-positive patients (2%) (162). The presence of anti-HBs in these settings does not appear to have clinical importance. Patterns of serological markers in various forms and phases of HBV infection are shown in Table 9 (163). Examples of discordant or unusual hepatitis profiles are given in Table 10. Tests with discordant results should be repeated, and testing for additional serological markers may be indicated to establish the correct diagnosis (164).

Recommendations:

- Tests for HBsAg, anti-HBs, and anti-HBc should be performed for diagnosis of current or past HBV infection. In suspected acute HBV infection, tests for IgM anti-HBc should be utilized (IB).

Table 10. Discordant or unusual hepatitis B serologic profiles requiring further evaluation.

- HBsAg positive/anti-HBc negative
- HBsAg, anti-HBs, and anti-HBc positive
- Anti-HBc positive only
- Anti-HBs positive only in a nonimmunized patient
- HBsAg negative/HBeAg positive
- Positive for HBeAg and anti-HBe
- Total anti-HBc negative/IgM anti-HBc positive

- HBeAg and anti-HBe should be measured only when indicated based on results of the initial tests (IIIB and IIIE).
- In patients with discordant results, tests should be repeated; persistently discordant results should be evaluated by a hepatologist or gastroenterologist (IIIB).

In the HBeAg-positive patient, loss of HBeAg and seroconversion to anti-HBe positivity typically is associated with loss of detectable HBV DNA by methods other than PCR, normalization of aminotransferases, and histologic improvement, implying a low replication state and significant clinical improvement (153). HBV DNA concentrations are useful in monitoring chronic hepatitis B patients receiving antiviral therapy. Loss of detectable HBV DNA by a solution-phase hybridization assay is an earlier indicator of response to antiviral therapy than loss of HBeAg (164). Several assays for detection of serum HBV DNA are available commercially; detection limits are given in Table 11. There currently is no standardization of HBV DNA assays between laboratories. Circulating HBV DNA can be found by sensitive PCR amplification methods in some patients with negative HBsAg and positive anti-HBs, anti-HBe, and anti-HBc months or years after clinical recovery from acute (165, 166) or chronic (167) hepatitis. The significance is not clear because most viral DNA is found in immune complexes (165) and may not represent the entire genome. Similarly, in patients with chronic HCV, HBV viral DNA can be found (using sensitive PCR methods) in both liver and serum, particularly in patients with anti-HBc as an isolated HBV marker (157, 158). Thus, there are few data on which to determine desirable lower limits of detection for HBV DNA measurements.

Recommendations:

- Quantitative HBV DNA, HBeAg, and anti-HBe measurements should be used for monitoring response to antiviral therapy (IB).
- An international standard for HBV DNA tests should be established and manufacturers should calibrate methods against it (IIIB).
- Tests for HBV DNA should be quantitative, and the clinically useful dynamic range for HBV DNA tests should be defined (IIIB).

HCV. Screening tests for HCV infection detect antibodies to HCV proteins, usually apparent by an average of 80

days (range, 33–129 days) after infection, measured by second-generation anti-HCV enzyme immunoassays (EIA-2) (168). Immunocompromised patients and those on dialysis may rarely lack detectable antibodies by EIA-2 despite other evidence of active viral infection (169). A third-generation EIA (EIA-3) for anti-HCV has been approved by the Food and Drug Administration (FDA) for screening of blood products; it contains reconfigured core and NS3 antigens and an additional antigen (NS5) not found in EIA-2. EIA-3 provides a slight increase in sensitivity but lower specificity than EIA-2, and shortens the time to detection of antibody to an average of 7–8 weeks after infection (170–172). The FDA has approved a method for home use for obtaining samples for anti-HCV testing. In patients who have cleared HCV from the circulation, titers of anti-HCV antibodies gradually fall (173, 174). In one study of persons with posttransfusion HCV infection, 6% were negative for all HCV markers, including anti-HCV antibodies, 17 years after infection (175). In evaluating possible perinatal transmission of HCV, maternal antibody clears by 12 months in 90% of uninfected infants and by 18 months in 100% (176). Approximately 90% of infected infants have detectable plasma HCV RNA by 3 months of age (177).

Supplemental tests for anti-HCV help resolve suspected false-positive EIA test results. Recombinant immunoblot assays (RIBAs) contain the same HCV antigens as do the EIA tests, along with superoxide dismutase (SOD; EC 1.15.1.1). A positive RIBA is defined as reactivity against two or more HCV antigens from different regions of the genome, without reactivity to SOD. Reactivity to a single HCV antigen or multiband reactivity with reactivity to SOD is considered indeterminate. Individuals with isolated positivity for either C100 or 5-1-1 antigen rarely have evidence of active HCV infection, whereas either C22- or C33-indeterminate patterns predict active HCV infection in 25–50% of individuals (178–180). In populations at high risk for HCV infection, <1% of EIA-2-positive specimens will be false positives. Additionally, in recently infected individuals, RIBA results are positive in only 85% of cases (181). Therefore, RIBA testing in high-risk populations is not necessary for the diagnosis of hepatitis C (182). Although the overall pattern does not differ among the common genotypes (183), antibody responses to two of the four antigens included (core and NS4) are significantly lower in patients infected with genotypes other than 1 (179). Isolated positivity for NS5 in the RIBA-3 is virtually never associated with presence of HCV RNA, suggesting that it is the cause of the reduced specificity with EIA-3 (184, 185). Indeterminate RIBA-3 results (attributable to antigens other than NS5) are associated with HCV viremia in ~50% of cases; HCV RNA-positive patients with indeterminate RIBA results are more likely to be immunosuppressed than those with positive RIBA results (180). At present, there are no commercially available antigen tests for HCV, although a

Table 11. Lower detection limits of HBV DNA assays.

Method	Detection limit, copies/mL ^a
Hybrid capture	3.0×10^6
Branched DNA	0.7×10^6
Liquid hybridization	4.0×10^4
PCR	10^2-10^3

^a Results can also be expressed as ng/L of HBV DNA by dividing by 2.85×10^5 .

highly sensitive assay for HCV core protein has been developed (186).

Detection of active HCV infection depends on detection of HCV RNA. HCV RNA can be detected in serum within 1–2 weeks after acute infection, weeks before ALT becomes abnormal and before the appearance of anti-HCV antibodies (173). The time course of typical HCV infection is illustrated in Fig. 7. Although not FDA-approved, reverse transcription-PCR assays for HCV RNA are used frequently in clinical practice; the most sensitive can detect <100 HCV RNA copies/mL. HCV RNA assays are not standardized, and quantitative assay results may vary significantly between different laboratories using different assays (187, 188). EDTA and sodium citrate plasma are preferred specimens for HCV RNA tests. Heparinized plasma is inhibitory for many nucleic acid amplification assays, and serum specimens provide suboptimal stability unless serum is frozen soon after specimen collection. HCV RNA is very susceptible to degradation by the high activities of RNase present in blood; therefore, serum specimens for HCV RNA should be centrifuged as soon as possible after clot formation. If centrifugation is performed immediately, <10% of HCV RNA is lost even if the plasma or serum is not separated from the formed elements for up to 6 h (189). If a serum separator tube is used, specimens are stable after centrifugation for up to 24 h (189). Short-term (<7 days) storage of serum or plasma at 4 °C is acceptable. Once frozen, samples are stable through at least three freeze-thaw cycles (189–191).

Quantitative HCV RNA assays often are less sensitive than qualitative RNA assays using the same technology,

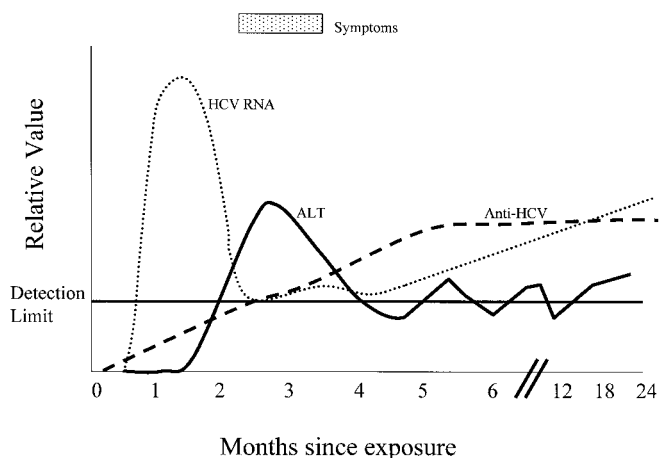


Fig. 7. Time course of serologic markers in acute hepatitis C infection. After infection, the first marker to appear is HCV RNA, usually detectable by 1–2 weeks after exposure to the virus. HCV RNA concentration increases, but it begins to fall with development of antibody response and may occasionally be negative. Anti-HCV appears at an average of 8–10 weeks after exposure; the time is shorter with third-generation than with second-generation anti-HCV assays. After the acute episode, which is clinically silent in most individuals, 70–90% will develop chronic infection with HCV. During the transition from acute to chronic infection, ALT (thick solid line) may fluctuate between normal and abnormal values, and 15–25% of chronically infected individuals have persistently normal ALT.

but this is not universal; in the case of PCR, this may be related to the larger sample size used for amplification. The current version of the branched DNA assay is the least sensitive, with a lower limit of detection of 200 000 copies/mL, although a third-generation assay with significantly lower cutoff values will soon be available. Results cannot be directly compared because different standards are used. A WHO HCV RNA international standard (genotype 1) for HCV RNA for nucleic acid amplification assays is now available (192). There are few data on clinically desirable lower detection limits for HCV RNA in untreated patients. In patients receiving treatment, lack of detectable HCV RNA in assays with lower detection limits <1000 copies/mL 6 months after treatment is associated with 90–95% likelihood of permanent clearance of the virus (193). In terms of upper detection limit, data from treatment studies show that risk of treatment failure is associated with HCV RNA $>3.2 \times 10^6$ copies/mL (194).

Recommendations:

- EIA screening tests for HCV antibody are adequate for diagnosis of past or current HCV infection in a patient population with a high prevalence of disease; supplemental testing is not needed in such patients. If confirmation of active infection is required, HCV RNA should be used (IIB and IIE).
- Supplemental anti-HCV tests (RIBAs) should be used in populations with low prevalence of disease or to confirm prior infection by HCV in a patient who is HCV RNA negative (IIIB and IIIE).
- Improved intermethod agreement and precision are needed for HCV RNA tests; methods should use a standard such as that developed by WHO (IIB).
- Specimens for HCV RNA should be collected either as EDTA or citrated plasma or be centrifuged promptly to prevent falsely low results (IIB).
- Assays for HCV RNA should ideally have a dynamic range from <1000 copies/mL to $>3.2 \times 10^6$ copies/mL (IIB).

There are six major genotypes and >90 subtypes of HCV, which vary in their world-wide distribution. In addition, HCV has a high rate of spontaneous mutation, producing discrete “quasispecies” that vary from one individual to the next (195). Genotypes 1a and 1b account for approximately two-thirds of HCV infections in the United States; genotype 1 represents 90–95% of infections in African Americans compared with ~60% in white patients (196, 197). Genomic amplification and sequencing followed by sequence comparison and phylogenetic tree construction is the reference method for genotype determination (198). A variety of genotype screening assays have been described, including PCR using genotype-specific primers (199–201), restriction fragment length polymorphism of amplified sequences (199, 202, 203), and a commercially available line probe assay (204–206). These methods compare

favorably with the commonly used reference methods for determining HCV genotypes (207).

Recommendation:

- Genotype assays should reliably differentiate all six major genotypes and distinguish genotype 1a from 1b (IIIB and IIIE).

HDV. HDV is a defective virus that replicates only in the presence of acute or chronic HBV infection; it requires HBV for maturation. Testing for evidence of HDV infection should be considered in HBsAg-positive patients with symptoms of acute or chronic hepatitis, particularly in those with fulminant hepatitis or where there is a high risk for HDV infection (such as in injection drug abusers). The only HDV serologic tests widely available commercially detect total anti-HDV. In patients in whom virus is cleared, antibody typically disappears between 1 and 5 years (208). In most clinical situations, HBsAg, IgM anti-HBc, and total anti-HDV are adequate to diagnose HDV infection. Patients with acute HDV co-infection usually are positive for IgM anti-HBc, whereas patients with HDV superinfection usually are negative for IgM anti-HBc. There is inadequate information on performance of HDV tests to make performance recommendations.

HEV. HEV is an enterically transmitted virus that causes sporadic and epidemic acute hepatitis in the developing countries of the world; it does not cause chronic hepatitis. In the United States, HEV infections have been seen rarely as a cause of hepatitis, predominantly among those who have traveled to endemic areas, although at least one case has occurred without a history of travel (209). Immunoassays have been developed for diagnostic use (210). An evaluation of multiple methods for detecting anti-HEV antibodies showed significant variation in titers reported and discordance between methods, although tests detecting antibodies to ORF2 were most accurate (211). Antibodies reactive with HEV antigens were found in 15–25% of homosexual men, intravenous drug users, and blood donors in Baltimore, MD, suggesting lack of specificity of assays (212); antibody to HEV antigens is found commonly in rats and pigs in the United States (213, 214).

Recommendation:

- Assays for HEV antibodies should detect antibodies to the ORF2 antigen to assure adequate clinical specificity (IIIB and IIIE).

Development and publication of these guidelines was supported by grants from Abbot Diagnostics; Diasorin, Inc.; Bayer Diagnostics (formerly Chiron Diagnostics); Innogenetics, Inc.; and Ortho Clinical Diagnostics. The following individuals reviewed the guidelines at various stages of their development and offered helpful com-

ments and modifications: Miriam Alter, Henry C. Bodenheimer, Thomas D. Boyer, Max A. Chernesky, Gary L. Davis, Jean C. Edmond, Stuart C. Gordon, Norman D. Grace, F. Blaine Hollinger, Donald M. Jensen, Lawrence A. Kaplan, Jacob Korula, Karen Lindsay, Brian J. McMahon, Jan M. Novak, Melissa Palmer, Eve A. Roberts, James R. Spivey, Thomas A. Shaw-Stiffel, and Myron Warshaw. Specific comments were provided by the following individuals during open discussion at the AACC Annual Meeting: Ed Ashwood, Bill Brock, Thomas Burgess, Jack Goldberg, Ajit Golwkar, Neal Greenberg, Michael Heinz, Richard Horowitz, Graham Johns, Ronald Lee, Steve Lobell, Greg Post, Phil Rosenthal, Norbert Tietz, Mark Walter, Earl Weissman, William Winter, and Jeffery Young.

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