Biochemistry & Analytical Biochemistry
ISSN: 2161-1009

Kainyu, et al., Biochem Anal Biochem 2018, 7:1
DOI: 10.4172/2161-1009.1000349

Research Article Open Access

Establishment of Reference Ranges for Liver Biochemistry Tests in Children in Meru County, Kenya

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Received date: January 03, 2018; **Accepted date**: January 25, 2018; **Published date**: February 05, 2018

Abstract

This study was aimed at determining the reference range values for eight liver function parameters that are routinely analyzed in the clinical chemistry laboratory of Meru Level 5 Hospital. The study was cross-sectional, population-based and carried out on the young population of ages one to seventeen years in Meru County, Kenya. A total of 768 samples were collected from the volunteers who participated in the study. Out of these, 740, comprising 360 females and 380 males that were found to be free from HIV, Hepatitis B and syphilis were used to construct the reference ranges. DRI - CHEM NX 500I Clinical Chemistry analyzer (Fujifilm, Europe) was used to analyze eight biochemical parameters. Determination of reference ranges was done in order to estimate the lower 2.5 and upper 97.5 percentiles of the distribution by use of parametric methods. The determined percentiles were considered the lower and upper reference limits respectively. Significant sex differences were observed in children reference values for total protein. Other parameters (alkaline phosphatase, gamma glutamyl transferase, direct bilirubin, total bilirubin, albumin, alanine aminotransferase, aspartate aminotransferase) did not show significant sex dependent differences. In conclusion, the findings of this study provide sex-specific reference range values for children from Meru County in Kenya. The study recommends the health care practitioners and facilities in the region under study to adopt the new reference values developed and for other regions in Kenya to carry out a similar study to determine their own reference values.

Keywords: Reference ranges; Liver; Sex

Introduction

Reference value refers to the value or test result obtained through observation or measurement of a particular type of analyte on an adequate number of individuals selected as representatives of the general population. The reference range is normally set as mean ± 2 standard deviations and encompasses 95% of the presumably healthy group of population studied. Clinicians order laboratory tests for a variety of reasons: screening for disease, diagnosis of disease, monitoring nous substances like electrolytes, determining prognosis, confirming a previous abnormal test, clinician education and medical legal purposes. When a test is used for disease screening, diagnosis or prognosis, the test result is normally compared with a normal range that is defined as usual value for a healthy population [1]. Clinical medicine practice requires that laboratory test results from a patient are compared against some pre-determined standard results so as to determine whether the patient is "normal" or is suffering from a certain pathological condition. Laboratories should therefore report test results along with the corresponding reference intervals since physicians and other health practitioners make their medical decisions based on available, appropriate and reliable reference intervals. Medical decision is also backed by the information gathered during medical interview as well as clinical examination. In the laboratory, the word "normal" has several meanings other than being used to describe the usual range of laboratory data for healthy populations. It is used to describe the health of individuals and is also synonymously used with the "Gaussian" when the shapes of distributions are described.

Factors specified when reference values are established include: (1) Make up of reference population in terms of age, gender and genetic and socio-economic factors. (2) The inclusion and exclusion criteria used. (3) The conditions, both physical and physiological under which the reference population is sampled and studied. (4) The procedure of collecting the specimen, including how the subject was prepared before collection and (5) the method of analyzing the sample used giving details of its precision and accuracy [2]. Since measured biochemical parameters are affected not only by individuals' factors such as age, sex, diet but also by population and ecological factors such as ethnic background, climate, geography and altitude, they are found to vary not just between individuals but between populations as well [3]. Presently most clinical laboratories in Meru County as well as the whole republic of Kenya use reference values as indicated on reagent kits or those that are published in medical and laboratory textbooks to interpret patient results. This can be of a grave mistake bearing in mind that parameters vary from region to region [4]. The patient's results can also vary due to the methods used in analyzing, that is either manual or by automation. Thus, it is important that clinical chemistry laboratories determine reference values that are specific to the populations they serve. To ensure the results are accurate and precise, there is need to establish reference values following the standard operating procedures (SOP) [5].

This study is designed to establish health associated reference ranges to be used by clinical chemistry laboratories in the area of study and determine whether there are significant variations between them and those used in the facilities as provided by the reagent manufacturers.

Materials and Methods

Selection of reference population

The reference population was selected based on the guidelines described by the CLSI, 2000. According to the guidelines, the reference individuals selected should be closely similar to the patient population under study and should be of the same age to be clinically significant. A priori sampling method was employed in this study. This is where selection of an adequate number of subjects who serve as reference individuals takes place first and then samples are drawn for analysis. Children between 1-17 years of age were randomly selected after holding community meetings with the leaders and parents/guardians to explain the objectives of the study.

Study design

This was a population based cross-sectional study involving 740 healthy male and female subjects of age 1 to 17 years.

Specimen collection

5 ml of blood was collected by venipuncture using a 23 gauge butterfly needle with a 5 ml syringe after sterilizing the area with 70% alcohol and dispensed into a plain vacutainer (without anticoagulant) tube then transferred in 2 ml tubes (vacutainer TM, Becton Dickinson, Franklin Lakes, NJ). Each of the tubes containing the specimens was labeled clearly with the subject's name, the study number and the date of collection of the sample. The specimens were arranged in Styrofoam cool boxes at 4°C and covered to protect them from heat and sunlight, awaiting transportation to the main analytical center.

Specimen transportation, processing and storage

The specimens collected in the field were ferried from the point of collection to the clinical chemistry laboratory of Meru Level Five Hospital in cool boxes within two hours of collection at room temperature. Upon arrival at the clinical chemistry laboratory, the blood specimens were centrifuged at a speed of 3000 rpm for two minutes to obtain serum. The serum was transferred to separate tubes labeled with subject's identification details. Laboratory analysis was done as soon as possible to avoid loss of sample viability. If analysis was not done immediately, the samples were stored at -20°C for a period not exceeding seven days.

Laboratory analysis

Serum samples were initially screened for human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2) by using HIV 1/2 Stat-Pak®, Chembio Diagnostic Systems that detects anti-HIV antibodies in human serum. HBsAg one step hepatitis B Surface Antigen Test Strip (HBsAg, Beijing, China) was used for screening hepatitis by a qualitative lateral flow immunoassay test. Screening for syphilis antibodies was by a syphilis ultra-rapid test using one step anti-Treponema pallidum test strip (Anti-TP Test Strip, China). Eight liver biochemistry tests were carried out on the serum samples: total protein (TP), albumin (ALB), alanine aminotransferase (ALT), direct bilirubin (DBIL), alkaline phosphatase (ALP), gamma glutamyl transferase (GGT), aspartate aminotransferase (AST) and total bilirubin (TBIL). All the assays were carried out as per the standard operating procedures (SOPs) followed at the hospital laboratory using DRI-CHEM NX500i dry chemistry clinical analyzer (Fujifilm, Europe).

Reagent preparation

The machine uses coded slides that are commercially acquired and specifically tailored for the equipment (Fuji DriChem slides). Each slide is impregnated with the reagent for a specific parameter and is labeled clearly. The slides for the various tests being carried out are inserted in the machine along with the sample and once the patient details are entered and the machine set to start, the tests run automatically and a print-out of the results is obtained.

Calibration of the test

Calibration was done using a magnetic card called QC card that comes with every reagent box. The card is passed through the QC card reader whenever slides from a new lot number are being used.

Quality control (QC) materials

To ensure accuracy and precision of the test results, all pre-analytical, analytical and post analytical precautions were taken into consideration. Internal QC materials from

Roche diagnostics; Precinorm and Precipath, were run daily or at any other time as deemed necessary. External QC materials were from BioRad, (Lyphochek® Unasssayed Chemistry Quality Control (USA). The multisera were supplied in lyophilized form and were reconstituted when being used as per the manufacturer's guidelines.

Data Management and Statistical Analysis

Data was categorized by sex and age as appropriate and each parameter was then examined as a histogram and as a normal probability plot. Kolmogorov-Smirnov Test was performed with significance at p=0.05 level to test fit of the data to Gaussian distribution. Since the results of the Kolmogorov-Smirnov test were not significant ($p>0.05$), the data was found to assume a normal distribution and was further subjected to parametric statistical methods. The lower and upper reference interval limits for each parameter were calculated from the arithmetic mean (X) ± 1.96 times standard deviation (X ± 1.96 SD) to obtain the 2.5 and 97.5% percentiles. Students T-test was used for comparison of means between sexes. The tests were conducted at 95% confidence interval and significance level of 5%. P values of less than or equal to 0.05 ($p \leq 0.05$) were considered statistically significant. Age and sex differences were tested using the Wilcoxon rank-sum test. The performance of analytical instruments and methods to analyze the levels of the selected analytes were achieved by using the paired T test. Quality control was observed throughout the study to make sure that all the results were in the recommended ranges of reporting. This was achieved by use of normal and pathological (PNU and PPU) pre-determined values respectively.

Results

Sex-specific reference values for ALT, AST, ALP, GGT, ALB, TBIL, TP and DBIL

From the study, it was noted that reference values that are used in management of children differ with those that were established,

therefore indicating that there is need to have each laboratory determine reference values that are specific to its population as required by the CLSI. 740 samples were analyzed in this study out of the 768 initially collected for the study. 28 samples were excluded because 6 (21.4%) of them were HIV positive and 22 (78.6%) were hemolyzed. Reference values for eight biochemical parameters were established for both males and females of age 1 to 17 years. Construction of these reference values was by use of the 2.5th and 97.5th percentiles as lower and upper limits respectively and at 95% confidence interval. This is in accordance with CLSI guidelines for reference interval determination (NCCLS 2000) [3].

Analyte (unit)	Sex	N	Mean	Percentiles		Reference Value	RI	Difference between M&F	
				2.5th	97.5th	F value		F Value	Sig
ALB (g/L)	M	380	38.47	27.98	48.96	27.98-48.96	20.98	0.120	0.949
	F	360	38.56	28.62	48.50	28.62-48.50	19.88		
	M&F	740	38.51	28.30	48.72	28.30-48.72	20.42		
AST (U/L)	M	380	32.07	10.16	54.30	10.16-54.30	44.14	0.167	0.919
	F	360	32.39	5.65	58.93	5.65-58.93	53.28		
	M&F	740	32.20	9.92	54.60	9.92-54.60	44.68		
ALP (U/L)	M	380	87.97	61.63	114.31	61.63-114.31	52.68	0.461	0.709
	F	360	87.97	58.21	114.23	58.21-114.23	56.02		
	M&F	740	87.48	61.63	114.31	61.63-114.31	52.68		
ALT (U/L)	M	380	32.23	11.18	57.20	11.18-57.20	46.02	0.896	0.443
	F	360	32.29	10.24	56.84	10.24-56.84	46.6		
	M&F	740	33.11	10.75	57.80	10.75-57.80	47.05		
D-BIL (µmol/L)	M	380	2.13	0.43	3.53	0.43-3.53	3.1	2.640	0.152
	F	360	2.20	0.21	4.19	0.21-4.19	3.98		
	M&F	740	2.17	0.46	3.88	0.46-3.88	3.42		
T-BIL (µmol/L)	M	380	42.17	12.98	71.78	12.98-71.78	58.8	0.158	0.924
	F	360	42.82	12.19	73.45	12.19-73.45	61.26		
	M&F	740	42.48	12.6	72.56	12.6-72.56	59.96		
TP (g/L)	M	380	43.25	30.78	55.72	30.78-55.72	24.94	2.809	0.039*
	F	360	42.26	29.95	54.57	29.95-54.57	24.62		
	M&F	740	42.77	30.38	55.18	30.38-55.18	24.8		
GGT (U/L)	M	380	73.58	18.82	128.34	18.82-128.34	109.52	0.399	0.753
	F	360	68.10	8.44	127.76	8.44-127.76	119.32		
	M&F	740	70.92	13.51	128.33	13.51-128.33	114.82		

Results are expressed as mean values for the number of subjects in column N. *Significant sex difference where p<0.05. The difference in sex is significant at p<0.05; Sig: Significance; RI: Reference Interval.

Table 1: Reference values by Sex for ALB, AST, ALP, ALT, D-BIL, T-BIL, TP and GGT.

Table 1 shows sex-specific reference values for every parameter depending on the p-values obtained from the difference between male and female subjects. Significant sex differences were observed in TP (p=0.039). In the course of the study, control value results as well as the standard deviation (SD) from the value were recorded every day. Table 2 compares reference range values established with those found in the literature. This was done by comparing the values for the lower and upper reference limits as well as the interval values for each analyte. From the study, it was noted that significant differences exist between the reference ranges developed and those in use at the hospital. Out of the eight parameters studied, five of them did not have distinct values for adults and children and the same reference values were found to be used when interpreting test results for both children and adults. ALT, AST and ALB had both adult and children values but these were found

to be significantly different from those established in the study. All the parameters studied except ALB portrayed differences in the reference range values obtained. ALT, AST, ALP, GGT and T-BIL all showed higher lower reference range limits than those of the manufacturers. ALT and AST had higher upper reference range limits as compared to those of manufacturers. The lower reference range limits of the enzymes ALT and AST were observed to be as high as ten times more than the manufacturer's. The enzyme ALP showed a significantly higher lower limit and a significantly lower upper limit. Thus, the reference interval for ALP was significantly shorter compared to that of manufacturer's. This phenomenon was also observed in GGT.

Parameter/Unit	Established Reference Intervals					Literature Reference Values	
	Male	Female	Male and Female			Mean values	RI
			Lower limit	Upper limit	Reference Interval		
ALB (g/L)	27.98-48.96	28.62-48.50	28.30	48.72	20.42	30-48	18
ALP (U/L)	61.63-114.31	58.21-114.23	61.63	114.31	52.68	47-406	359
ALT (U/L)	11.18-57.20	10.24-56.84	10.75	57.80	47.05	0-50	50
AST (U/L)	10.16-54.30	5.65-58.93	9.92	54.60	44.68	0-50	50
D-BIL (µmol/L)	0.43-3.53	0.21-4.19	0.46	3.88	3.42	≤ 3.4	3.4
T-BIL (µmol/L)	12.98-71.78	12.19-73.45	12.60	72.56	59.96	1.7-21	19.3
GGT(U/L)	18.82-128.34	8.44-127.76	13.51	128.33	114.82	1.0-132	131
TP (g/L)	30.78-55.72	29.95-54.57	30.38	55.18	24.8	33-56	23

Table 2: Comparison of Established Biochemical Parameters of children 1-17 years with those in literature.

Discussion

The results of this study provide the pioneer clinical chemistry reference ranges for the population of 1 to 17 years in Meru County, Kenya using 740 samples, 380 males and 360 females. The number of participants in each category was more than the minimum number of 120 participants per subgroup required to determine reference ranges as recommended by CLSI 2000. The tests were done using the same analytical methods and results expressed in the same units as those found in literature for easy comparisons. External and internal quality control methods were closely followed and monitored throughout the study [6] in addition to following the SOP at Meru Level Five Hospital.

In the study, it was established that there exists no reference ranges in Meru County for children and adolescents of 1-17 years for five out of the eight parameters studied. During clinical trials, this group of the population was found to be considered together with the adult population. Out of the eight parameters studied only three were found to have reference ranges specific to children, though the ages covered by the reference values used are not defined. These parameters include ALT, AST and ALB. There was no reference range values available for T-BIL, D-BIL, ALP, GGT and TP therefore the reference values developed in this study were compared against those for the adult population available. Assuming that adults and children exhibit the same reference ranges is a big mistake since reference ranges are known to vary with age among many other factors. Most studies carried out in Africa show reference intervals for direct bilirubin and albumin that are similar with those published from populations derived from the United States [7]. However, certain parameters have upper reference limits that are significantly higher. A good example of such a parameter is total bilirubin; T-BIL (manufacturer's: 1.7-2.1 µmol/L; established: 12.6-72.56 µmol/L). The reason for these high T-BIL levels in the African populations may be due to RBC hemolysis as a result of malnutrition, physical exertion or malaria infection, conditions that are pre dominant in sub Saharan Africa. However, even within the African continent, disparities in theses parameters are observed because of large differences in climate, location, diet and human genetics [8].

Male adolescents had higher values for ALT, D-BIL, AST, TP and T-BIL than female adolescents. These differences in sex were particularly significant for T-BIL and TP. AST was found to have significant difference as the age of the child progressed. It is known that muscle mass affects AST, therefore the differences observed in this category of the population could be as a result of the production of this metabolite by male children's developed muscles. The sex difference observed in serum TP in this study contrasts the one found in literature where males and females have the same reference values but agrees with the study done in Rwanda for adult humans [6]. ALB, ALT, AST, T-BIL and D-BIL have demonstrated higher values compared to manufacturers' ranges. This could be due to differences in diet, genetics and analytical methods. Males had higher values of GGT than females; this could be as a result of extra production of the enzyme from the prostate gland in males as compared to females who lack the organ. This result was reported in studies carried out in other East African states [8,9]. ALT and AST have demonstrated higher values for both lower and upper reference values compared to those of manufacturer's range and other locations (ALT: 10.75-57.8 U/L; 0-50 U/L ;AST: 9.92-54.6 U/L; 0-50 U/L). Different lifestyles and genetic composition of the population could also explain the differences [10].

Generally, reference ranges have been shown to vary between different populations due to differences in genetics, physical, environmental and socio-economic conditions and diet [11,12]. The reference values for the parameters analyzed in this study differ from those used to service the population. This clearly indicates the necessity to determine sex- and age-based reference values for specific populations instead of taking a set of reference values determined for

one population and use them on another population. This will decrease the frequency of values reported as abnormal in otherwise healthy children and adolescents.

Conclusion

From the study it was observed that no reference ranges are available for the study population in Meru County, Kenya and physicians and other health workers rely on adult values available from some Caucasian populations to interpret laboratory results for this group of population. This is a grave mistake because serum biochemistry parameters are known to vary with age, thus adult values cannot adequately represent children reference ranges. The importance of serum biochemistry normal ranges in the diagnosis and monitoring of disease cannot be underestimated; therefore establishment and use of local reference ranges should be encouraged because it enhances patient care and health research. The results of the study show that reference values obtained vary with those from literature and those that are used at Meru Level Five Hospital. The upper limits of serum transaminases, bilirubin, total protein and albumin for the children sampled in this study were higher than those from the Caucasian children. Significant sex- specific differences were observed in TP ($p=0.039$). Most parameters like ALP had a much shorter reference range compared to that found for Caucasian population (Established: 61.63-114.31 U/L; Manufacturer's: 47-406 U/L). This clearly indicates the need to determine population specific ranges instead of using a general range developed using a different population. There was little or no information found in literature for this population thus comparisons were done with those of adult populations. This was also observed in the clinical setting for most of the biochemical parameters studied, with adult values being used to interpret the results for this group of the population.

Clearly, similar studies of children in Africa should be carried out so as to broaden the present findings thus enabling improved care and conduct of clinical trials. Population-specific reference ranges/values obtained will be useful to achieve accurate, clinically relevant results that will provide true information about the patient's state of health in the region.

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