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Sources of variation analysis and derivation of reference intervals for ALP, LDH, and amylase isozymes using sera from the Asian multicenter study on reference values

Yoshihisa Shimizu, Kiyoshi Ichihara *, on behalf of the Asia-Pacific Federation of Clinical Biochemistry

Department of Laboratory Sciences, Faculty of Health Sciences, Yamaguchi University Graduate School of Medicine, Ube, Japan

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ABSTRACT

Background: Sources of variation (SV) of ALP, LDH, and amylase isozymes were explored.
Methods: We analyzed 3511 sera from well-defined healthy individuals recruited during the 2009 Asian project for derivation of common reference intervals (RIs). Up-to-date electrophoresis auto-analyzer and reagents were employed for high resolution and reproducibility. SVs including sex, age, body mass index (BMI), ABO blood groups, and levels of drinking, smoking, and exercise were analyzed by multiple regression analysis. RIs were determined by parametric methods after refining healthy individuals by use of latent reference values exclusion method.
Results: Age-related changes in ALP2–3 were different in females: ALP2, linear increase from 20–64 y; ALP3, lowering until 45 y and rising steeply thereafter. ALP2 increased with BMI especially in females. ALP5 was barely detectable except in blood-types O and B. Age-related increases in LDH1–LDH3 were noted in females, whereas BMI-related increases were found only for LDH2–LDH5 in both sexes. Pancreatic amylase showed age-related increase in females and was slightly higher in blood-type O. RIs for absolute and relative activities of each isozyme were derived in consideration of sex and age.
Conclusions: Investigation of these isozymes revealed various age-, BMI-, and blood-type-related changes that are all relevant in clinical interpretation of enzyme test results.

1. Introduction

Most of the enzymes commonly analyzed in laboratory tests comprise different isozymes that show characteristic tissue distributions. Therefore, the altered isozyme profile provides an important clue to the etiology of pathological conditions. The most commonly tested and clinically important isozymes are those of lactate dehydrogenase (LDH), alkaline phosphatase (ALP), and amylase (AMY). As for LDH isozymes, characteristic changes are observed in myocardial infarction and hemolytic states (↑LDH1); in liver or skeletal muscle damage (↑LDH4, 5); in hematological malignancy or in advanced stages of any malignancy (↑LDH2, 3) [1,2]. Isozymes for ALP in healthy adult consist of liver-derived ALP2, bone-derived ALP3, and intestine-derived ALP5, while ALP1 from liver is elevated only under cholestatic conditions; ALP4 from placenta is elevated during pregnancy. These characteristic changes in ALP isozymes are of great relevance for differential diagnosis of liver, bone and intestinal disorders [3]. Meanwhile, AMY is composed of isozymes from pancreas (pAMY) and various tissues such as salivary gland and lung (sAMY). Although pAMY is specific to the pancreas and dependent on renal clearance, the etiology of elevated sAMY is diverse including acute parotitis, and failure in circulation [4].

For diagnostic interpretation of altered isozyme profile, before considering these pathological conditions, it is of great importance to exclude biological sources of variation (SV), such as age, sex, and body mass index (BMI). However, these SVs have not been well characterized. In fact, most previous studies characterizing the isozymes have limitations due to the imprecision of their assays and insufficient sample sizes used for study [1–11]. Further, assay standardization of these enzymes had not been achieved at the time of the previous studies.

In the Southeast Asian multicenter study on collaborative derivation of reference intervals (RIs) conducted in 2009, 3541 well-defined healthy individuals from 7 different countries were recruited [12,13]. We have performed isozyme analysis for ALP, LDH, and AMY for the

Abbreviations: BG, blood group; DrkLvl, level of drinking; HCMC, Ho Chi Minh City; HK, Hong Kong; KL, Kuala Lumpur; MRA, multiple regression analysis; pAMY, pancreatic AMY; sAMY, salivary AMY; SDR, standard deviation ratio; SmkLvl, level of cigarette smoking; stdβ, standardized partial regression coefficient; SV, sources of variation; tALP, total enzyme activity for ALP; tAMY, total AMY activity; tLDH, total enzyme activity for LDH; 3N-ANOVA, three-level nested ANOVA.

* Corresponding author at: Department of Clinical Laboratory Sciences, Faculty of Health Sciences, Yamaguchi University Graduate School of Medicine, Minami-Kogushi 1-1-1, Ube 755-8505, Japan. Tel.: +81 836 22 2884; fax: +81 836 35 5213.
E-mail address: ichihara@yamaguchi-u.ac.jp (K. Ichihara).

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entire set of specimens. We used an up-to-date, efficient, automatic electrophoresis analyzer, which allows high throughput processing and much improved precision. Separation of the peaks has become clearer especially for ALP through preprocessing of specimens using neuraminidase.

We performed multiple regression analysis (MRA) to explore sources of variation (SV) of the activities of both the total and individual isozymes, especially with regard to regionality, sex, age, BMI, ABO blood groups, and levels of daily alcohol consumption, smoking, and regular exercise based on a health-status questionnaire. We also derived RIs for absolute and relative activities of each isozyme in consideration of sex and age.

2. Materials and methods

2.1. Specimens obtained from the Asian study

The selection criteria for volunteers and the recruitment, specimen processing, and assays method are described in detail elsewhere [11,12]. The target population, study protocol, and assay methods for the total activities of ALP, LDH, and AMY are briefly described as follows.

A total of 3541 healthy subjects were recruited based on the criteria shown below. However, the actual number of specimens available after testing for the main analytes was 3511 due to insufficient sample volumes. They comprised 2055 specimens from 13 areas throughout Japan and 1456 from 7 Asian cities: Seoul, Beijing, Taiwan (Taipei, Tainan), Hong Kong (HK) including Macau, Kuala Lumpur (KL), Ho Chi Minh City (HCMC), and Jakarta.

Exclusion criteria were (1) BMI ≥ 28, (2) ethanol consumption ≥ 75 g/day, (3) smoking > 20 cigarettes/day, (4) taking regular medication, (5) ≤2 weeks after recovery from acute illness or surgery requiring hospitalization, and (6) pregnancy or within 1 y postpartum.

With the subjects in the full basal condition described in the protocol, blood was drawn into a vacuum tube containing clot activator and centrifuged within 1 h after clotting. Serum samples were then aliquoted into Nunc® CryoTubes® (Thermo Scientific, Denmark) and deep-frozen at −80 °C until the time of assay. This study was approved by the Ethical Review Board of the Faculty of Health Sciences, Yamaguchi University Graduate School of Medicine (2008-4). All volunteers who donated their serum specimens signed informed consent forms.

2.2. Analytical methods

2.2.1. Total activities for ALP, AMY, and LDH

Total enzyme activities for ALP (tALP) and LDH (tLDH) were measured by the IFCC-recommended method [12] by use of a UniCel DxC 800 auto-analyzer (Beckman Coulter), whereas total AMY activity (tAMY) was measured by use of the JSCC method using blocked-p-nitrophenyl-α-maltopentaoside as its substrate. The reagent for tAMY had been standardized in reference to Japanese certified enzyme reference material JCCLS CRM-001b [14]. Within-day and between-day coefficients of variation (CVs) for total enzyme activities were 1.91% and 2.60% for tALP, 1.42% and 0.29% for tLDH, and 0.89% and 0.61% for tAMY, respectively.

2.2.2. Isozyme analyses

For all of the isozyme analyses, we used an automatic electrophoresis analyzer (Epalyzer-2; Helena Laboratories, Co., Ltd.) which features a ready-to-use prepackaged kit called the QuickGel plate (Helena) that contains an agarose gel bed pre-absorbed with buffer, which are optimized for each enzyme. Ten to 20 specimens can be run in parallel simultaneously by use of the single plate.

2.2.2.1. ALP isozymes. The system requires pretreatment of specimens with proteinase to convert the high-molecular form of ALP-5 into the small-molecular form. In addition, it uses neuraminidase (derived from *Vibrio cholerae*) for better separation of ALP-2 and ALP-3. However, neuraminidase degrades other ALP enzymes. Therefore, each serum specimen was divided into two 30-μL aliquots: one to be treated just with proteinase and the other to be treated with both proteinase and neuraminidase. This pretreatment with both enzyme solutions was done at room temperature for 30 min. Subsequently, electrophoresis was performed by use of a QuickGel ALP kit (Helena) after automatic application of 10 specimens (1 μL each) for 23 min under constant voltage of 230 V.

After completion of the electrophoresis, the QuickGel plate was stained with nitrotetrazolium blue for 24 min at 45 °C, washed with 1.25% methanol solution, added with a reagent for cessation of the dye reaction, and re-washed for 10 min. Subsequently, the plate was dried, and the image of isozyme peaks was analyzed by a scanner.

2.2.2.2. AMY isozymes. For isozyme analysis of AMY, a QuickGel AMY plate (Helena) was used in which 1 μl of serum was automatically applied to the gel plate and electrophoresed at 320 V for 40 min. Soon after the run, a solution of maltopentaose [15], a newly developed substrate with the property of color development by AMY, was poured onto the plate for staining at 45 °C for 15 min. The plate was washed by use of 2.0% acetic acid in water, and then the isozyme patterns on the plate were analyzed by the scanner.

2.2.2.3. LDH isozymes. LDH isozyme analysis was performed by use of a QuickGel LD plate and Titan gel S-LD, a staining reagent (both, Helena). One microliter of serum was automatically applied to the plate and electrophoresed at 250 V for 13 min. After the run, DL-lactic acid lithium was added for the formation of pyruvate. Staining with nitrotetrazolium blue was done at 40 °C for 14 min. The plate was washed by use of 2.0% acetic acid solution, and the isozyme peaks were then analyzed by the scanner.

2.3. Quality control sample handling and measurement

To monitor stability of the isozyme assays, 50 ml of sera freshly taken from two healthy individuals were aliquoted into 100 polystyrene sample containers of 0.5-ml capacity. All of the specimens were deep frozen at −80 °C until the time of assay for quality control monitoring of every 100 serum specimens. In addition to this practice, we also monitored quality control specimens of ALP and LDH isozymes with Gel ALP Isoenzyme Control and CK/LD Isoenzyme Control (Helena), again at the interval of every 100 specimens.

2.4. Statistical analysis

2.4.1. MRA

MRA was performed for each sex separately to explore the following SVs: country (city); age; BMI; level of drinking (DrkLvl), cigarette smoking (SmkLvl), and regular exercise; and ABO blood group. The activity of each enzyme or isozyme was set as an object variable, and a group of SVs were set as explanatory variables. The effect of the difference in countries (cities) was analyzed by introducing dummy variables by setting Japan as the reference category. Therefore, 7 dummy variables for Seoul, Beijing, Taiwan, HK, HCMC, KL, and Jakarta were introduced. ABO blood group (BG) type A was set as the reference category, and therefore dummy variables for type AB (BG-AB), type B (BG-B), and type O (BG-O) were introduced. Hence, a regression model composed of 14 exploratory variables was always used irrespective of the enzyme or isozymes concerned.

The magnitude of association between the object and each explanatory variable was expressed as the standardized partial regression