Research Article

Rapid screening model for identifying patients with suspected intravascular hemolysis to improve patient care and reduce sample rejection rates in clinical chemistry

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The importance of developing a rapid screening model is critical for identifying patients with intravascular hemolysis to improve patient care and reduce sample rejection rates. Specimen hemolysis is a leading cause of spurious test results in clinical chemistry. Intravascular hemolysis releases cell-free hemoglobin into the serum and haptoglobin irreversibly binds hemoglobin to form a hemoglobin-haptoglobin complex, which is cleared in the circulation by the monocyte-macrophage scavenger receptor CD163 in the liver and spleen. The depleted serum haptoglobin in a hemolyzed sample is used to diagnose intravascular hemolysis. However, haptoglobin testing may take hours or days to confirm intravascular hemolysis. The aim of this study was to investigate the relationship between intravascular markers of hemolysis and haptoglobin in hemolyzed specimens. This study retrospectively mined archived data from the laboratory database between February 2017 and February 2018 from patients 6 months of age and above. The Statistical Package for Social Science was used for data analysis. The partial plot results showed a strong relationship between hemolysis markers and haptoglobin levels. Multiple regression models that predict serum haptoglobin levels in intravascularly hemolyzed samples were analyzed. Although the results showed a strong relationship between dependent and independent variables, the data did not demonstrate a clinically significant relationship or establish cause and effect. The use of a larger sample size along with adequate controls in preanalytical, analytical, biological and environmental variables would likely improve the clinical significance of the model. With proper modifications and validations, this model has the potential to provide a rough estimate of the haptoglobin levels and reduce cost as well as sample rejection rates. Due to the numerous hemolytic diseases, this model could be used to direct the clinicians to select the appropriate test for diagnosis of intravascular hemolysis.

Key words: haptoglobin, intravascular hemolysis, extravascular hemolysis, turnaround time, *in vivo* hemolysis, *in vitro* hemolysis, hemolysis markers.

Introduction

The importance of developing a rapid screening model is critical for identifying suspected patients with intravascular hemolysis (IVH). Hemolysis is defined as the breakdown of the red blood cell (RBC) membrane to release free hemoglobin (Hb) and its contents into the extracellular fluid/ plasma.[1] Hemolyzed specimens account for 40%-70% of rejected specimens in the clinical chemical and represent 3.3% of routine specimens received in the laboratory.[2] The hemolysis can be categorized into *in vivo* and *in vitro* hemolysis. hemolysis is caused by preanalytical factors such as blood collection technique, specimen handling and delivery, or specimen storage.[3-5] In contrast, *in vivo* hemolysis, is caused by pathological conditions such as biochemical, immunological mechanisms, physical, chemical, and/or infections inside the body.[6] Biochemically, *in vitro* hemolyzed specimens are characterized by red coloration of plasma or serum, high Hb, potassium (K), lactate dehydrogenase (LD), aspartate aminotransferase (AST), and normal haptoglobin (HP) and reticulocytes (retics).[7] In contrast, *in vivo* hemolyzed samples are

characterized by elevated Hb, Bili, (unconjugated bilirubin) retics, low HP, normal K and a lack of red coloration of the plasma/serum.[8,9]

In vivo hemolysis can be further classified into IVH and extravascular hemolysis (EVH) depending on the site of RBC destruction. While both hemolytic pathways can overlap and converge during the process, it is worth noting the key difference between the two. The RBC's bound by IgM and IgG are marked by the reticuloendothelial system (monocyte-macrophage) in the spleen and liver as cells are targeted for the IVH and EVH pathways, respectively.[10]

The IVH releases free Hb and RBC content into the circulation during excessive hemolysis while in EVH, the whole RBC is lysed inside the macrophage and no RBC content is released into the plasma.[11] During normal IVH, HP irreversibly binds all free Hb to form a HP-Hb complex. The HP-Hb complex binds to the transmembrane scavenger receptor CD163 on the monocytes and macrophage in the liver and spleen where the complex is destroyed.[12,13] This suggests that, in negligible IVH instances, the IVH pathway merges into the EVH pathway. However, during increased IVH, the HP clearance mechanism is overwhelmed by excess free Hb and becomes depleted. The depleted serum HP level in a hemolyzed sample is used to diagnose IVH.[14]

Without sufficient information on the clinical notes to indicate whether the sample was a difficult collection and the lack of available guidelines, it is understandable why there is heterogeneity across all laboratories regarding the proper management and handling of hemolyzed specimens.[15,16] The dilemma that laboratory staff frequently face is whether to reject the sample to avoid an erroneous lab result resulting in an error in the diagnosis or a rejection that may lead to delay and treatment of the patient during recollection. [17-19] Rejecting *in vitro* hemolyzed specimens may be appropriate because the hemolysis interferes with analytical processes and yields spurious results which can have a deleterious impact on the quality of patient care and the laboratory's reputation.[20]

However, the identification of hemolysis related to mechanisms *in vivo*, may be important and clinically relevant to adequate patient management and care.

It appears there is no single screening and definitive test that can identify IVH with high aacuracy.[21] The routine laboratory tests used to screen for patients suspected with IVH include complete blood count, peripheral blood smear revision, total and unconjugated bilirubin, LD, retic counts, HP, ferritin, and urinalysis.[22,23] The presumptive test often requires a Coombs' test, serological testing, enzymatic testing, osmotic fragility test, hemoglobin analysis, and genetic testing to rule out/in IVH.[24-26] This testing process can be costly, time consuming and often inconvenient to the patients.

The aim of this study is to investigate the relationship between the activities of markers for hemolysis (HM) and serum HP levels in hemolyzed samples with the goal of developing a model that rapidly screens and identifies a patient suspected of experiencing intravascular hemolysis. The HM used in this study include unconjugated bilirubin (Bili), C-reactive protein (CRP), AST, Retics, LD, and alkaline phosphatase (ALP), which are often elevated in intravascularly hemolyzed specimens. The model derived from the relationship between HM and HP would provide a rapid estimated HP level.

Materials and Methods

Ethical approval

The study was conducted in the Pathology Queensland Laboratory at the Queensland Children's Hospital, Queensland, Australia and was approved by the Children's Health Queensland Hospital and Health Services Human Research Ethics Committee (Approval No: HREC/17/QRCH/244). The Charles Sturt University Human Research Ethic Committee also approved (Protocol No: H18017). The consent to

Accepted: October 21, 2020

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use the data was given by data custodians, the Pathology Queensland and Public Health Act (CT_3098 & CT_2926***).***

Sample collection

The research retrospectively mined archived pathology data for tests performed between February 2017 and February 2018. The data were retrieved from patients aged over 6 months who visited or were admitted at 34 public hospitals serviced by Pathology Queensland Laboratory. The 6 months threshold was selected since HP level is detectable at six months of age.[27] The data came from patients aged from 4 to 93 with a mean age of 62, median = 65 and mode of 74. The database search input for hemolysis markers returned results of HP, Bili, CRP, AST, retics, LD, and ALP with a hemolysis index greater than two.

The first criterion for including the parameters into the study was, the analyte must be a hemolysis marker and part of a hemolytic disease screening/diagnostic test or often elevated in hemolyzed samples with a hemolysis index of 2 and above. The second selection criterion was the linear relationships between the independent variables and dependent variable (HP). The units of measurements for these parameters include: LD (U/L), HP (g/L), ALP (U/L), CRP (mg/L), retics (%), AST (U/L) and Bili (µmol/L). The data were downloaded and cleaned, transformed and processed as described by Pujari, 2001.[28] The data were de-identified; thus, no patient' identifiable information was retrieved. The analytes were measured in a continuous scale (scale ranging from 0 to over 100).

Data analysis

The statistical package for social sciences (SPSS) software was used to produce multiple linear regression coefficient[29] and correlation coefficient analyses between HP and each of the independent variables as described. A linear regression was chosen because it permits the assessment of the extent of each relationship among the independent variables and dependent variable.[30] The following regression equation was expected: $\hat{Y} = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_4X_4 + \beta_5X_5 + \beta_6X_6 + \varepsilon$. Where β_0 is y-intercept, $\hat{Y}$ is predicted serum HP level and $\beta_{1\text{-}6}$ are the slopes of Bili, LD, CRP, Retic, ALP and AST, respectively. The R^2 and adjusted R^2 were reported and used to determine the level of variance in the HP that is explained by the Bili, LD, CRP, retic, ALP and AST as described.[31]

The *t*-test and F-test determined the significance of the predictors while the beta coefficients determined the magnitude and direction of the relationship.[32] F-test was the preferred tool to test the strength of the impact of LD, ALP, CRP, Retics, AST and Bili results on HP values.

For statistically significant models, for every 1 unit increase in the independent variable, the dependent variable increases or decreases by the number of unstandardized beta coefficients. The assumptions of linearity and homoscedasticity were assessed by examining the partial plots. The multiple regression equation derived from this study can be used to: (a) predict new values for the serum HP when the independent variables results are known; and (b) determine the variation in the HP explained by the independent variable.

Results

The study examined 175 archived pathology data. The preliminary review resulted in excluding 11 data with very high values and leaving 164 data (87 males, 77 females). The data was from patients aged 4- 93 years old. The patients mean age was 62, mode = 65 and median = 74. The results came from 34 different public hospitals spread across the state. The criteria used to exclude a patient data set was based on the distance away from the statistical mean, mode, median that fell out of three standard deviations (3SD). After removal of these outliers, the statistical analysis was completed. The data was then screened to meet multiple regression assumptions. Dependent and independent variables were measured on a continuous scale (from 0 to over 100). The sample residuals were independent from the sample mean model. There were linear partial correlations between each of the predictors and the HP level. The study shows presence of homoscedasticity of residuals around the model and no evidence of multicollinearity among independent variables. There were no significant outliers, high leverage points or highly influential points in the data as attested by Cook's distances, tolerance, and variance inflation factors (VIF) values. The errors (residuals) around the mean model were normally distributed.

Hemolysis markers and haptoglobin

The study investigated the presence of multicollinearity among the independent variables. Visual inspection of the collinearity statistics shows that none of the independent variables have correlation values greater than 0.7, which is the minimum threshold required to prove the presence

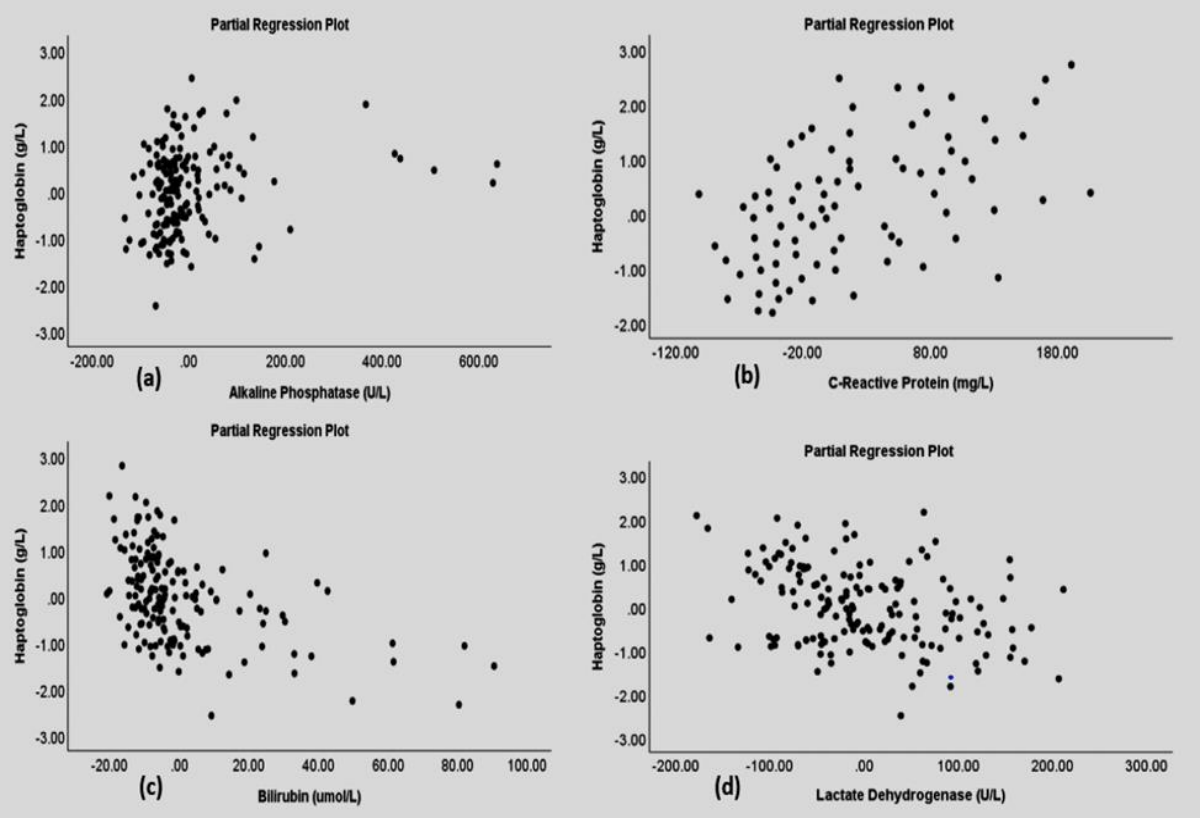

Figure 1: Haptoglobin Correlation Studies

of multicollinearity. The criteria to rule out multicollinearity is tolerance (>0.1) and VIF (<10).

The SPSS Statistics produced tolerance values greater than 0.1 (the lowest is 0.919) and VIF values are <2. The following partial correlation depicted a linear relationship between HP and CRP, Bili, LD and ALP (Figure 1. (a), (b), (c) and (d)). The partial correlation plots showed a strong relationship between HP and ALP, CRP, Bili, and LD, respectively. ALP and CRP have strong positive relationship with HP (Figure 1. (a) & (b). The ALP surges as HP levels increase. The serum elevation of Bili and LD contributed a negative impact on serum HP level (Figure 1. (c) and (d).

Normality test of sample residuals

To determine statistical significance of the model, the study used two methods: histogram with superimposed normal curve and P-P plot. The histogram with superimposed normal distribution showed the standardized residuals to be more-or-less distributed normally around the mean (Figure 2) the Distribution of Residuals Around the Mean Model. The mean of the model is on point zero on the histogram. The residuals are within ±3 standard deviation (SD). Any value outside the ±3SD were excluded from the model. In Figure (2.b), the P-P plot depicted the behaviors of residuals along the line of best fit. The observed and expected cumulative probability of residuals are lined up on a straight line, which shows that residuals are normally distributed along the mean model (Figure 2.b).

Hypothesis testing

The null hypothesis (H_0) states that the elevated retic, CRP, Bili, ALP, LD, and AST level in hemolyzed samples have no effect on the serum HP level. Low serum HP level in hemolyzed specimens may be due to a random effect and this implies that the relationship between HP and each of the independent variables is equal to zero; H_0: $\beta_1=\beta_2=\beta_3=\beta_4=0$, where β is the slope of the line of best fit. The alternative hypothesis argued that at least one of the slopes is greater than zero i.e. H_0: $\beta_1 = \beta_2 = \beta_3 = \beta_4 \neq 0$. In this study, the standardized beta coefficients are: CRP = 0.511, Bili = 0.407, LD = 0.274 and ALP = 0.165. The study set the level of significance at $\alpha = 0.05$; the mean critical value of 0.05 is 1.65. The *t*-statistic results for CRP = 7.875, Bili = 6.925, LD = 3.837 and ALP = 2.432, demonstrated that these critical values are greater than 1.65. The null hypothesis was rejected at a critical value greater than 1.65. The SPSS generated four models numbered 1 to 4. The model 1 is based on a relationship between a CRP and HP. This model has lower *R*2, adjusted *R*2 and high standard error of estimate (table 1).

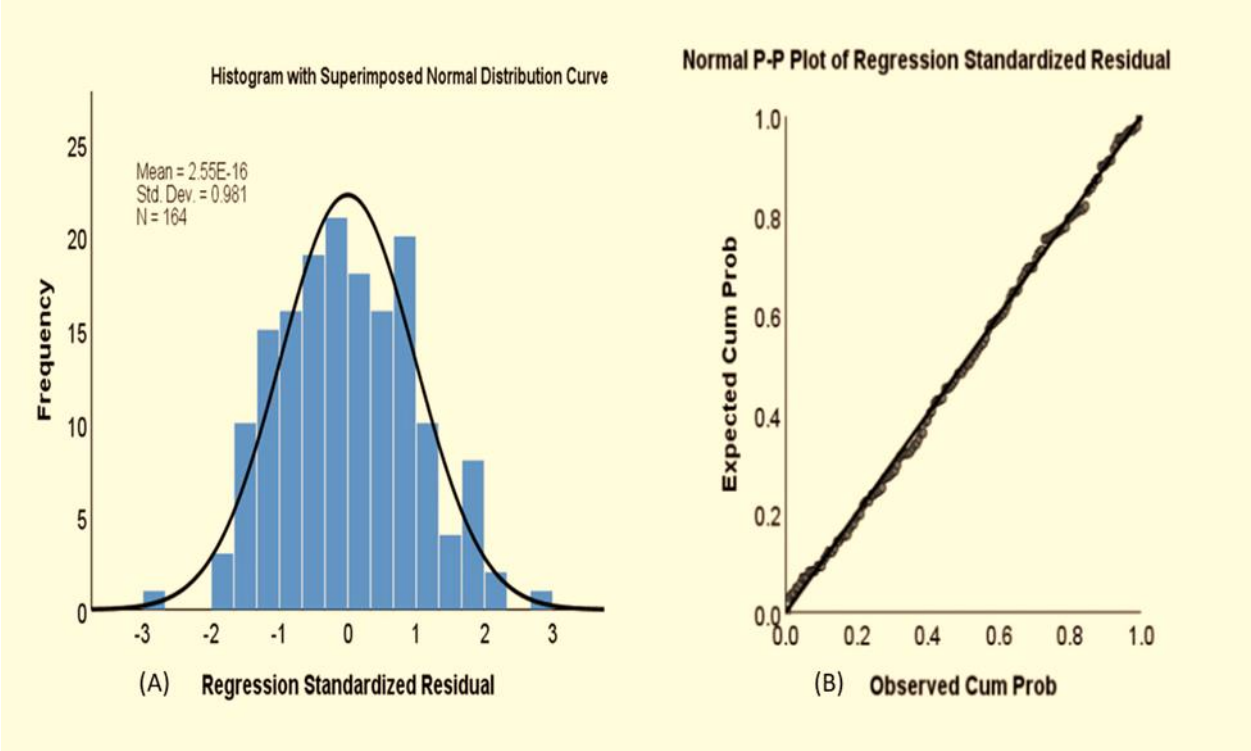

Figure 2: Test for the normality of sample residuals. (a) Histogram Depicting the distribution of residuals around mean model. (b) shows residuals lining along the line of best fit. Both demonstrated that the residuals are normally distributed.

In model 2, Bili was added to CRP and the effect on HP prediction was observed. Model 3 has CRP, Bili, and LD added as predictors. As more independent variables were added to model 1 and their effects on model 3 are observed, prediction of serum HP improved. CRP, Bili, LD, and ALP were added in Model 4 and yielded less standard error of estimates, improved *R*, *R*2 and adjusted *R*2. The model 4 has better prediction than model 1, 2 and 3. Thus the model 4 is the preferred model.

Table 1: Multiple regression models and predictors represented by superscript letters

Model Summary[e]						
Model	**Constant**	**R**	**R^2**	**Adjusted R^2**	**SEE**	**Durbin-Watson**
1	1.374	0.419[a]	0.175	0.170	1.043	
2	1.838	0.588[b]	0.345	0.337	0.932	
3	2.840	0.646[c]	0.418	0.407	0.882	
4	2.678	0.666[d]	0.444	0.430	0.864	2.151

a. Predictors: (Constant), C-Reactive Protein (mg/L); b. Predictors: (Constant), C-Reactive Protein (mg/L), Bilirubin (µmol/L); c. Predictors: (Constant), C-Reactive Protein (mg/L), Bilirubin (µmol/L), Lactate Dehydrogenase (U/L); d. Predictors: (Constant), C-Reactive Protein (mg/L), Bilirubin (µmol/L), Lactate Dehydrogenase (U/L), Alkaline Phosphatase (U/L); e. Dependent Variable: HP (g/L); SEE -standard error of estimate. The smaller the SEE, the closer the residuals are to the line of best fit.

Table 2: Analysis of variance

ANOVA[a]						
	Model	**Sum of Squares**	**df**	**Mean Square**	**F**	**Sig. F**
1	Regression	37.451	1	37.451	34.454	.000[b]
	Residual	176.088	162	1.087		
	Total	213.539	163			
2	Regression	73.772	2	36.886	42.490	.000[c]
	Residual	139.766	161	0.868		
	Total	213.539	163			
3	Regression	89.161	3	29.720	38.232	.000[d]
	Residual	124.378	160	0.777		
	Total	213.539	163			
4	Regression	94.818	4	23.704	31.747	.000[e]
	Residual	118.721	159	0.747		
	Total	213.539	163			

Key: df -degree of freedom, F -F-test, Sig -significant. The df is calculated by Nk-1, where k is number of variables. All four equations have been tested and have been found to statistically predict HP level in hemolysed samples.

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The decision to retain the null hypothesis (H_0) was set at alpha (α) greater than 0.05 and the decision to reject null hypothesis was set at p-value <0.05. The study found the p-values for CRP, ALP, LD and Bili to be less than 0.05 and therefore the null hypothesis was rejected at p-value <0.005. The standardized beta coefficient for each marker were: Bili = -0.407, CRP = +0.511; LD = -0.274 and ALP = +0.165. On the other hand, the unstandardized beta coefficient results suggest that for every unit change in CRP, ALP, Bili and LD, the HP level decreases by 0.009, 0.002, 0.025 and 0.004 g/L, respectively, provided that all the other variables are constant. The overall model produced is: Predicted HP – 2.678 + 0.009* CRP 0.025 * Bili-0.004* LD + 0.002 * ALP.

Discussion

The results from this research support the alternative hypothesis that the increase in levels and activities of serum Bili, CRP, LD, and ALP, respectively in intravascularly hemolyzed samples have a significant impact on serum HP levels. The results are significant at F (4,159), 31.75; p <0.005. The null hypothesis was rejected using critical and p-value approaches. Therefore, these findings provide an alternative technique for estimating serum HP results from pre-existing HM results and in the absence of IVH, EVH can be inferred from the results. The Bili, CRP, LD, and ALP have shown to be valuable parameters to assess reduction of HP level in hemolysis.

The study found CRP level and ALP activity to have positive correlation with HP. Like HP, CRP and ALP are acute phase proteins (APP), produced in the hepatocytes under the influence of cytokines. The role of CRP and ALP in tissue injuries/damages are to mediate and modulate inflammatory responses, respectively.[33] CRP has been shown to activate complement pathways, which exacerbate the intravascular hemolysis.[34] For instance, a sudden increase in serum CRP level at the start of IVH suggests that immune systems are responding to cell injury and can contribute to a higher rate of hemolysis. The cytokines-APP interactions trigger a surge in serum HP level with clinical and pathological consequences. The HP on the other hand controls the accumulation of cell-free hemoglobin levels. A sharp increase in the level of HP in a hemolyzed sample suggests a pending inflammatory situation. CRP and ALP alone would not necessarily suggestion present of IVH according to the model.

In contrast, the study found that high LD and Bili levels have a negative impact on serum HP level. Bilirubin and LD activity surge several days after the initiation of a hemolytic episode. LD has five isoenzymes and the most predominant isoenzymes in the RBC membrane are LD1 and LD2. [35,36] These isoenzymes spill into peripheral blood during IVH and increase the serum LD activity. In addition, high serum LD level strongly correlates with high level of cell-free Hb.[37,38] As free Hb level is catabolized, it yields globin and heme. The heme breakdown leads to a high Bili concentration. By the time the Bili and LD start to surge, the serum HP level is already significantly declining. The results showed that high LD and Bili concentration contribute to the rapid depletion of serum HP level. These results suggest that the Bili and LD model have a high probability of showing the extent of IVH impact on patients. In other words, the variables that negatively decrease HP level, are better candidates for a model that can accurately estimate HP level in hemolyzed samples and diagnose IVH.

The next plausible question is how best these variables accurately estimate HP level. This study showed that 44% coefficient of determination (R^2 = 0.44) of HP results variation above the HP mean model were accounted for by independent variables. For instance, a unit rise of serum Bili level, contributes to a 0.025 g/L drop of serum HP level provided other independent variables are held constant (Table 1, model 4). Bilirubin has the greatest impact on HP level compared with the other independent variables. When all the independent variables are added into the model, the *R2* dropped slightly from 44% to 43%. This slight decrease of adjusted R^2 improves the error of prediction of the model. The remaining variance of the model can be accounted for by systemic and random errors due to mechanical, preanalytical or biological factors.[39] These results are consistent with a previous study which showed that positive biases are expected in a normal population.[40] The study assessed the cross-talks (multicollinearity) between independent variables and no evidence of such relationship was found among the independent variables. This suggests that there is high degree of certainty in the model to accurately estimate serum HP in hemolyzed samples.[41]

The tools used to assess multicollinearity include tolerance, variance inflation factors (VIF) and Pearson's r. All the Pearson's (r) results are below 0.7, which suggest that there is no multicollinearity effect on the model. In addition, the tolerance and VIF results are greater than 0.1 and less than 2, respectively. The tolerance and VIF values are below the minimum threshold required to confirm multicollinearity among the independent variables, which suggested that model has not been affected by any collinearity.[43,44] The model is likely to accurately estimate the serum HP level in hemolyzed samples. After addressing the question of multicollinearity, the student attempted to address the question of closeness of results to reference or normal population.

The study found the residuals to be normally distributed around the mean model as depicted by histogram with superimposed normal curve and P-P plot (Figure 2.a & 2. b). These residuals' graphs

demonstrated that the residuals behave naturally and are free from external biases, implying that the model produced by this study is free of statistical noises. The

research excludes retics and AST from the model as they contributed no effect on the model. As expected, retics and AST elevation are often associated with bone marrow compensation and hepatocellular conditions, respectively. It is likely that AST and retic elevations in hemolyzed samples and concomitant decrease of HP level are due to extravascular factors other than IVH. The data were sampled from 34 public hospitals spread across the state and from diverse patient demographics and conditions.

Although, the research found statistically significant impact of Bili, LD, CRP and ALP on HP level, the results did not provide clinical significance or establish a cause and effect relationship. This model can be improved to demonstrate clinical significance by carrying out further studies with a larger sample size and controls applied to the preanalytical, analytical, biological, and environmental variables.

Conclusion

The present study shows a strong association exists between elevated independent variables and dependent variable in hemolyzed samples. The results also indicated that high levels of Bili and LD have negative relationship with HP while CRP and ALP significantly increase serum HP level in hemolyzed specimens. Negative association variables demonstrate an excellent model for the prediction of the HP level and diagnosing IVH. The parameters produced multiple regression model that can predict serum HP level from levels of hemolysis markers. Due to great variation of hemolytic diseases, the results of this model should be interpreted in the context of the patient's medical condition. With further study, the model has the potential to produce an estimated level of HP, which can be used to make diagnostic decisions, send sample for a confirmation of the HP level and to reduce sample rejection rates. The study utilized the data from a diverse patient's pool, which supports the validity of the study.

Declaration

The datasets generated during and/or analyzed during the current study are not publicly available due to sensitive nature of the data but are available from the corresponding author on reasonable request.

Acknowledgements

GP conceived the idea, designed the project, mined the data, performed data analysis using SPSS and wrote the manuscript. GD assisted in the navigation of ethical approval processes while PB and UN provided professional advice and guidance. We thank the technical assistance provided by BS at Pathology Queensland Laboratory, Queensland Children's Hospital, Queensland, Australia.

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