ISSN 1024 – 6193

PAKISTAN JOURNAL OF PATHOLOGY

An Official Journal of Pakistan Association of Pathologists and all Societies of Pathology Specialties

Quarterly

Vol. 35, No. 1, Jan - Mar 2024

Indexed with Indexus Medicus for the Eastern Mediterranean Region (IMEMR), EBSCO Host, Register with International Standard Serial Number (ISSN-France), Indexed with PASTIC, Asian Digital Library, PakMediNet, , NLM Catalog (ID 9425966), Recognized by the Pakistan Medical and Dental Council (PM&DC) and Higher Education Commission (HEC) Islamabad (Category-Y)

Website: http://www.pakjpath.com

Pakistan Journal of Pathology

Vol. 35, No. 1, Jan - Mar 2024

An Official Journal of Pakistan Association of Pathologists

Recognized by Higher Education Commission in Category **'Y'**

CONTENTS

ORIGINAL ARTICLES

Original Article

Glycated hemoglobin (HbA1c) in diagnosis of gestational diabetes mellitus

Muhammad Younas, Asif Ali, Muhammad Qaiser Alam Khan, Sajida Shaheen, Arooj Ishtiaq, Maimoona Roghani

Armed Forces Institute of Pathology (National University of Medical Sciences), Rawalpindi Pakistan

ABSTRACT

Objective: To evaluate the diagnostic accuracy of glycated hemoglobin for diagnosing gestational diabetes mellitus.

Material and Methods: This cross-sectional comparative study was conducted at the Department of Chemical Pathology and Endocrinology, Armed Forces Institute of Pathology (National University of Medical Sciences), Rawalpindi Pakistan from June 2023 to October 2023. The study included women having gestational amenorrhea between 24th to 28th weeks, who gave informed written consent for a 75-gram oral glucose tolerance test (OGTT) at AFIP, Rawalpindi. Women with diabetes, hypertension, hemoglobin< 10g/dl, on steroid treatment, with gastrointestinal or thyroid diseases were excluded. The medical history and anthropometric measurement (height, weight, body mass index (BMI) and blood pressure were recorded on a predesigned proforma. Blood sample for glucose was taken in sodium fluoride tubes and HbA1c in potassium EDTA tubes. American Diabetes Association criteria 2023 was used to diagnose gestational diabetes mellitus (GDM). Study participants were divided into two groups on basis of 75g- OGTT results; Group 1 included GDM and Group 2 Normal Glucose Tolerance (NGT).

Results: A total of 100 pregnant ladies were enrolled whose ages ranged from 15-45 (mean & SD 29±5 & 31±6 years in groups 1& 2 respectively). Mean fasting plasma glucose (FPG) was 5.11±0.6 mmol/l & 4.57±0.4mmol/l, and HbA1c was 6.12%±1 & 5.26% ±0.48 in groups 1 & 2 respectively. GDM was found in 19 (group 1) of 100 patients, while 81 responded normally. HbA1c at 5.6% has 84.2% sensitivity, 87.6% specificity, 61.5% PPV, 95.9% NPV and 87% diagnostic accuracy. FPG, 1hr glucose and HbA1c exhibited an Area Under Curve (AUC) by Receiver Operating Characteristic (ROC) Curve of 0.762, 0.801 and 0.894 respectively.

Conclusion: HbA1c has shown higher diagnostic yield for gestational diabetes mellitus and can be used as a screening test.

Keywords: Glycated hemoglobin, Oral glucose tolerance test, Gestational diabetes mellitus

This article can be cited as: Younas M, Ali A, Khan MQA, Shaheen S, Ishtiaq A, Roghani M. Glycated hemoglobin (HbA1c) in diagnosis of gestational diabetes mellitus. Pak J Pathol. 2024; 35(1): 1-6.

DOI: https://doi.org/10.55629/pakjpathol.v35i1.805

INTRODUCTION

Gestational diabetes mellitus (GDM) is a complicated condition that poses significant health risks to both mothers and neonates, characterized by elevated blood glucose levels during pregnancy. The incidence of GDM is increasing in parallel to improvements in life standards and health awareness. The estimated global prevalence has been reported to be 14% by International Diabetes Federation and reported prevalence in Pakistan is 9.47% [1,2].

GDM presents unique challenges to heath care providers, as it typically arises during pregnancy and often settles postpartum, however it carries prolonged health implications for mothers and their babies. Maternal complications may include an increased risk of developing Type 2 Diabetes Mellitus (T2DM) post-partum [3], while babies have an increased risk of neonatal hypoglycemia, macrosomia and an increased vulnerability to obesity and T2DM during their own lifetimes. GDM, if not managed, can lead to adverse pregnancy consequences, such as pre-eclampsia and preterm birth [4].

Correspondence: **Dr. Muhammad Younas, Department of Chemical Pathology and Endocrinology, Armed Forces Institute of Rawalpindi (National University of Medical Sciences), Rawalpindi**

Email: muhammadyounaschempath360@gmail.com

Receiving Date: **02 Jan 2024**
Revision Date: **02 Feb 2024**
Acceptance Date: **22 Feb 2024**

Accurate and timely diagnosis of GDM is vital to alleviate these risks and guarantee the best possible outcome to both mothers and infants. Traditionally, GDM diagnosis has relied mostly on the OGTT, a diagnostic test requiring fasting and multiple blood glucose measurements [5]. This method, while effective, has limitations, including patient inconvenience, potential false-positive results, and variable international diagnostic criteria. Similarly, there is requirement for a minimum 8 hours medical fast, 3 blood samples, risk of vomiting, and increased chances of variability. Similarly, over 10% of pregnant women fail to finish the OGTT process [6]. Despite these challenges, OGTT remains an important diagnostic tool for GDM due to its ability to identify pregnancies with unfavorable outcomes. International associations have made efforts to standardize diagnostic criteria, such as those proposed by the ADA, to improve the accuracy and consistency of GDM diagnosis [7].

HbA1c, on the other hand, is a widely used biomarker in the management of diabetes, reflecting average plasma glucose over the preceding 2-3 months [8]. Its application in diagnosing GDM is a topic of growing interest and research [9,10]. Latest research indicates that HbA1c may serve as a useful screening tool for GDM, although it should not be relied upon as a complete substitute for OGTT [11]. Retnakaran *et al* have indicated that assessing HbA1c levels before pregnancy, on average 1.4 years earlier, strongly predicted GDM. Each 0.1% rise in pregravid HbA1c raised the risk of GDM in a next pregnancy by 22% [12]. Similarly diagnostic role of HbA1c in GDM has been proved by different researchers [13,14]. However, Liu X *et al* have emphasized that further research and clinical practice support are still needed for the application of HbA1c in GDM [15]. Furthermore, there were constant Hba1c variations throughout pregnancy between women diagnosed with GDM and control group [16]. Other studies have reached similar conclusions, stating that HbA1c levels, which are used to diagnose pre-diabetes in non-pregnant individuals, are also linked to the development of GDM. These findings support the use of HbA1c as a tool for predicting GDM [17].

Keeping in view the prevalence of GDM in our country, its dreadful effects and usefulness of timely diagnosis, present study was planned to evaluate the diagnostic performance of HbA1c among the females at risk of GDM.

MATERIAL AND METHODS

The cross-sectional study was conducted in the Department of Chemical Pathology and Endocrinology, Armed Forces Institute of Pathology, Rawalpindi from June 2023 to October 2023 after approval from the Institutional Review Board reference number: Cons-CHP-3/READ-IRB/23/2219.

Sample size calculation was performed using the World Health Organization sample size calculator, which came out to be 100, keeping in view the prevalence of GDM at 9.47% in Pakistan [2]. Pregnant ladies between 18-45 years of age, having gestational amenorrhea between 24th to 28th weeks who were referred to AFIP for 75g OGTT were enrolled in the study after obtaining informed written consent. Known diabetics, hypertensives, gastrointestinal and thyroid disorders were excluded. Ladies with Hemoglobin <11 g/dl or taking hematinics were also excluded. History and measurements of anthropometric indices like height, weight, BMI & blood pressure were recorded. Blood samples were taken in sodium fluoride and Potassium EDTA tubes for blood glucose and HbA1c, respectively.

Plasma glucose analysis was performed using the Hexokinase Method and HbA1c by Turbidimetric inhibition immunoassay (TINIA) on the Cobas Pure by Roche Diagnostics, (a fully automated Chemistry Analyzer). The diagnosis of GDM was confirmed using the diagnostic criteria established by the ADA 2023 [18]. Based on the findings, participants were categorized into two groups: Group 1 with GDM, and Group 2 with NGT.

The data analysis was conducted using the Statistical Package for Social Sciences (SPSS) program version 25.0. Data distribution was tested by Shapiro-Wilk test and found to be

normally distributed. The results were reported as the mean ± standard deviation (SD). An independent t-test was utilized to compare the HbA1c and OGTT results. The Area under the curve for FPG, 1-hour post-OGTT, 2-hour post-OGTT, and HbA1c were compared using ROC curve analysis.

RESULTS

Participants were between 18-45 years of age (mean & SD 29±5 & 31±6 years in groups 1 & 2, respectively). Of the 100 individuals, GDM was found in 19 cases (group 1) and HbA1c was also raised (> 5.6%) in 16 cases in this group, whereas 81 cases showed normal responses. Biochemical results showed mean FPG 5.11±0.66 and 4.57±0.4 mmol/l, HbA1c was 6.12 ±1 and 5.26±0.48 % in groups 1 and 2 respectively.

Results revealed that OGTT had a good association with a previous history of miscarriage/ GDM and significant difference was found among the group's participants who had a history of GDM in previous pregnancies, as depicted in Table-I.

Comparison of the age, BMI, gestational amenorrhea between the GDM and non GDM groups showed no significant difference. The fasting, 1hr, 2 hr. glucose and HbA1c among the groups had statistically significant differences as shown in Table-II.

The diagnostic yield of HbA1c was evaluated using OGTT as a gold standard method for the diagnosis of GDM. Table-III shows the comparison of HbA1c with OGGT as a positive response HbA1c at 5.6% has shown sensitivity, specificity, PPV, NPV& Diagnostic efficacy of 84.2%,87.6%, 61.5%, 95.9% and 87% respectively.

In addition, we conducted ROC curve analysis to compare the AUC values of FPG, 1-hour post OGTT result, 2-hour post-OGTT result, and HbA1c by taking ADA 2023 diagnostic criteria as gold standard.

In ROC curve analysis, the BMI variable demonstrated a moderate discriminatory capacity, as reflected by an AUC value of 0.617 (Figure-I. This suggests that BMI alone may not reliably discern between positive and negative cases. Conversely, FPG variable exhibited a robust discriminatory capacity, with an AUC of 0.762, indicating its strong ability to differentiate GDM. Furthermore, the 1hr glucose outperformed the others, boasting an AUC of 0.801, signifying excellent discriminatory power in identifying GDM. HbA1c has shown exceptional discriminatory performance, with an AUC of 0.894. This means high accuracy of HbA1c at 5.6% to distinguish between positive and negative cases, establishing it as a powerful diagnostic parameter in workup of GDM.

Table-I: Association of OGTT with history of miscarriages and GDM in previous pregnancies (n=100).

		OGTT – Normal Response Count (%)	OGTT – GDM Count (%)	Sig
Miscarriage History	No	32 (82.1%)	7 (17.9%)	0.046
	Yes	49 (80.3%)	12 (19.7%)	
GDM History	Yes	(4.2%)	8(57.1%)	>0.05
	No	69 (95.8%)	6 (42.9%)	

Table-II: Independent samples t-Test between GDM and Non-GDM group (n=100).

Variable	Mean ± SD		p-value
	Normal Response (n= 81)	GDM (n= 19)	
Age (years)	28.94 ±5.023	30.68± 0.51881	.442
Gestational Amenorrhea (weeks)	25.41± 4.623	26.68± 5.100	.601
BMI (kg/m^2)	25.4432 ±3.6124	27.1947±5.010	.170
Fasting Glucose (mmo/l)	4.57±0.40	5.11± 0.66	0.017
Glucose_1hr (mmo/l)	7.24±1.20	9.08±1.67	0.018
Glucose_2hr (mmo/l)	5.95±1.01	7.85±1.77	0.002
HbA1c (%)	5.26±0.48	6.12±1.01	0.001

Table-III: Relationship of GDM with HbA1c at cut-off 5.6% (n=100).

		OGTT	
		Positive 19 (GDM)	**Negative 81 (No GDM)**
HbA1c	Positive (n=26)	16 (TP)	FP (10)
	Negative (n=74)	3 (FN)	TN (71)
Diagnostic Yield			
Sensitivity		TP/TP+FN	84.2%
Specificity		TN/TN+FP	87.6%
PPV		TP/ TP+FP	61.5%
NPV		TN/TN+FN	95.9%
Diagnostic Efficacy		TN +TP/TN+TP+FN+FP	87%

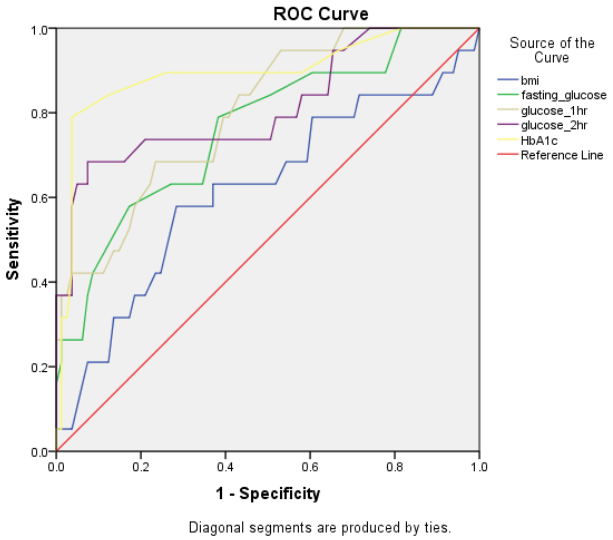

Figure-I: ROC curve analysis of BMI kg/m², glucose (fasting, 1hr, 2hr mmol/l) and HbA1c%.

DISCUSSION

Diagnosis of GDM remains a challenge and imperative to save mothers and babies. Among the various methods for assessing glucose metabolism in pregnant women present study has focused on the comparison of HbA1c and OGTT as diagnostic tools.

In present study (n=100) GDM was found in 19 cases, out of which 16 cases also had raised HbA1c (> 5.6%). Results of present study are in accordance with Valdan *et al* (n=700) who found 115 (16.4%) ladies had GDM. The sensitivity, specificity, NPV and PPV for ruling out GDM at even lower HbA1c of 4.85% was 92.2%,32.8%, 95.5% & 21.2% respectively. In addition, at HbA1c cut-off value of 5.45% sensitivity, specificity, NPV and PPV for ruling out GDM was 54.8%, 96.8%, 91.5% and 76.8% respectively. ROC analysis revealed HbA1c to be the most precise parameter for diagnosis, followed by glucose at the 2-hour. Fasting glucose and glucose after 1 hour, both showed good, refined ability. Whereas BMI performed less in this scenario, which is in accordance with present study.

Similarly, Khan SH *et al* discovered that those who showed a delayed peak in their blood glucose levels in OGTT had the highest levels of HbA1c. The highest AUC for the diagnosis of GDM was exhibited by the cumulative sum of all glucose readings. They determined that modifying HbA1c levels can support in decreasing the requirements of OGTT thus advocating use of HbA1c [19].

Similarly Bozkurt L *et al* have reported HbA1c at ≥5.7% during early pregnancies showed higher FPG (90.4±13.2 vs 79.7±7.2 mg/dL, p<0.001), mean plasma glucose (145 ±31 vs 116.2±21.4 mg/dL, as well as highest glucose and tended to a delay in reaching the extreme plasma glucose values in contrast to normal HbA1c, which is also in agreement with present study with mean FPG 4.57±0.4mmol/l and 5.11 mmol/l, HbA1c 5.26% 4±0.48 and 6.12 %±1 in group 1 & 2 respectively [20].

Similarly, Osmundson *et al* (n=2812) found that the risk of GDM was 50% higher in females with a first trimester HbA1c level between 5.7 to 6.4% compared to females with a normal HbA1c level, which also supports present study [21].

Negrea MC *et al* (n=312) studied HbA1c analysis in addition to OGTT in workup of GDM, and found 149 women had GDM. The area under the ROC curve for GDM detection by HbA1c was 0.73 (95% CI 0.68-0.79, p < 0.0001) at cutoff value of HbA1c of 5.5%. The sensitivity, specificity, PPV and NPV for this cutoff were 12.0%, 99.4%, 20 and 0.88, respectively, which supports present study [22].

Another researcher Singh V *et al* (n=200) have found that women who developed GDM had a substantially higher HbA1c level (5.4 ± 0.4%) compared to those who did not develop GDM (4.9 ± 0.2%). In the ROC analysis of HbA1c values for predicting the development of GDM, a threshold value of HbA1c ≥5.25% had 84.8% sensitivity and 62.7% specificity, irrespective of the individual's risk status. Similarly, within the high-risk group, a threshold value of HbA1c ≥5.15% had a sensitivity and specificity of 83.3% & 97% respectively to predict GDM. They concluded that HbA1c could be considered as a potential biomarker for predicting GDM, whose results are also comparable to present study where we used a cutoff of 5.6% in accordance with most of international studies [23].

However, Siricharoenthai P *et al* (n=114) have found The AUC for HbA1c detection of GDM was 0.725 (95% confidence interval 0.621-0.829) at Cut-off value of HbA1c 5.8%. Sensitivity, specificity, PPV, NPV, and accuracy were 17.1%, 100%, 100%, 73.2%, and 74.6%, respectively. They concluded that HbA1c could not replace OGTT for the diagnosis of GDM. However, HbA1c might be a useful tool to reduce the number of OGTT, associated costs and patient inconvenience [24]. Similarly, Yi Lai *et al* (n=19261) found of 3,547 (18.42%) women were diagnosed with GDM. HbA1c was positively, but only weakly correlated with FPG, 1-hour glucose, and 2-hour glucose (r=0.31, 0.24, and 0.25, respectively, P<0.001). The AUC of the HbA1c level for detecting GDM was 0.664 (95% CI: 0.653-0.674, P<0.01) at 5.0%, which yielded a sensitivity, specificity, PPV, and NPV of 60.1%,65.3%, 28.1%, 87.9% respectively, which are different from present study [25].

Participants in present study were between 15-45 years of age (mean & SD 29±5 & 31±6 years in group 1&2 respectively) which is in accordance with earlier studies [11,12,19,21].

LIMITATIONS OF STUDY

It was a single center study which may be influenced by potential confounding variables, such as dietary habits, physical activity, and family history.

CONCLUSION

HBA1C has shown reliable performance in workup of gestational diabetes mellitus and can be used as a screening test.

RECOMMENDATIONS

Longitudinal multicenter studies are required to provide a precise understanding of the relationship between HbA1c and GDM. Additionally, assessing insulin resistance, a hallmark of GDM and T2DM, can provide valuable insights into the condition's pathophysiology and diagnosis.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

GRANT SUPPORT & FINANCIAL DISCLOSURE

Declared none

AUTHORS CONTRIBUTION

Muhammad Younas: Idea conception, Write up, literature review, sample collection and analysis

Asif Ali: Literature, data interpretations, critical review

Muhammad Qaiser Alam Khan: Overall supervision and approval of the study

Sajida Shaheen: Proof reading, literature review, revision of the study

Arooj Ishtiaq and Maimoona Roghani: Data collection and statistical review

REFERENCES

1. Wang H, Li N, Chivese T, Werfalli M, Sun H, Yuen L, Hoegfeldt CA *et al*. IDF diabetes atlas committee hyperglycaemia in pregnancy special interest group. IDF Diabetes Atlas: Estimation of Global and Regional Gestational Diabetes Mellitus Prevalence for 2021 by International Association of Diabetes in Pregnancy Study Group's Criteria. Diabetes Res Clin Pract. 2022; 183: 109050. DOI:https://doi.org/10.1016/j.diabres.2021.109050
2. Inam I, Madnia E, Ammar A, Sajjad S. Prevalence of gestational diabetes mellitus in Pakistan: A cross sectional study. Pak J Med Health Sci 2022; 16; 241-3. DOI: https://doi.org/10.53350/pjmhs221610241
3. Bellamy L, Casas JP, Hingorani AD, Williams D. Type 2 diabetes mellitus after gestational diabetes: a systematic review and meta-analysis. Lancet 2009; 373(9677): 1773-9. DOI:https://doi.org/10.1016/s0140-6736(09)60731-5

4. Mou SS, Gillies C, Hu J, Danielli M, Al Wattar BH, Khunti K. Association between HbA1c levels and fetal macrosomia and large for gestational age babies in women with gestational diabetes mellitus: A systematic review and meta-analysis of 17,711 Women. J Clin Med. 2023; 12(11): 3852. DOI: https://doi.org/10.3390/jcm12113852
5. American Diabetes Association. 2. Classification and diagnosis of diabetes: standards of medical care in diabetes-2020. Diabetes Care 2020; 43(Suppl1): S14–s31. DOI: https://doi.org/10.2337/dc20-S002
6. Hes RC, Rowan J, Florkowski CM. Is there a role for HbA1c in pregnancy? Curr Diab Rep. 2016; 16(1): 5. DOI: https://doi.org/10.1007/s11892-015-0698-y
7. Metzger BE, Gabbe SG, Persson B, Buchanan TA, Catalano PA, Damm P, *et al*. International association of diabetes and pregnancy study groups recommendations on the diagnosis and classification of hyperglycemia in pregnancy. Diabetes Care. 2010; 33: 676-82. DOI: https://doi.org/10.2337/dc09-1848
8. American Diabetes Association. Standards of Medical Care in Diabetes. Diabetes Care 2022; 45(Supplement_1), S1-S198.
9. Balani J, Hyer S, Johnso A, Shehata H. Predicting gestational diabetes mellitus by first trimester HbA1c: A retrospective study in women with moderate to severe obesity. Pract Diab. 2023, 40: 22-27. DOI: https://doi.org/10.1002/pdi.2466
10. Deng, Y., Cheng, D., Liao, G. Tan X, Yang J. Trimester-specific reference intervals for hemoglobin A1c in non-diabetic pregnancy in a Chinese population. BMC Pregnancy Childbirth 2023; 23; 671. DOI: https://doi.org/10.1186/s12884-023-05980-0
11. Valadan M, Bahramnezhad Z, Golshahi F, Feizabad E. The role of frst-trimester HbA1c in the early detection of gestational diabetes. BMC Pregnancy Childbirth. 2022; 22:71. DOI: https://doi.org/10.1186/s12884-021-04330-2
12. Retnakaran R, Shah BR. Pregravid HbA1c and glucose measurement to rule out future gestational diabetes mellitus and reduce the need for oral glucose tolerance testing in pregnancy. Diabetes Care. 2020; 43: e93–5. DOI: https://doi.org/10.2337/dc20-0785
13. Kattini R, Hummelen R, Kelly L. Early gestational diabetes mellitus screening with glycated hemoglobin: A systematic review. J Obstet Gynaecol Can. 2020; 42(11): 1379–84. DOI: https://doi.org/10.1016/j.jogc.2019.12.015
14. Fong A, Serra AE, Gabby L, Wing DA, Berkowitz KM. Use of hemoglobin A1c as an early predictor of gestational diabetes mellitus. Am J Obstet Gynecol. 2014; 211(6): 641.e1–641.e7. DOI: https://doi.org/10.1016/j.ajog.2014.06.016
15. Liu X, Wu N, Al-Mureish A. A review on research progress in the application of glycosylated hemoglobin and glycated albumin in the screening and monitoring of gestational diabetes. Int J Gen Med. 2021; 14: 1155-65. DOI: https://doi.org/10.2147%2FIJGM.S296316
16. Hinkle SN, Tsai MY, Rawal S. HbA1c measured in the first trimester of pregnancy and the association with gestational diabetes. Sci Rep. 2018; 8: 12249. DOI: https://doi.org/10.1038/s41598-018-30833-8
17. Amylidi S, Mosimann B, Stettler C, *et al*. First-trimester glycosylated hemoglobin in women at high risk for gestational diabetes. Acta Obstet Gynecol Scand. 2016; 95: 93–7. DOI: https://doi.org/10.1111/aogs.12784
18. ElSayed NA, Aleppo G, Aroda VR, Bannuru RR, Brown FM, Bruemmer D, *et al*. On behalf of the american diabetes association. management of diabetes in pregnancy: Standards of care in diabetes-2023. Diabetes Care. 2023; 46 (Supplement_1): S254–S266. DOI: https://doi.org/10.2337/dc23-S015
19. Khan SH, Manzoor R, Baig AH, Tariq B, Ayub N, Sarwar S, *et al*. Role of HbA1c in diagnosis of gestational diabetes mellitus. J Pak Med Assoc. 2020; 70(10): 1731-36. DOI: https://doi.org/10.5455/jpma.24080
20. Bozkurt L, Göbl CS, Leitner K. HbA1c during early pregnancy reflects beta-cell dysfunction in women developing GDM. BMJ Open Diab Res Care. 2020; 8: e001751. DOI: https://doi.org/10.1136/bmjdrc-2020-001751
21. Osmundson SS, Zhao BS, Kunz L. First trimester hemoglobin A1c prediction of gestational diabetes. Am J Perinatol. 2016;33:977–82. DOI: https://doi.org/10.1055/s-0036-1581055
22. Negrea MC, Oriot P, Courcelles A, Gruson D, Alexopoulou O. Performance of glycated hemoglobin A1c for the diagnosis of gestational diabetes mellitus during the SARS-CoV-2 pandemic in Belgium (2020-2021). Eur J Obstet Gynecol Reprod Biol. 2023; 289: 36-41. DOI: https://doi.org/10.1016/j.ejogrb.2023.08.368
23. Singh V, Warman S. Association of early pregnancy values of glycosylated hemoglobin and the development of gestational diabetes mellitus. Cureus. 2023; 15(10): e46685. DOI: https://doi.org/10.7759/cureus.46685
24. Siricharoenthai P, Phupong V. Diagnostic accuracy of HbA1c in detecting gestational diabetes mellitus. J Matern Fetal Neonatal Med. 2020; 33(20): 3497-3500. DOI:https://doi.org/10.1080/14767058.2019.1576169
25. Lai Y, Chen H, Du Z, Zhou S, Xu W, Li T. The diagnostic accuracy of HbA1c in detecting gestational diabetes mellitus among Chinese pregnant individuals. Ann Transl Med. 2020; 8(16): 1014. DOI: https://doi.org/10.21037/atm-20-5464

Unveiling colistin resistance in extensively drug-resistant microorganisms among ICU patients of tertiary care hospital Karachi, Pakistan

Sadia Talib[1], Abeera Ahmed[2], Syeda Hira Abid[1], Tahira Assad[1], Muhammad Nizamuddin[3], Shaista Sharif[4]

[1]Karachi Institute of Medical Sciences, Karachi Pakistan
[2]The Pakistan Navy Ship Rahat Hospital, Karachi Pakistan
[3]Dow International Medical College, Karachi Pakistan
[4]Teshil Headquarters Hospital, Shahpur Pakistan

ABSTRACT

Objective: To determine the prevalence of extensively drug-resistant (XDR) microorganisms in the intensive care unit (ICU) of a tertiary care hospital in Karachi and to identify the presence of colistin resistance (CLR) among these XDR isolates.

Material and Methods: A cross sectional study was carried out in the ICU of a tertiary care hospital from August 2022 to February 2023 and various clinical samples of XDR Gram negative bacilli (GNB) were collected from ICU. These specimens were processed by following the guidelines established by the Clinical and Laboratory Standards Institute (CLSI), Agar and broth microdilution methods were used to assess the colistin susceptibility of XDR isolates.

Results: The study focused on 100 extensively drug-resistant (XDR) specimens. *K. Pneumoniae* dominated (32%), followed by *E. coli* (23). Among the 100 XDR, 89% were colistin susceptible, and 11% were resistant, tested by agar and broth microdilution. Of the 11% CLR, *P. aeruginosa* was most common, with the highest resistance in urine specimens. Colistin resistance was highest for *P. aeruginosa* (45%), followed by *A. baumannii* (18%), *E. coli* (18%), *K. Pneumoniae* (9%), and *K. oxytoca* (9%). CLR isolates were mainly (27%) from urine.

Conclusion: Prolonged hospital stays and antibiotic pressure can lead to CLR development. Labs should monitor XDR closely, implementing measures to minimize antibiotic resistance. Controlling colistin resistance through stewardship is crucial.

Keywords: Extensively drug resistant**,** Colistin resistant, Minimum inhibitory concentration, Nasobronchial lavage

This article can be cited as: Talib S, Ahmed A, Abid S, Assad T, Nizamuddin M, Sharif S. Unveiling colistin resistance in extensively drug-resistant microorganisms among ICU patients of tertiary care hospital Karachi, Pakistan. Pak J Pathol. 2024; 35(1): 7-12.

DOI: https://doi.org/10.55629/pakjpathol.v35i1.800

INTRODUCTION

Antimicrobial resistance is escalating globally, in both hospital and community communities. ICUs are particularly prone to nosocomial infections, with the majority of infections caused by gram-negative bacteria, including *ESBL-E* and/ or *CPE*, *P. aeruginosa*, and *A. baumannii* [1]. Nosocomial infections typically involve gram-negative bacteria and these bacteria may become (XDR) extensively drug-resistant due to their propensity to acquire antibiotic resistance [2]. Gram-negative XDR bacteria offer a significant threat to the economy of developing nations like Pakistan, where rates of antibiotic resistance are significantly greater as a result of the overuse and improper prescription of antibiotics [3]. Strict monitoring of antibiotic resistance is necessary to create prompt management strategies and remedies to this grave public health issue [4]. Clinicians have a tremendous challenge when treating infections brought on by some infectious agents, such as extensively drug-resistant (XDR) Gram-negative bacteria notably in situations with limited resources like in most labs of Pakistan. In order to track the increasing incidence of antibiotic

Correspondence: **Dr. Sadia Talib, Department of Pathology Karachi Institute of Medical Sciences, Karachi Pakistan**

Email: sadiasonoo@gmail.com

Receiving Date: **08 Dec 2023** Revision Date: **14 Feb 2024** Acceptance Date: **13 Mar 2024**

resistance and create effective strategies for the management and control of these infections, there are still little national data available on the epidemiologic traits of CR and XDR Gram-negative bacilli in Pakistan. For this purpose, the phenotypic characteristics of XDR strains of GNB isolates obtained from different samples were studied [5]. A polymyxin E called colistin was discovered in Japan in 1949. Bacillus polymyxa is the true source of polymyxin E [6].

Not because of an improved safety profile, polymyxins had returned as a last-resort treatment against MDR and XDR Gram-negatives by the mid-1990s due to the emergence of XDR Gram-negative superbugs, particularly *P. aeruginosa*, *A. baumannii, and K. pneumonia,* which are resistant to all other available antibiotics, as well as the lack of novel antimicrobials available to treat MDR bacterial infections. Unfortunately, colistin has been over- and misused in animal and human medicine, leading to the widespread evolution of infections resistant to it [7]. A quick and accurate approach for testing colistin's antimicrobial susceptibility is required due to the rise in multi-resistant gram-negative bacterial infections and concurrent rise in colistin resistance [8].

A variety of laboratory methods can be used to evaluate or screen the in vitro antimicrobial activity of an extract or a pure compound. The broth or agar dilution are the most popular and fundamental techniques [9].

The micro- or macro-dilution of broth is one of the most straightforward methods for assessing an antimicrobial's susceptibility. The agar dilution method is one of the pioneering AST approaches used by researchers to establish the MIC of an antimicrobial agent accessible since the 1940s, along with the broth dilution method. “The MIC, which is typically given in mg/L (ug/mL), is the lowest concentration of a medicine that suppresses the growth of visible bacteria on agar or in broth [10]. It is still used nowadays, especially for newly developed drugs, as a quantitative measure in AST [11].

Hence, there is a need to delineate the prevalence of XDR microorganisms in ICUs and further investigate the occurrence of colistin resistance in these isolates. By studying the prevalence and identification of CLR in XDR isolates collected from a tertiary care hospital in Karachi, this research aims to provide valuable insights into the resistance patterns of these microorganisms and contribute to the understanding of antimicrobial resistance in ICU settings.

MATERIAL AND METHODS

This study was carried out in the Microbiology Department of the Combined Military Hospital (CMH) Karachi from August 2022 to March 2023 with authorization from the Institutional Ethical Review Board of CMH Mair Cantt (File No. 85/2020/Trg/ERC). We collected 100 specimens from CMH Malir Cantt patients in the intensive care unit (ICU) for the cross-sectional investigation. Using openepi.com, the sample size for 85 isolates was calculated while taking into account the 5.81 percent CT-resistant bacterial frequency in Pakistan.[11] Achieving a 95% confidence interval with a 0.5% margin of error served as the foundation for the computation. Consecutive non-probability sampling was the technique used for sampling.

The XDR gram-negative rods were selected during a three-month sampling process. With the exception of urine samples, which were placed on cled agar, all ICU specimens were placed on 5% Sheep Blood Agar (SBA) and MacConkey agar. After overnight incubation, samples showing growth of Gram-negative bacilli were identified using standard laboratory protocols, including Gram staining and API 20E. XDR isolates from various sources were confirmed using the Kirby-Bauer method. The research tool, Performa, was designed for reliability. Colistin sulfate, known as Polymyxin E, was used in the study, and its potency was determined following CLSI guidelines. Colistin susceptibility was assessed by agar and broth microdilution, with MICs expressed as the lowest concentration preventing visible growth under controlled conditions. The CLSI guidelines were followed in order to evaluate both approaches using the quality-control (QC) strain of *P. aeruginosa* with ATCC 27853. A range of 0.5 to 2µg/ml was

deemed appropriate for quality control.[12]Data were statistically analyzed using SPSS software (version 24), The Categorical data is reported as Frequencies and percentages and quantitative in mean ± SD or Median (IQR). The significance level was set at a p-value of ≤0.05, and the Cohen's kappa test was applied for agreement determination. For difference among the nature of specimen and microbe Fishers exact test was applied.

RESULTS

A total of 100 XDR (resistant to at least one drug in all classes except one or two drugs) isolates from various clinical specimens were examined in the current investigation. Urine samples from intensive care unit patients included the bulk of the isolates. The polymyxin propensity of these bacteria was evaluated using two methods (AD & BMD), and the findings were compared to one another using BMD as the reference method, as indicated in table-I. The most prevalent isolates was *K. pneumoniae* [32]. 1 ug/ml and 2 ug/ml, respectively, were corresponding MIC 50 and MIC 90 values. (Table-I). A comparison between the broth and agar dilution procedures was carried out on 100 XDR isolates.

The isolates with the highest frequencies that were relevant to the sample were *K. pneumonia* in pus (31%), and *E. Coli* in urine (83%) *P.aeruginosa* in urine (35%), A. baumanii in NBL(35%) . E. cloacae in sputum (50%), *K. oxytoca* in pus (60%), and *C. ferrundii* in NBL (100%). Agar dilution technique revealed that two of the 32 *K.pneumoniae* strains were resistant. Out of the 23 isolates of *E. Coli* tested in the present study, two were resistant. This study included 17 *A.baumannii*, 4 of which were resistant. There were two resistant *P. aeruginosa* among the 14 isolates examined in this study. *E. cloacae* were 6 in this study, none of them was resistant. There were five *K.oxytoca* in this research, and one of them was resistant. Of the three *C. freundii* none were resistant, (Table-II) The p-value shows statistical significance difference, among the distribution of microbes in specimen type, (<0.001).

The study on CLR isolates indicates a diverse range of bacterial strains from different clinical samples (Table 1.3). P. aeruginosa comprises 45% of the total isolates, with two from double lumen, and one each from urine, sputum, and pus. *A.baumannii* represents 18% with one isolate from tissue and another from NBL. *E. coli* contributes 18%, originating primarily from urine. *K.pneumoniae and K. oxytoca* each accounts for 9% from NBL and urine samples respectively. This comprehensive analysis highlights the varying frequencies of bacterial strains in clinical specimens, providing crucial insights into microbial prevalence in healthcare settings.

Table-I: Clinical sources of XDR (n=100) isolates.

Clinical samples% N=100		MIC Range (≤0.5-32µg/ml	Isolates (n=100) %
Urine	31 (31%)		*K. Pneumonia* 32 (32%)
Pus	22 (22%)	MIC 50 = 1	*E. Coli* 23 (23%)
Blood	15 (15%)	MIC 90= 2	*A. Baumannii* 17 (17%)
NBL	14 (14%)	Susceptibility% = 90%	*P. Aeruginosa* 14 (14%)
Tissue	6 (6%)		*E. Cloacae* 6 (6%)
Sputum	3 (3%)		*K. Oxytoa* 5 (5%)
Tip for C/S	3 (3%)		*C. Ferundii* 3 (3%)
CVP	4 (4%)		

Table-II: Analysis of nature of specimens.

Name of microbe	Nature of specimen (N %)							P-Value
	Urine	Blood	I/V Catheter	NBL	Pus	Sputum	Tissue	
Klebsiella pneumoniae	5 (16%)	8 (25%)	4 (12%)	5 (15%)	10 (31%)			
E.coli	19 (83%)	2 (8.7%)			2 (8.7%)			**<0.001**
Acenito bacterbaumani		2(11.8%)		6 (35%)	3 (17.6%)		6 (35%)	

Pseudomonas aeruginosa	5 (35%)	3 (21%)		4 (28.6%)	2 (14%)
Enterobacter cloacae					3 (50%)
Klebsiella oxytoca	2 (40%)			3 (60%)	
Citrobacter ferundi			3(100%)		

Table-III: Colistin resistant isolates isolated from various specimens.

Name of CLR isolate	No. of CLR isolate	Nature of isolated sample	Number of samples	% of each CLR isolate
P. Aeruginosa	2	Double lumen	2	45%
P. Aeruginosa	1	Urine	1	
P. Aeruginosa	1	Sputum	1	
P. Aeruginosa	1	Pus	1	
A. Baumani	1	Tissue	1	18%
A. Baumani	1	NBL	1	
E. Coli	2	urine	2	18%
K. Pneumoniae	1	NBL	1	9%
K. Oxytoca	1	urine	1	9%
Total CLR isolates	**11**			

DISCUSSION

The global challenge of antimicrobial resistance (AMR) is evident in recent studies of Brown et al emphasizing its pervasive impact across healthcare settings and communities [13]. Over the past five years, infections from drug-resistant bacteria have surged posing a considerable threat, especially in intensive care units (ICUs) susceptible to nosocomial infections, as supported by recent research. Most recent results are dominated by gram-negative bacteria, especially those that produce carbapenem-producing Enterobacteriaceae (CPE) and extended-spectrum β-lactamases (ESBL-E) [14].

Our study revealed 11% colistin resistance among XDRs, compared to Qamar et al.'s study in Pakistan showing 15% (using 251 strains, exceeding our sample size [15]. Similarly, Abd El-Baky RM et al. reported 87% colistin-resistant XDR *P. aeruginosa*, aligning with our study, where *P. aeruginosa* also exhibited significant colistin resistance [16]. The study by Abd El Baky et al. found 87% XDR *P. aeruginosa* with 45% colistin resistance. Notably, *P. aeruginosa* emerged as the most common colistin-resistant isolate, aligning with Coșeriu's et al findings, emphasizing the need for heightened vigilance in monitoring urinary tract infections [17].

Bir et al. reported 15% total colistin resistance in Carbapenem-resistant Enterobacterales (CRE) with 16% *E. coli* resistance, comparable to our findings of 11% CLR isolates among 100 XDR, with 18% *E. coli* resistance [18].

Our study's findings of *K. pneumoniae* resistance among 100 mixed isolates are consistent with an Italian investigation that found 43% CLR among 96% of carbamenase-producing *K. pneumoniae* [19]. Our study agrees with Fatima et al., having two resistant *A. baumannii* isolates. However, our small sample size and mixed isolates are study limitations[20].

Matthaiou and Kontopidou rarely stated colistin resistance in *K. pneumoniae*, *A. baumannii*, and *P. aeruginosa*, contrary to our study, which included various isolates [21,22]. Ling et al.'s study observed 2.52% resistance in *K. Oxytoa* MDR samples. The collective results indicate a significant increase in colistin resistance over time (2013–2018, 2019–2021), possibly due to increased antibiotic use [23,24].

LIMITATIONS OF STUDY

Our findings reveal emerging in vitro colistin resistance in extensively drug-resistant (XDR) strains from Pakistan, limiting therapeutic options and underscoring the global health concern of antibiotic resistance. Therapeutic use of broad-spectrum antibiotics should be

reserved for severe infections, emphasizing the need for antimicrobial surveillance and antibiotic stewardship programs. The study suggests further investigation into plasmid-mediated colistin resistance in gram-negative bacteria (GNB) to understand its definitive cause.

CONCLUSION

Limitations include a single-center study in Karachi, advocating for broader national research to establish a more reliable antibiotic susceptibility pattern against XDR. Genetic studies are crucial for confirming resistant strains, and future research should focus on the clinical significance of acquiring MCR genes and the implications of hetroresistance in colistin susceptibility testing.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

GRANT SUPPORT & FINANCIAL DISCLOSURE

Declared none

AUTHORS CONTRIBUTION

Sadia Talib: Conception, Analysis
Abeera Ahmed: Design, data analysis, interpretation, literature review
Syeda Hira Abid: Administrative support, overall supervision of study
Tahira Assad: Proofreading, literature review
Muhammad Nizamuddin and Shaista Sharif: Critical review

REFERENCES

1. Endimiani A, Ramette A, Rhoads DD, Jacobs MR. The evolving role of the clinical microbiology laboratory in identifying resistance in Gram-negative bacteria: an update. Infect Dis Clin North Am. 2020; 34(4): 659-76.
DOI: https://doi.org/10.1016/j.idc.2020.08.001
2. Pogue JM, Cohen DA, Marchaim D. Editorial commentary: Polymyxin-resistant *Acinetobacter baumannii:* urgent action needed. Clin Infect Dis. 2015 May 1;60(9):1304-7.
DOI: https://doi.org/10.1093%2Fcid%2Fciv044
3. Ayukekbong JA, Ntemgwa M, Atabe AN. The threat of antimicrobial resistance in developing countries: causes and control strategies. Antimicrobial Resistance & Infection Control. 2017; 6(1):1-8.
DOI: https://doi.org/10.1186/s13756-017-0208-x
4. Abbas S, Sabir AU, Khalid N, Sabir S, Khalid S, Haseeb S, *et al*. Frequency of extensively drug-resistant gram-negative pathogens in a tertiary care hospital in Pakistan. Cureus. 2020; 12(12). e11914.
DOI: https://doi.org/10.7759%2Fcureus.11914
5. Bilal H, Khan MN, Rehman T, Hameed MF, Yang X. Antibiotic resistance in Pakistan: a systematic review of past decade. BMC Infect Dis. 2021; 21(1):244.
DOI: https://doi.org/10.1186/s12879-021-05906-1
6. World Health Organization. Antimicrobial stewardship programmes in health-care facilities in low-and middle-income countries: A WHO practical toolkit.
7. Mlynarcik P, Kolar M. Molecular mechanisms of polymyxin resistance and detection of mcr genes. Biomed Pap Med Fac Palacky Univ Olomouc Czech Repub. 2019; 163(1).28-38.
DOI: https://doi.org/10.5507/bp.2018.070
8. Pfennigwerth N, Kaminski A, Korte-Berwanger M, Pfeifer Y, Simon M, Werner G, *et al*. Evaluation of six commercial products for colistin susceptibility testing in Enterobacterales. Clin Microbiol Infect. 2019; 25(11): 1385-9.
DOI: https://doi.org/10.1016/j.cmi.2019.03.017
9. Balouiri M, Sadiki M, Ibnsouda SK. Methods for in vitro evaluating antimicrobial activity: A review. J Pharmaceutical Analysis. 2016; 6(2): 71-9.
DOI: https://doi.org/10.1016/j.jpha.2015.11.005
10. Wiegand I, Hilpert K, Hancock RE. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nature Protocols. 2008; 3(2): 163-75.
DOI: https://doi.org/10.1038/nprot.2007.521
11. Furqan W, Ali S, Usman J, Hanif F, Naeem A, Nasrullah A, Tayyab N. Assessing Colistin resistance by phenotypic and molecular methods in carbapenem-resistant enterobacterales in a tertiary care hospital in Pakistan. Infect Drug Resist. 2022: 5899-904.
DOI: https://doi.org/10.2147/idr.s376490
12. Clinical and Laboratory Standards Institute (CLSI). Methods for dilution of antimicrobial susceptibility tests for bacteria that grow aerobically; Approved standard, 11th ed. Document M07- A11. Wayne, PA. CLSI; 2018; 1-11
13. Browne K, Chakraborty S, Chen R, Willcox MD, Black DS, Walsh WR, et al. A new era of antibiotics: The clinical potential of antimicrobial peptides. Int J Mol Sci. 2020; 21(19): 7047.
DOI: https://doi.org/10.3390%2Fijms21197047
14. Garcia-Vidal FJ, Fernández-Domínguez AI, Martin-Moreno L, Zhang HC, Tang W, Peng R, et al. Spoof surface plasmon photonics. Rev Mod Phys. 2022; 94(2): 025004.
DOI:https://doi.org/10.1103/RevModPhys.94.025004
15. Qamar S, Shaheen N, Shakoor S, Farooqi J, Jabeen K, Hasan R. Frequency of colistin and fosfomycin resistance in carbapenem-resistant

Enterobacteriaceae from a tertiary care hospital in Karachi. Infect Drug Resist. 2017: 231-6.

16. Abd El-Baky RM, Masoud SM, Mohamed DS, Waly NG, Shafik EA, Mohareb DA, *et al*. Prevalence and some possible mechanisms of colistin resistance among multidrug-resistant and extensively drug-resistant *Pseudomonas aeruginosa*. Infect Drug Resist. 2020: 323-32. DOI: https://doi.org/10.2147%2FIDR.S238811
17. Coșeriu RL, Mare AD, Toma F, Vintilă C, Ciurea CN, Togănel RO, *et al*. Uncovering the resistance mechanisms in extended-drug-resistant *pseudomonas aeruginosa* clinical isolates: insights from gene expression and phenotypic tests. Microorganisms. 2023; 11(9): 2211. DOI:https://doi.org/10.3390/microorganisms11092211
18. Bir R, Gautam H, Arif N, Chakravarti P, Verma J, Banerjee S, et al. Analysis of colistin resistance in carbapenem-resistant Enterobacterales and XDR *Klebsiella pneumoniae*. Ther Adv Infect Dis. 2022; 9: 20499361221080650. https://doi.org/10.1177/20499361221080650
19. Rojas LJ, Salim M, Cober E, Richter SS, Perez F, Salata RA, et al. Colistin resistance in carbapenem-resistant *Klebsiella pneumoniae*: laboratory detection and impact on mortality. Clin Infect Dis. 2017; 64(6): 711-8. DOI: https://doi.org/10.1093/cid/ciw805
20. Sana F, Satti L, Zaman G, Ikram A, Gardezi AH, Khadim MT. In Vitro comparison of disk diffusion method and agar dilution method for sensitivity of polymyxin B against Multi Drug Resistant *Acinetobacter Baumannii*. Pak Armed Forces Med J. 2019; 69(5): 998-1003.
21. Matthaiou DK, Michalopoulos A, Rafailidis PI, Karageorgopoulos DE, Papaioannou V, Ntani G, *et al*. Risk factors associated with the isolation of colistin-resistant gram-negative bacteria: A matched case-control study. Crit Care Med. 2008; 36(3): 807-11. DOI:https://doi.org/10.1097/ccm.0b013e3181652fae
22. Kontopidou F, Giamarellou H, Katerelos P, Maragos A, Kioumis I, Trikka-Graphakos E, *et al*. Infections caused by carbapenem-resistant *Klebsiella pneumoniae* among patients in intensive care units in Greece: A multi-centre study on clinical outcome and therapeutic options. Clin Microb Infect. 2014; 20(2): O117-23. DOI: https://doi.org/10.1111/1469-0691.12341
23. Ghasemian A, Mobarez AM, Peerayeh SN, Abadi AT, Khodaparast S, Nojoomi F. Report of plasmid-mediated colistin resistance in *Klebsiella oxytoca* from Iran. Rev Med Microbiol. 2018; 29(2): 59-63. DOI:https://doi.org/10.1097/MRM.0000000000000134
24. Uzairue LI, Rabaan AA, Adewumi FA, Okolie OJ, Folorunso JB, Bakhrebah MA, *et al*. Global prevalence of colistin resistance in *Klebsiella pneumoniae* from bloodstream infection: A systematic review and meta-analysis. Pathogens. 2022; 11(10): 1092. DOI: https://doi.org/10.3390/pathogens11101092

The clinical significance of CRP and IL-6 in early recognition of neonatal sepsis

Farheen Aslam[1], Muhammad Wajid Khurshid Sipra[1], Faizan Ahmed Zakir[1], Zain Ul Abeden Anwar[2], Asma Shaukat[1]

[1]Quaid-e-Azam Medical College, Bahawalpur Pakistan
[2]Bahawal Victoria Hospital, Bahawalpur Pakistan

ABSTRACT

Objective: The aim of this study was to determine serum CRP and IL-6 levels in confirmed and clinically suspected groups of neonatal sepsis and these biochemical parameters are being compared with blood culture of neonates with clinical signs and symptoms of sepsis.

Material and Methods: The samples of 180 suspected cases of neonatal sepsis were sent to pathology department. Out of them 92 were proven positive for blood culture and were considered as confirmed septic group while rests were included in clinically suspected group. Blood culture was performed on BACT/ALERT® 3D. Serum CRP was performed by immunoturidimetric method on AU 680 Beckman Coulter and IL-6 measurement was done on Access 2.

Results: The serum CRP and IL-6 levels were higher in confirmed sepsis group and their levels were in correspondence to severity of infection. At cut off values of >10mg/L, CRP showed sensitivity of 94%, and specificity of 74% while at cut off of >35ng/L, IL-6 had sensitivity and specificity of 70% and 88% respectively.

Conclusion: The results of this study suggested that CRP was a highly sensitive marker and IL-6 was a more specific for detection of neonatal sepsis.

Keywords: Neonatal sepsis, CRP, IL-6

This article can be cited as: Aslam F, Sipra MWK, Zakir FA, Anwar ZA, Shaukat A. The clinical significance of CRP and IL-6 in early recognition of neonatal sepsis. Pak J Pathol. 2024; 35(1): 13-19.

DOI: https://doi.org/10.55629/pakjpathol.v35i1.775

INTRODUCTION

Neonatal sepsis is a condition in which signs and symptoms of systemic infection are manifested in first month of patient's life [1]. It is one of major contributing factors that threatens the survival of neonate and causes permanent organ damage and disability in the child. As the immune system of neonates is fragile and weak so they are vulnerable of being infected by bacterial, fungal and viral infections and are susceptible to develop neonatal sepsis earlier [2]. The prognosis of neonatal sepsis largely relies on early diagnosis and effective treatment strategies [3]. The under developed countries like Pakistan that spend inadequate financial resources on health care system face difficulties in early identification and dealing with complications of neonatal infection [3,4].

The blood culture is a routinely performed procedure for recognition of neonatal septicemia. Although with advent of new techniques and introduction of automation in blood culture analysis, it is still a tedious process [4,5]. Early recognition of sepsis will prevent excess use of antibiotics, treatment cost and reduce probability of development of drug resistant strains. The different biomarkers in the form of acute phase proteins, adhesion molecules, cell surface receptors, chemokines and pro-inflammatory cytokines are employed for early identification of neonatal sepsis. Tumour necrosis factor- alpha (TNF-α), Interleukin-6 (IL-6), Interleukin-8 (IL-8), C-reactive protein (CRP), high sensitive C-reactive

Correspondence: **Dr. Farheen Aslam, Department of Pathology, Quaid-E-Azam Medical College, Bahawalpur Pakistan**

Email: farheenaslam75@yahoo.com

Receiving Date: **20 Jul 2023**
Revision Date: **03 Jan 2024**
Acceptance Date: **26 Feb 2024**

protein (hs-CRP) and pro-calcitonin (PCT) are commonly used and their effectiveness as diagnostic markers either alone or in combination is being studied [2,6].

The previous studies applied different inclusion and exclusion criteria, sampling protocols, inflammatory biomarkers and analytical techniques for timely and rapid detection of neonatal sepsis and monitoring efficacy of treatment [6,7,8].

CRP is an acute-phase reactant that is produced in liver within 4-6 hours as a result of raised levels of different cytokines i.e. IL-1 and TNF that are produced under influence of tissue injury and inflammatory response of the body. CRP has doubling time of 8 hours and its peak is reached within 36 hours [3,8].

Interleukin 6 (IL-6), a chemokine produced by the T and B lymphocytes. IL-6 level starts to rise in early stage of bacterial infections and can be measured for early identification of neonatal sepsis. As it has short life, it cannot be used as a sole marker of sepsis [9,10].

The aim of this study was to determine serum IL-6 and CRP levels in confirmed and clinically suspected groups of neonatal sepsis and these biochemical parameters are being compared with blood culture results in neonates with clinical signs and symptoms of sepsis.

MATERIAL AND METHODS

This cross-sectional study was carried out in Pathology Department of Quaid-e-Azam Medical collage Bahawalpur in collaboration with Pediatric Department of Bahawal Victoria Hospital (BVH) Bahawalpur. The study was conducted on neonates (<28 days of age) of either sex who presented with sign and symptoms of sepsis in Pediatric wards of Bahawal Victoria Hospital, Bahawalpur. The sampling technique was non probability convenience. Sample size was calculated by Raosoft, Inc [10]. Blood samples were taken from suspected cases of neonatal sepsis for duration of 6 months from October 2022 to March 2023. The samples were taken from neonates who showed at least three clinical manifestations. These are shortness of breath, more than 70 breathing rate per minute, temperature instability, feeding intolerance, weakness, hepatosplenomegaly, irritability, tachycardia (HR>190bpmin) or bradycardia (HR<90bpmin). All the neonates having congenital anomalies, taking antibiotics or having surgical intervention were excluded from the study. The parents of study participants gave informed consent. The participants were divided on basis of blood culture reports into sepsis proven (positive blood culture report) and clinically suspected (blood culture negative report) groups. The study was conducted after taking approval from the Institution Ethical committee.

The venous blood was collected and about one ml of blood was transferred under complete aseptic conditions into the specified blood culture bottle and inserted in BACT/ALERT® 3D of BioMerieux. The culture bottles were then incubated at 37°C for upto 5 days for growth of microorganisms. On receiving signal from instrument, positive samples were stained and sub cultured according to manufacturer's recommendation under controlled environmental condition. The subcultures were done on blood agar, chocolate agar, Bile Esculin agar, MacConkey agar Sabouraud dextrose agar. Gram staining and specified biochemical methods were used for isolation of microorganisms. Anaerobic culture was not performed. Coagulase-negative staphylococci were not included in culture positive sepsis group due to suspicion of contamination. Rest of blood was collected in clot activator tube and CRP and IL -6 were measured after samples were centrifuged for ten minutes at 3000 rpm. Serum CRP levels were determined by turbidimetric method on AU 680 Beckman Coulter fully automated chemistry analyzer. In this procedure, the rate of decline in transmitted light signal after striking with suspended particles are produced due to immunological interaction between CRP in sample and coated rabbit anti-CRP-antibodies present on latex particles was measured. The IL- 6 was estimated by simultaneous one-step immune enzymatic ("sandwich") assay on Access 2 hormone Analyzer of Beckman Coulter.

Mean and standard deviation were calculated by using SPSS software version 21. The difference between the proven sepsis group and clinically suspected sepsis group for CRP and IL-6 were assessed using student t-test and p-value less than 0.05 was considered statistically significant. The sensitivity, specificity, Positive Predictive Value (PPV), Negative Predictive Value (NPV) for CRP and IL-6 were calculated by using Graph Pad prism software (version 5, 2007, San Diego, California, USA). Receiver Operating Characteristics (ROC) curve was constructed to determine the sensitivity and specificity of selected analytes. Area under the Curve (AUC) was computed and analyzed by SPSS software version 21.

RESULTS

The samples of 180 suspected cases of neonatal sepsis were sent to pathology department. Out of them 92 were proven positive on blood culture and were considered as confirmed sepsis group while rest were included in clinically suspected group. On stratification of the total subjects into three age groups; 1-10 days, 11-19 days, 20-28 days it was observed that 105 (58%) neonates belong to 1-10 days age group, 65 (36%) were in 2nd group and rest of them (6%) fell in third group. More than half (60%) of study participants were male. On analysis, blood culture positive and negative groups did not show significant difference in age, sex distribution or birth weight (Table-I). Among the culture positive cases, Staph Aureus was the most common organism and was isolated in 35 culture positive cases while Pseudomonas species were detected in 21 cases.

Serum levels of IL-6 were significantly raised in cases of Staph Aureus and Pseudomonas Aeruginosa infections as compared to others septic cases. Similarly, neonates infected by Micrococci and candida Albicans showed higher concentrations of CRP in comparison to other microbial infection. The serum CRP levels in culture positive cases were raised (44.02±24.90mg/L) as compared to clinically suspected neonatal group (11.88±18.12mg/L) and there was statistically significant difference (p-value <0.0001) between two groups. The IL-6 level in the serum of proven sepsis group was significantly elevated (87.04±68.95ng/L) than clinically suspected group (25.68±32.05) (p-value <0.0001) (Table).

The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of CRP were 94%, 74%, 78% and 93% while IL6 showed 70%, 88%, 80% and 73%. The cut off values of these parameters were also estimated. The optimal cut-of values of CRP and IL-6 in proven cases of sepsis were >10mg/L and > 35 ng/L versus clinically suspected cases.

ROC of the CRP [Fig-2] and IL-6 [Fig-3] shows the sensitivity of 94% and 70%, and specificity of 74% and 88% respectively. ROC analysis shows AUC of nCRP is 0.89 with standard error 0.024 at 95% Confidence Interval for lower and upper level are 0.81to 0.92 while that of IL-6 is 0.80 with lower and upper bound from 0.79 to 0.90.

Table-I: Demographic characteristics of both groups.

Groups	Confirmed Sepsis	Clinically Suspected Sepsis
Individuals	92	88
Gender wise distribution		
Male	57 (62%)	50(57%)
Female	35 (38%)	38(43%)
Age wise distribution(days)		
1-10	54 (59%)	51 (58%)
11-19	33(36%)	32(36%)
21-28	05(5%)	05 (6%)
Weight (kg)	2.58± 0.36	2.50±0.38

Table-II: Comparison of analytes between two groups.

Analytes	Confirmed Sepsis (Mean ± SD)	Clinically Suspected Sepsis (Mean ± SD)	p-value
CRP (mg/L)	44.02±24.90	11.88±18.12	<0.0001
IL-6 (ng/L)	87.04±68.95	25.68±32.05	<0.0001

Table-III: The diagnostic accuracy of CRP and IL-6

Analytes	Sensitivity %	Specificity %	PPV %	NPV %
CRP	94	74	78	93
IL-6	70	88	80	73

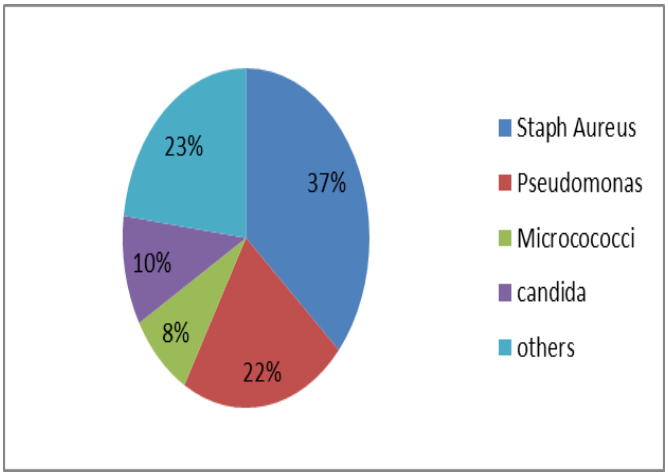

Figure-I: Organisms isolated on basis of blood culture.

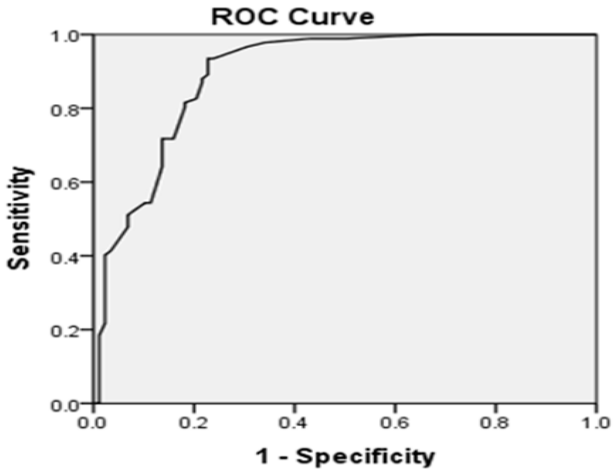

Figure-II: Receiver operating characteristics curve of CRP at Cutoff >10 mg/L.

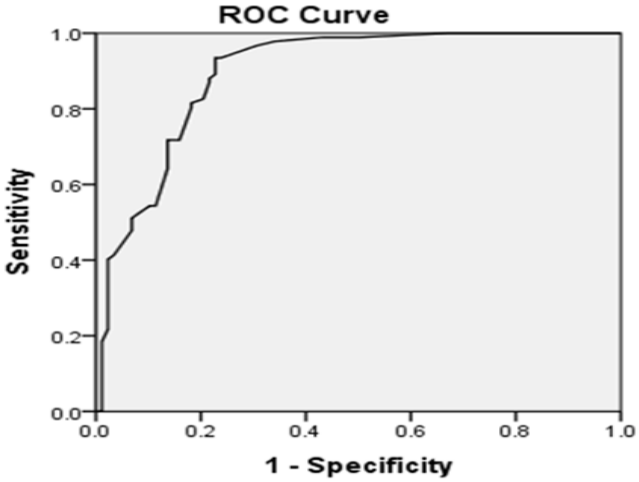

Figure-III: Receiver Operating Characteristics Curve of IL-6 at Cutoff >35 ng/L

DISCUSSION

Neonatal sepsis has major contributory role in neonatal mortality due to delay in appearance of symptoms and non-specific clinical manifestation [7]. In our study, Staphylococcus Aureus and Pseudomonas Aeruginosa were the common microorganisms detected from blood culture positive group but other studies isolated the growth of Klebsiella Pneumoniae, Coagulase negative Staphylococcus, Group B Streptococcus and Escherichia Coli in culture positive group. [6, 11, 12] Although blood culture is most commonly used for the diagnosis of sepsis but it is lengthy and laborious procedure and is often negative due to inadequate blood sample volume, prior administration of antibiotics and low level bacteraemia [5]. Total leukocyte count (TLC) is also helpful in detection of neonatal septicemia but its large range and unavailability of population based and age-related reference ranges in neonates make it difficult to interpret the results.

Various chemical and immunological biomarkers i,e CRP, IL-6, tumor necrosis factor (TNF), procalcitonin, and E-selectin are raised in the blood during neonatal sepsis. But IL-6 and CRP are most commonly used for the diagnosis of neonatal sepsis. In this study, IL-6 was evaluated in culture positive confirmed cases and clinically suspected group to find out sensitivity and reliability for detection of neonatal sepsis. The marker showed significant

difference between two groups. IL-6 levels rises earlier in response to infection. On exposure to bacterial products, IL-6 is produced by lymphocytes, macrophages and fibroblasts that promotes secretion of various other inflammatory mediators and acute phase proteins [13].

IL-6 has short half-life, reaches peak within 3 hours, its level significantly decreases 24–48 hours after the emergence of signs and symptoms of disease. As a result, its diagnostic sensitivity correlates with timing of sample collection but if its level is persistently elevated then it is indicator of poor prognosis. Therefore, it can be used as diagnostic marker and to assess the intensity and prognosis of disease process [14].

The results of our study were in harmony with other studies showing higher levels of the serum in the culture positive confirmed septic group as compared to clinical suspected septic group [11,12,13]. The result of one study showed higher levels of IL-6 even in umbilical blood samples of neonates suspected to have sepsis [15].

In our study the sensitivity and specificity of IL-6 was 70% and 88% at cut off of 35ng/L which correlated well with that of study by Küng *et al,* who observed early predictability of IL-6 at cut off level of 80 ng/L for the sepsis detection with sensitivity of 75% and a specificity of 81% [12]. The sensitivity and specificity of 75% and 72.8% with an area under the curve of 0.804 at a cut-off of 40 ng/L were recorded in retrospective nested case-control study [14]. However, an Indian study reported 100% sensitivity and specificity of 62.86% at cut off of 51.29 pg/ml [16].

The different results reported in globally diverse studies were due to timing and type of sample collection, age at time of infection, difference in selection criteria and cut off values used for measurement of IL-6.

The C-reactive protein is commonly employed marker for early detection of various infective conditions. CRP is produced by the liver within 6–8 hours of infection and serves as late acute phase reactant, peaks within 48 hour and its level declines as the inflammation subsides. The study conducted in East India reported significantly higher CRP levels in the suspected septic group as compared to normal subjects [17]. Our study reported higher level of CRP in both confirmed and suspected groups but statistically significantly raised levels were recorded in confirmed septic group. The results are consistent with study of Zeng *et al* that exhibited significantly higher results in organ dysfunction septic group [15]. The results of 1098 suspected septic neonates revealed higher CRP levels in confirmed septic group and there was direct relationship between severity of sepsis and serum CRP concentration [18]. These findings supported the results of our study.

CRP levels may correlate with degree of infection and tissue damage and its level is not dependent on gender, age and body temperature. Similarly, degree of anemia, obesity and pregnancy do not show any impact on its level [17,19]. Meta-analysis of 22 globally different studies on CRP reported median sensitivity and specificity of 62% and 74% respectively. This study suggested that CRP should not be used as a biomarker for early diagnosis and also had limited role in predicting duration of antibiotic therapy in neonatal sepsis [19]. The results of a Pakistani study also showed low validity of CRP at cut off level of 5 mg/L with sensitivity of 35.53% [20]. Alternatively, some studies proposed that CRP could be used as a screening marker for rapid detection of neonatal sepsis supporting our results. Kaur *et al* evaluated different parameters of neonatal sepsis to create strategy for early diagnosis and found that CRP had highest sensitivity (87%) at > 6 mg/L [21] while Ganesan *et al* based-on CRP >13.495 mg/L reported sensitivity of 80% and specificity of 65.7% [16]. One study showed that serial monitoring of CRP along with cytokines IL-6 and IL-8 would be helpful in improving diagnostic accuracy of sepsis and evaluating the antibiotic response [10]. In our study, at cut off level of >10mg/L CRP had sensitivity of 93% with specificity of 76% showing that nCRP is better predictor of neonatal sepsis. The outcome of another study supported our findings that CRP

had high sensitivity with NPV of 98-100% in early detection of neonatal sepsis [22]. So, both CRP and IL-6 should be used for rapid and early detection of neonatal sepsis as one has high sensitivity and other shows high specificity.

CONCLUSION

The study concluded that CRP has better sensitivity while IL-6 has better specificity for diagnosis of neonatal sepsis. As the sample size of this study is small so multicenter trials should be conducted to standardize the selection criteria of neonates, optimum cut-off levels, and methodology for measurements of various inflammatory markers like CRP and IL-6.

LIMITATIONS OF STUDY

Limitations to the study include the fact that it is a single-center study. The diagnostic validity of combine IL-6 and CRP parameters is not estimated. Study outcome would have been improved if Sequential Organ Failure Assessment (SOFA) Score or any other scientific criteria were used to label sepsis. Serial estimation of CRP and IL-6 should be performed to monitor prognosis of disease and to modify treatment accordingly.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

GRANT SUPPORT & FINANCIAL DISCLOSURE

Declared none

AUTHORS CONTRIBUTION

Farheen Aslam: Concept, data collection, drafting and data analysis.

Muhammad Wajid Khurshid Sipra: Data interpretation and work design

Faizan Ahmed Zakir: Questionnaire design, data collection, revision and finalization of results

Zain Ul Abeden Anwar: Study design, Data collection

Asma Shaukat: Reviewed and finally approved the manuscript for submission to the journal

REFERENCES

1. Fleischmann SC, Goldfarb DM, Schlattmann P, Schlapbach LJ, Reinhart K, Kissoon N. The global burden of paediatric and neonatal sepsis: a systematic review. Lancet Respir Med. 2018; 6: 223-30. DOI:https://doi.org/10.1016/s2213-2600(18)30063-8
2. Tzialla C, Manzoni P, Achille C, Bollani L, Stronati M, Borghesi A. New diagnostic possibilities for neonatal sepsis. Am J Perinatol. 2018; 35(6): 575-7. DOI: https://doi.org/10.1055/s-0038-1639361
3. Sun D, Wang Q, Zhang X, Zhao X, Zhang H, Liu A. Clinical application of serum inflammatory factors combined with dynamic detection in the diagnosis and treatment of neonatal sepsis. Iran J Public Health. 2021; 50(2): 325-32. DOI:https://doi.org/10.18502%2Fijph.v50i2.5347
4. Miller JM, Binnicker MJ, Campbell S, Carroll KC, Chapin KC, Gilligan PH, *et al*. A Guide to utilization of the microbiology laboratory for diagnosis of infectious diseases: 2018 Update by the Infectious Diseases Society of America and the American Society for Microbiology. Clin Infect Dis. 2018; 67(6): e1-e94. DOI: https://doi.org/10.1093/cid/ciy381
5. Scheer CS, Fuchs C, Gründling M, Vollmer M, Bast J, Bohnert JA, *et al*. Impact of antibiotic administration on blood culture positivity at the beginning of sepsis: A prospective clinical cohort study. Clin Microbiol Infect. 2019; 25(3): 326-31. DOI: https://doi.org/10.1016/j.cmi.2018.05.016
6. Ershad M, Mostafa A, Cruz DM, Vearrier D. Neonatal sepsis. Curr Emerg Hosp Med Rep. 2019; 7(3): 83-90. DOI: https://doi.org/10.1007/s40138-019-00188-z
7. Opal SM, Wittebole X. Biomarkers of infection and sepsis. Crit Care Clin. 2020; 36(1): 11–22. DOI: https://doi.org/10.1016/j.ccc.2019.08.002
8. Eschborn S, Weitkamp JH. Procalcitonin versus C-reactive protein: Review of kinetics and performance for diagnosis of neonatal sepsis. J Perinatol. 2019; 39(7): 893-903. DOI: https://doi.org/10.1038/s41372-019-0363-4
9. Xu XJ, Luo ZB, Xia T, Song H, Yang SL, Xu WQ, *et al*. Comparison of interleukin-6, interleukin-10, procalcitonin and C-reactive protein in identifying high-risk febrile illness in pediatric cancer patients: A prospective observational study. Cytokine. 2019; 116: 1-6. DOI: https://doi.org/10.1016/j.cyto.2019.01.004
10. Tessema B, Lippmann N, Willenberg A, Knüpfer M, Sack U, König B. The diagnostic performance of interleukin-6 and C-reactive protein for early identification of neonatal sepsis. Diagnostics (Basel). 2020; 10(11): 978. DOI: https://doi.org/10.3390/diagnostics10110978
11. Kuzniewicz MW, Mukhopadhyay S, Li S, Walsh EM, Puopolo KM. Time to positivity of neonatal blood cultures for early-onset sepsis. Pediatr Infect Dis J. 2020; 39(7): 634-40. DOI:https://doi.org/10.1097/INF.0000000000002632
12. Küng E, Unterasinger L, Waldhör T, Berger A, Wisgrill L. Cut-off values of serum interleukin-6 for

culture-confirmed sepsis in neonates. Pediatr Res. 2023; 93(7): 1969-74.
DOI: https://doi.org/10.1038/s41390-022-02329-9

13. Eichberger J, Resch B. Reliability of interleukin-6 alone and in combination for diagnosis of early onset neonatal sepsis: Systematic review. Front Pediatr. 2022; 10: 840778.
DOI: https://doi.org/10.3389/fped.2022.840778
14. Ebenebe CU, Hesse F, Blohm ME, Jung R, Kunzmann S, Singer D. Diagnostic accuracy of interleukin-6 for early-onset sepsis in preterm neonates. J Matern Fetal Neonatal Med. 2021; 34(2): 253-8.
DOI: https://doi.org/10.1080/14767058.2019
15. Zeng G, Chen D, Zhou R, Zhao X, Ye C, Tao H, *et al*. Combination of C-reactive protein, procalcitonin, IL-6, IL-8, and IL-10 for early diagnosis of hyperinflammatory state and organ dysfunction in pediatric sepsis. J Clin Lab Anal. 2022; 36(7): e24505.
DOI: https://doi.org/10.1002/jcla.24505
16. Ganesan P, Shanmugam P, Sattar SB, Shankar SL. Evaluation of IL-6, CRP and hs-CRP as early markers of neonatal sepsis. J Clin Diagn Res. 2016; 10(5): DC13-7.
DOI: https://doi.org/10.7860/JCDR/2016/19214.7764
17. Chatterjee K, Mandal PK, Rahaman SR, Malathi R, Ray SK, Dutta A. Raised IL-6 and C-reactive protein in neonatal sepsis in Eastern India. Int J Contemp Pediatr. 2017 Sep;4(5):1590-4.
DOI:https://doi.org/10.18203/2349-3291.ijcp20173124
18. Li X, Li T, Wang J, Feng Y, Ren C, Xu Z, Yang J, *et al*. Clinical value of C-reactive protein/ platelet ratio in neonatal sepsis: A Cross-Sectional Study. J Inflamm Res. 2021; 14: 5123-9.
DOI: https://doi.org/10.2147/JIR.S334642
19. Burstein B, Beltempo M, Fontela PS. Role of C-reactive protein for late-onset neonatal sepsis. JAMA Pediatr. 2021;175(1):101–102.
DOI:https://doi.org/10.1001/jamapediatrics.2020.2129
20. Khan F. C-reactive protein as a screening biomarker in neonatal sepsis. J Coll Physicians Surg Pak. 2019; 29(10): 951-53.
DOI: https://doi.org/10.29271/jcpsp.2019.10.951
21. Kaur S, Singh K. Early-onset neonatal sepsis: Role of C-reactive protein, micro-ESR, and gastric aspirate for polymorphs as screening markers. Int J Pediatr. 2021; 2021:1544553.
DOI: https://doi.org/10.1155/2021/1544553
22. Chaudhuri PK, Ghosh A, Sinha V, Singh BK, Singh M, Lugova H, *et al*. The role of C-reactive protein estimation in determining the duration of antibiotic therapy in neonatal sepsis. Cureus. 2022 12; 14(10): e30211.
DOI: https://doi.org/10.7759/cureus.30211

.

Frequency of loss of calretinin expression in clinically susceptible cases of Hirschsprung disease in rectal biopsies

Ayesha Haider[1], Unaiza Jamil[1], Iqra Ahmad Shah[1], Maria Aslam[2], Kiran Mumtaz[1], Syed Naeem Raza Hamdani[1]

[1]Combined Military Hospital, Multan Pakistan
[2]Combined Military Hospital, Abbottabad Pakistan

ABSTRACT

Objective: To determine the frequency of loss of calretinin expression in clinically susceptible cases of Hirschprung disease in rectal biopsies.

Material and Methods: This descriptive, cross-sectional study was conducted at Department of Histopathology, Combined Military Hospital, Multan for a period of 6 months (17th Oct 2021 to 16th April 2022). A total 93 suspected cases of Hirschprung disease were taken. Specimens received in 10 % buffered formalin and fulfilling inclusion criteria were included in the study. The cases diagnosed as Hirschprung disease were taken and calretinin immunohistochemical stain was applied and result recorded after evaluation by consultant histopathologist. Data was analyzed using (Statistical Software for Social Sciences (SPSS) version 19).

Results: Out of 93 cases, 67 (72.0%) were male patients, whereas 26 (28.0%) were female patients. The average age of the study participants was 7.95 months, with a minimum age of 1 month and a maximum age of 12 months. Twenty-eight (30.1%) of the 93 study cases were from rural areas, while 65 (69.9%) were from metropolitan areas. Twenty-five families (26.9%) reported monthly incomes of up to Rs. 35000, while 68 families (73.1%) reported incomes of over Rs. 35,000. Of these 93 study cases, failure to pass meconium was noted in 24 (25.8%) patients, constipation was noted in 75 (80.6%) and abdominal distension was noted in 62 (66.7%) patients. Loss of calretinin expression was noted in 34 (36.6%) of our study cases.

Conclusion: According to the findings of our investigation, calretinin is very helpful in identifying suspicious cases of Hirschsprung disease. When acetylcholinesterase enzyme histochemistry is not accessible, it can be a helpful, cost-effective diagnostic assistance in centers with limited resources. It is a reliable and efficient diagnostic tool for early diagnosis of the illness. Loss of Calretinin expression was significantly associated with age and residential status.

Keywords: Calretinin expression, Frequency, Hirschsprung disease, Immunohistochemistry

This article can be cited as: Haider A, Jamil U, Shah IA, Aslam M, Mumtaz K, Hamdani SNR. Frequency of loss of calretinin expression in clinically susceptible cases of Hirschprung disease in rectal biopsies. Pak J Pathol. 2024; 35(1): 20-25.

DOI: https://doi.org/10.55629/pakjpathol.v35i1.784

INTRODUCTION

The enteric nervous system is the part of the autonomic nervous system that controls the coordination of gastrointestinal tract [1]. It originates from a population of multipotent migratory cells known as neural crest cells. Neurocristopathies are conditions that occur from abnormal neural crest cell development. One of such disorders is Hirschprung disease. Hirschprung illness, which affects 1 in 5000 live births, causes the patient to present with abdominal distension and an inability to pass meconium; this condition typically necessitates surgical removal of the aganglionic intestine [1]. A frequently encountered congenital illness, Hirschsprung disease (HD) is brought on by a lack of parasympathetic ganglion cells in the intramucosal and submucosal plexuses [2]. It is

Correspondence: **Dr. Maria Aslam, Assistant Professor, Consultant Histopathologist, Combined Military Hospital Abbottabad, Pakistan**

Email: drchickywicky@gmail.com

Receiving Date: **02 Oct 2023** Revision Date: **14 Feb 2024** Acceptance Date: **19 Mar 2024**

mostly diagnosed during first year of life in children. The definitive diagnosis of HD is made by histologic demonstration of absence of ganglion cells in both the myenteric (Auerbach) plexus which lies in longitudinal and inner circular smooth muscle fibers layers in intestine and the sub-mucosal (Meissner) plexus, even though anorectal manometry or radiologic investigations are also employed to establish the diagnosis [3]. Hirschsprung disease is identified when there are no ganglion cells present in rectal biopsies. There may also be hypertrophied submucosal nerve trunks that are larger than 40 microns in diameter [4].

There are several reasons for the development of HD. It can both run in families or manifest spontaneously. Boy babies have been observed to be more susceptible than girls to develop this illness. The symptoms of HD can range from a persistently increasing constipation in older children to a sudden intestinal blockage in newborn. In the first few months of life, over 80% of patients show growing abdominal distention, poor feeding, and problematic bowel movements.

Because it necessitates specific tissue handling, acetylcholinesterase histochemistry (AChE) helps diagnosis but is not widely accepted [5]. This technique requires frozen tissue samples and there are chances of false-negative results mostly due to the young age of patients [3]. Since the loss of calretinin immunoreactive neurons is correlated with aganglionosis, calretinin immunohistochemistry (IHC) may be a beneficial alternative [5]. A study conducted in India indicated loss of calretinin expression in 40.45% cases [6]. In this study, any issues arising due to the combination of histology and acetylcholinesterase labeling are resolved by calretinin immunohistochemistry, which reliably detected almost all HD cases without producing any false positive results [6]. It indicated calretinin sensitivity as 91.4% and a 100% specificity in HD cases.

In this study loss of calretinin expression will be observed in rectal biopsy specimen using immunohistochemistry. This study is conducted with the purpose of diagnosing clinically suspicious cases of Hirschsprung disease and providing a reliable diagnostic tool. As very little information at the national level is available on this topic, the findings of this study may lead to establishing a baseline data of local population.

MATERIAL AND METHODS

This descriptive cross-sectional study was conducted at the Department of Histopathology, Combined Military Hospital, Multan, from October 17th 2021 to April 16th 2022 for a period of 6 months, following approval of the ethics review committee. Using the WHO sample size calculator, a sample size of 93 was determined with a confidence level of 95, an anticipated population proportion of 40.45%, a margin of error of 10%, and a sample size of 93. After receiving informed consent patients were chosen using a non-probability consecutive sampling technique.

Biopsy samples received in 10% buffered formalin taken from patients of both genders of any age were included in the study. Inadequate sample (Biopsy not including submucosa and muscularis mucosae), unfixed tissues and poorly stained slides were excluded from this study.

The samples were processed after being embedded in a cassette and fixed in 10% buffered formalin. Hematoxylin and eosin (H&E) were used to stain the sections after they were cut at a thickness of 3-5 microns. The cases diagnosed as Hirschsprung's disease were taken and calretinin immunohistochemical stain was applied and the result was recorded after evaluation by a consultant histopathologist. The bias for inter-observer variation was minimized by showing all cases to two different consultant histopathologists. Confounding variables were minimized by strictly adhering to inclusion and exclusion criteria. Endpoint of the study was the presence or absence of calretinin immunohistochemical stain.

Data was analyzed by using Statistical Software for Social Sciences (SPSS) version 19. Mean $\pm$ SD were calculated for quantitative variables like age. Frequency and percentages were calculated for qualitative variables like gender, age groups, residential status, socioeconomic status, failure to pass meconium,

abdominal distension, constipation and diagnosis. Chi-square test after stratification was used for analysis with a significant p-value of <0.05.

RESULTS

A total of 93 patients who met the inclusion criteria for our study were included. Out of these patients, 67 (72.0%) of the patients were male and 26 (28.0%) were female (Table-I). Mean age of the patients was 7.95 ± 3.00 months (ranging from 1 month till 12 months). Mean age of the male patients was 7.45 ± 2.96 months, while female patients was 9.23 ± 2.77 months.

Twenty-eight (30.1%) of the 93 study cases were from rural areas, while 65 (69.9%) were from urban areas. Twenty-five families (26.9%) reported monthly income of up to 35000.00 PKR, while 68 families (73.1%) reported income of over 35,000.00 PKR. Of these 93 study cases, failure to pass meconium was noted in 24 (25.8%), constipation was noted in 75 (80.6%) and abdominal distension was noted in 62 (66.7%) as shown in Table-I.

In 34 (36.6%) of the study cases, there was a loss in calretinin expression (Table-II). Association and significance of calretinin expression was checked with gender, age, monthly family income, residential status, inability to pass meconium, constipation, and abdominal distension. A significant association (p-value <0.05) was seen with age and residential status. There was not any significant association of loss of calretinin expression with gender, monthly family income, residential status, failure to pass meconium or constipation.

Table-I: Frequency and percentages of qualitative variables.

Variables		Frequency	Percentage (%)
Age	< 6 months	38	40.9
	>6 months	55	59.1
Gender	Male	67	72.0
	Female	26	28.0
Monthly income	< 35000	25	26.9
	>35000	68	73.1
Residential status	**Rural**	28	30.1
	Urban		
Failure to pass meconium	Yes	24	25.8
	No	69	74.2
Constipation	Yes	75	80.6
	No	18	19.4
Abdominal distention	Yes	62	66.7
	No	31	33.3
Loss of Calretinin	Yes	34	36.6
	No	59	63.4

Table-II: Association of loss of calretinin with regards to gender, age, monthly family income, residential status, failure to pass meconium, constipation and abdominal distension.

Variables		Yes (loss of calretinin) n=34	No (No loss of calretinin) n=59	p-value
Gender	Male (n=67)	23	44	0.483
	Female (n=26)	11	15	
Age	Up to 6 months (n=38)	30	08	0.000
	More than 6 months (n=55)	04	51	
Residential status	Rural (n=28)	16	12	0.010
	Urban (n=16)	18	47	
Monthly family income	Up to 35000.00 (n=25)	07	18	0.341
	More than 35000.00 (n=68)	27	41	
Failure to pass meconium	Yes (n=24)	11	13	0.328
	No (n=69)	23	46	
Constipation	Yes (n=75)	27	48	1.000
	No (n=18)	07	11	

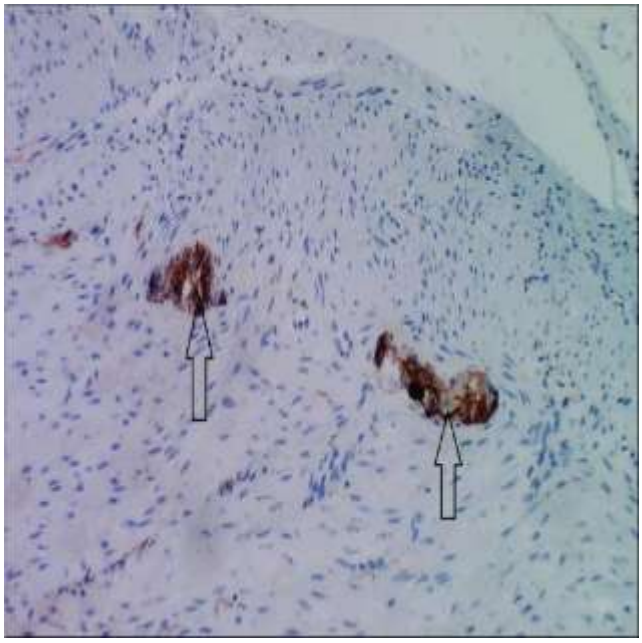

Figure-I: Calretinin positivity in rectal biopsy (IHC x40).

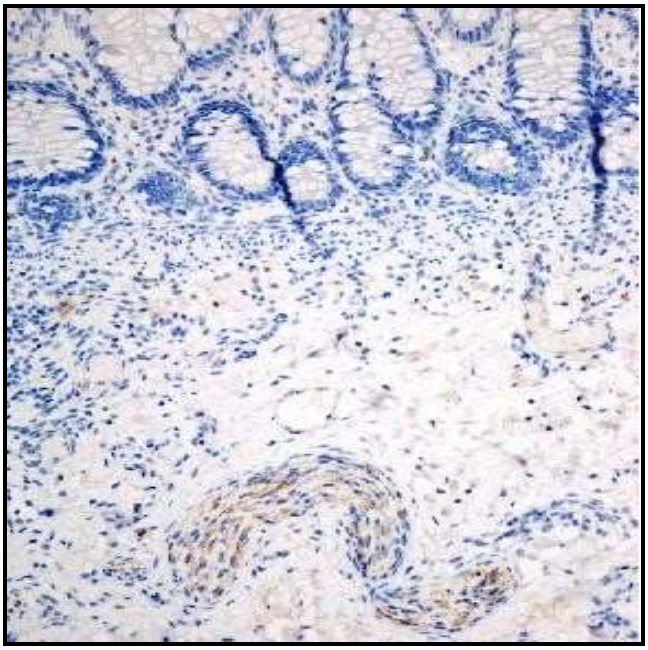

Figure-II: Calretinin negative in Hirschsprung disease (IHC x40).

DISCUSSION

Hirschsprung disease, often referred to as congenital megacolon or congenital colonic aganglionosis, is a developmental illness that is characterized by a lack of ganglion cells in the submucosal (Meissner's) and myenteric (Aurbach's) plexuses in the distal bowel extending proximally for various distances. This leads to a functional intestinal blockage brought on by the afflicted segment's dysmotility. With a frequency of around 1 in 5,000 live births, it is one of the most prevalent surgical diseases in children. Because they are unable to migrate cephalocaudally via the neural crest between the fourth and the 12th week of pregnancy, ganglion cells are lacking in all or part of the colon as a result of Hirschsprung disease. The aganglionic segment typically projects proximally from the anus. The recto-sigmoid portion of the colon is where short-segment disease is most prevalent. Beyond this area, long-segment illness can infect the entire colon. The large and small intestines are infrequently involved.

The main modalities of diagnosis include radiographic examinations, anorectal manometry, and histological investigation of rectal wall samples [18]. A total of 93 biopsy samples were examined in this study. Among the 93 study cases, 67 (72.0%) patients were male and 26 (28.0%) were female. Calretinin positivity was also observed in male patients predominantly. Numerous other researches have also revealed similar male gender predominance. A study from Peshawar by Khan *et al* [19] similarly revealed a 75% male gender predominance, which is comparable to our research's findings. Another study by Henna *et al*. from Lahore also showed a 4:1 male to female ratio. A substantial male gender predominance was also found by Hussain *et al* [21] from Karachi. Additionally, Zamir *et al* [22]. reported a 3:1 male to female ratio, which is in line with the outcomes of the study we conducted.

Mean age of this study´s cases was 7.95 ± 3.00 months. About 55 of the study's patients, or 59.1%, were older than 6 months. Similar age group was observed in a study conducted by Henna *et al*. Though Mabula *et al* from Tanzania has reported a mean age of 24 months in their study population which may be due to the fact that study population in this study did not exceed the age of 12 months. Calretinin positivity was observed more in patients up to 6 months of age as compared to patients older than 6 months. Of the 93 study cases, 28 (30.1%) were from rural areas and 65 (69.9%) were from metropolitan areas. Twenty-five households (26.9%) reported monthly family income of up to Rs. 35,000, while 68 families (73.1%) reported monthly family

income of beyond Rs. 35,000. The same results were also observed by Zamir *et al*.

Of these 93 study cases, the most common symptom observed was constipation, noted in 75 (80.6%) patients followed by abdominal distension in 62 (66.7%) patients. Failure to pass meconium was noted to be the least frequent symptom, observed in 24 (25.8%) patients. A study conducted at Lahore by Henna *et al* [20] has also reported similar frequencies of these associated symptoms. They also reported constipation to be the most common symptom, in 88.9% patients, abdominal distension in 77.8 % and delayed passage of meconium in 33% of patients. Mabula *et al* [23] from Tanzania has reported constipation in 94.5 %, abdominal distension in 92.7% and failure to pass meconium in 61.8% patients.

Twenty-eight rectal biopsy samples from 2010 to 2011 were examined in a prospective study at the University of Texas in Houston [4]. In the muscularis mucosae, superficial submucosa, and lamina propria, thin nerve fibrils that were positive for calretinin were consistently linked with the presence of ganglion cells establishing positive predictive value of this IHC stain. Another study conducted retrospectively between 2008 and 2010, tissue samples from children with a histological diagnosis of HD who underwent radical surgery at the Dr. Sheikh Children Hospital in Iran were examined [3]. In this study, there were 20 blocks in the control group, 30 blocks from the ganglionic zone, and 30 blocks from the aganglionic zone. Based on immunostaining for calretinin in the submucosa, there were no false-negative or false-positive results in this investigation. Loss of calretinin expression was noted in 34 (36.6%) patients in this study. In India, a study carried out 2013 on 131 suspected cases of HD, loss of expression of calretinin was noted in 53 (40.45%) patients [6]. These findings are closer to current study´s outcome.

CONCLUSION

In conclusion, this study indicated that calretinin is an extremely useful IHC stain which can be very reliably used in diagnosing suspicious cases of Hirschsprung disease. It can serve as a valuable as well as cost-effective diagnostic aid in resources-limited centers where acetylcholinesterase enzyme histochemistry is not available. We do encourage that more studies be conducted using this IHC stain for better predictability of diagnosis as well as an aid to start timely treatment of affected patients.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

GRANT SUPPORT & FINANCIAL DISCLOSURE

Declared none

AUTHORS CONTRIBUTION

Ayesha Haider: Entire research work, sample collection and data analysis

Unaiza Jamil: Literature review, drafting and analysis

Iqra Ahmad Shah: Sample collection, data analysis and revision of manuscript

Maria Aslam: Result interpretation, data analysis and revision of manuscript

Kiran Mumtaz: Sample collection and data analysis

Syed Naeem Raza Hamdani: Concept, design and overall supervision

REFERENCES

1. Butler RT, Jaden NE, Trainor PA. The developmental etiology and pathogenesis of Hirschsprung disease. Transl Res. 2013; 162(1): 1-15.
   DOI: https://doi.org/10.1016/j.trsl.2013.03.001
2. Rosai J. Hirschprung's disease and related disorders. In Rosai J, Ackerman L, editors. Rosai and Ackerman's surgical pathology.10th ed. St .Louis: Mosby; 2011.p.732-34
3. Hiradfar M, Sharifi N, Khajedaluee M, Zabolinejad N, Jamshidi ST. Calretinin immunohistochemistry: An aid in the diagnosis of Hirschprung's disease. Iran J Basic Med Sci. 2012; 15(5): 1053-9.
4. Alexandrescu S, Rosenberg H, Tatevian N. Role of calretinin immunohistochemical stain in evaluation of Hirschsprung disease: An institutional experience. Int J clin Exp Pathol. 2013; 6(12): 2955-61.
5. Kapur RP, Reed RC, Finn LS, Patterso K, Johanson J, Rutledge JC. Calretinin immunohistochemistry versus acetyl-cholinesterase histochemistry in the evaluation of

suction rectal biopsies for Hirschprung disease. Pediatr Dev Pathol. 2009; 12(1): 6-15.
DOI: https://doi.org/10.2350/08-02-0424.1

6. Samuel VG, Bonnard A, Lagausie PD, Philippe-Chomette P, Albert C, Ghoneimi AE, *et al*. Calretinin immunohistochemistry: A simple and efficient tool to diagnose Hirschprung disease. Mod Pathol. 2009; 22: 1379–84
DOI: https://doi.org/10.1038/modpathol.2009.110
7. Kannaiyan L, Madabhushi S, Malleboyina R, Are NK, Reddy KR, Rao B. Calretinin immunohistochemistry: A new cost-effective and easy method for diagnosis of Hirschprung's disease. J Indian Assoc Pediatr Surg. 2013; 18(2): 66–8.
DOI: https://doi.org/10.4103%2F0971-9261.109355
8. van den Berg MM, Benninga MA, Di Lorenzo C. Epidemiology of childhood constipation: A systematic review. Am J Gastroenterol. 2006; 101: 2401-9.
DOI:https://doi.org/10.1111/j.1572-0241.2006.00771.x
9. Rubin GP. Childhood constipation. Am Fam Physician. 2003; 67: 1041-2.
10. Di Lorenzo C. Pediatric anorectal disorders. Gastroenterol Clin North Am. 2001; 30: 26987.
11. Constipation Guideline Committee of the North American Society for Pediatric Gastroenterology, Hepatology and Nutrition. Evaluation and treatment of constipation in infants and children: Recommendations of the North American Society for Pediatric Gastroenterology, Hepatology and Nutrition. J Pediatr Gastroenterol Nutr. 2006; 43: e113.
12. Hyman PE, Milla PJ, Benninga MA, Davidson GP, Fleisher DF, Taminiau J. Childhood functional gastrointestinal disorders: Neonate/ toddler. Gastroenterology. 2006; 130: 1519-26.
DOI: https://doi.org/10.1053/j.gastro.2005.11.065
13. Benninga MA, Büller HA, Heymans HS, Tytgat GN, Taminiau JA. Is encopresis always the result of constipation? Arch Dis Child. 1994; 71: 186-93.
14. Thompson WG, Longstreth GF, Drossman DA, Heaton KW, Irvine EJ, Müller-Lissner SA. Functional bowel disorders and functional abdominal pain. Gut. 1999; 45 Suppl 2: II43-7.
DOI: https://doi.org/10.1136/gut.45.2008.ii43
15. Partin JC, Hamill SK, Fischel JE, Partin JS. Painful defecation and fecal soiling in children. Pediatrics. 1992; 89: 1007-9.
16. Di Lorenzo C. Childhood constipation: Finally some hard data about hard stools! J Pediatr. 2000; 136: 4-7.
DOI:https://doi.org/10.1016/s0022-3476(00)90039-8
17. Abrahamian FP, Lloyd-Still JD. Chronic constipation in childhood: A longitudinal study of 186 patients. J Pediatr Gastroenterol Nutr. 1984; 3: 460-7.
18. Dingemans AJ, van der Steeg HJ, Rassouli-Kirchmeier R, Linssen MW, van Rooij IA, de Blaauw I. Redo pull-through surgery in Hirschsprung disease: short-term clinical outcome. J Pediatr Surg. 2017;52(9):1446-50.
DOI: https://doi.org/10.1016/j.jpedsurg.2016.09.059
19. Khan K. Two stage management of patients with Hirschsprung`s disease. J Surg Pak Int. 2005; 10(4): 7-10.
20. Henna N, Sheikh A, Shaukat M, Nagi AH. Children with clinical presentation of hirschsprung's disease - a clinicopathological experience. Biomedica. 2011; 27(1): 1-4.
21. Hussain S, Mazhar A, Siddiqui A. Transanal endorectal pull-through: An effective alternative to staged management of Hirschsprung's disease. Pak J Surg. 2013; 29(3): 17982.
22. Zamir N, Akhtar J. Hirschsprung`s disease: Pattern of clinical presentation. J Surg Pak (Int). 2014; 19(1): 40-4.

Phlebotomy- A gateway to laboratory diagnostics

Shehla Ambreen Alizai[1], Rabia Sadaf[2], Maliha Atif[2], Kanwal Shehzadi[3], Naima Noor[4], Muhammad Saeed[4]

[1]Islamabad medical & Dental College, Islamabad Pakistan
[2]Akhtar Saeed Medical College, Rawalpindi Pakistan
[3]Qaid-E-Azam University, Islamabad Pakistan
[4]Rawal General & Dental Hospital, Islamabad Pakistan

ABSTRACT

Objective: To assess the knowledge, practice and attitude about venipuncture amongst nursing staff of a tertiary care hospital.

Material and Methods: This questionnaire-based survey was done at Rawal General and dental hospital, Islamabad, from January to April 2022. The Questionnaire was developed as per the CLSI H3-A6 and WHO's venipuncture guidelines. It was distributed to nursing staff by hand. Most were filled inside the classes in front of the teacher.

Results: The frequency of correct responses in the knowledge section varied from 4(4.6%) about the number of tube inversions to 79(90.8%) about wearing of gloves before taking a sample while 47(54%) selected the correct option of identifying a patient. Regarding the standard phlebotomy protocols, the response was quite low which was 26(29.9%), 18(20.7%) and 8(9.2%) about the correct angle of needle insertion, what to inspect of the supplies and the location of applying the tourniquet respectively. Recapping with two hands is still done by 13% and 79% still put the sample by holding tube in the other hand. In spite of all this 66% still think that one can learn venipuncture by practicing the techniques without going through a proper course.

Conclusion: Nursing staff in Rawal General and dental hospital is not fully aware about basic laboratory protocols and their importance. This unawareness can lead to generation of erroneous lab reports. Formal education, training with summative assessments should be a part of the curriculum of nursing courses followed by rigorous implementation monitoring.

Keywords: Awareness, Nurses, Phlebotomy, Venipuncture, Sample

This article can be cited as: Alizai SA, Sadaf R, Atif M, Shehzadi K, Noor N, Saeed M. Phlebotomy- A gateway to laboratory diagnostics. Pak J Pathol. 2024; 35(1): 26-33.

DOI: https://doi.org/10.55629/pakjpathol.v35i1.790

INTRODUCTION

The importance of medical laboratory tests, especially the blood examination as the baseline investigation as well as the specified tests for proper diagnosis is undeniable [1].

The quality of results of the blood examinations majorly depends upon the skills and knowledge of the phlebotomist. It is important that any medical laboratory should have a well-trained phlebotomist, as even minor negligence in the standard operating procedure of blood sampling may give false results leading to misdiagnosis and improper management of the disease [2]. In most of the hospitals in Pakistan, it has been observed that blood sample collection in admitted patients is mostly done by the nurses, who are not properly trained according to the standard guidelines of blood sampling set by WHO [3]. Moreover, there are no standard operating procedures of blood sampling and infection control practices displayed in the wards thus leading to many pre-analytical errors. The most common error is that the blood sample is hemolysed due to improper vigorous mixing of blood or when the blood is collected through intravenous catheter of size less than 20G [3,4]. It has also been observed that many samples that come for the blood

Correspondence: **Dr. Shehla Ambreen Alizai, Associate Professor, Department of Pathology, Islamabad Medical and Dental college, Islamabad Pakistan**

Email: alizaishehla@gmail.com

Receiving Date: **11 Oct 2023**
Revision Date: **01 Mar 2024**
Acceptance Date: **21 Mar 2024**

cultures are already contaminated with the normal flora of the skin of the patient due to lack of awareness of proper patient preparation before blood collection [5]. Many other studies from Asian and African countries have also shown the inappropriate knowledge of health care professionals especially related to patient preparation, tourniquet application time, order of draw, identification of the appropriate collecting tubes required for different blood tests, sample transportation and storage [1,6]. To avoid all these errors and to provide accurate and reproducible results, it is of vital importance to regularly evaluate the knowledge of phlebotomy procedures of our nursing staff to update their training according to the standardized procedures set by World Health Organization [7].

Since we were facing the problems related to pre-analytical errors in our reports, we conducted this study to assess the knowledge, skill and attitude regarding awareness on the blood sampling procedure among the nurses and to provide references for improving the sampling practice at our hospital.

MATERIAL AND METHODS

This descriptive cross-sectional was conducted at the Department of Microbiology, RG&DH Islamabad form January to April 2022. Sample size was calculated through WHO calculator using the following formula

$$N=z^2\ 1-\alpha/2\ P(1-P)/d^2$$

Where P= Expected proportion in population based on previous studies [8] and with 95% level of confidence, the calculated sample size was 73. Due to possible lack of response, we oversampled by 16%, making the final sample size 86. Non-probability consecutive sampling technique was used to collect data. The questionnaire was based on KAP (Knowledge, Attitude and Practice) having 26 questions on knowledge, 9 questions on practice and 3 questions on attitude regarding awareness of blood sampling procedure among nurses. For the knowledge questions, incorrect responses were given a 0 score, while 1 point was assigned for choosing the correct answer. The expected minimum and maximum total knowledge score were 0 and 26, respectively. Attitude towards blood sampling procedure was measured by 3 questions. A statement with options yes and no were given 1 and 0, respectively. The expected maximum total attitude score was 3. Practice was scored 1 for standard practice and 0 for other. Participants' KAP levels were defined as "good" or "poor" based on Bloom's cut off point. Participants with knowledge scores above 60% were regarded as having good knowledge, while those with score below 60% were considered having poor knowledge. Participants with attitude scores of 59% and below were considered as having a unacceptable attitude, while those within the range of 60-79% moderate and score above 80% were regarded as having a good attitude. For practice section, participants with scores >80% and <80% were classified as taking acceptable and unacceptable blood sampling measures, respectively.

The collected data were analyzed using SPSS version 22.0. Descriptive statistics were used to summarize the demographic characteristics of the sample data. The KAP assessment was conducted out by assigning scores to the variables. Bivariate statistics (Pearson's Chi square) was conducted to check the association of participant's knowledge scores with their attitude and practice scores.

RESULTS

A total of 86 questionnaires were distributed. There were 57(66%) females and 29(34%) males. Almost all of the investigated nurses were aware that patient identification should be confirmed prior to venous blood sampling. However, the frequency of nurses who knew the right procedures to identify a conscious patient was 47(54%). Few,18(20.7%) knew what to check about the phlebotomy supplies. Although 72.4% of the nurses knew proper tourniquet releasing time, the correct rates on the tourniquet applying location were quite low, 8(9.2%). In addition, a good number of the investigated nurses knew that gloves should be worn during phlebotomy and the proper time

to put on gloves 79 (90.8%) & 71(81.6%) respectively. The knowledge about correct angle of insertion of the needle was known to very few, 26(30%). The rest of the results on knowledge of the pre-sampling phase are shown in Table-I.

The knowledge of mix by inverting blood collection tubes is shown in Table-II. The tube inversions were lower than those of recommended times. In the practice section (Table-III), the frequency of nurses who still recap the needle with two hands is 12(13.8%) while 69(79.3%) put the sample by holding tube in the other hand. The correct rates on where to dispose needles were relatively high 72 (82.8%) while the rest used the medical waste bin for disposal. A good percentage perform hand hygiene before putting on gloves i.e 73(83.9%). During sampling 45(51.7%) of the nurses were not practicing the right order of draw during multi-tube sampling. The rest of the answers' detail is shown in the table.

In the Attitude section (Table-IV) quite a high number of nurses (58-66%) believed that not performing hand hygiene when wearing gloves, taking the sample even before the antiseptic is dry and no need for a formal phlebotomy training was acceptable.

The p-values were 0.329 and 0.152, indicating no significant association of Knowledge with Attitude and Practice for the given study.

Table-I: Knowledge of the investigated nurses about the pre-sampling phase.

Questions	Wrong answer	Correct answer
K1. Patient identity should be confirmed prior to venous blood sampling?		
True	3(3.4%)	84(96.6%)
False		
K2. What is the right procedure to identify a patient who is conscious?		
Ask a patient to give his/her full name	40(46%)	47(54%)
Nurse states a patient's full name or bed number		
Check a patient's bed tag		
K3. What should be inspected about the supplies? (multi-choice)		
Expiry dates of phlebotomy devices		
Looseness or defects of the tube cap		
Appropriate tubes according to the test requests		
All of the above	69(79.3%)	18(20.7%)
K4. Which vein is preferred for venipuncture		
Median cubital vein / median vein	24(27.6%)	63(72.4%)
Basilic vein		
Other veins		
K5. Where is the proper location to apply a tourniquet?		
3.5 - 5.0 cm above the venipuncture site		
5.0 - < 7.5 cm above the venipuncture site		
7.5 - 10.0 cm above the venipuncture site	9(90.8%)	8(9.2%)
10.0 - < 12.5 cm above the venipuncture site		
K6. How long can tourniquet application last?		
≤ 60 seconds	24(27.6%)	63(72.4%)
>60 seconds		
K7. Gloves should be worn during phlebotomy?		
True	8(9.2%)	79(90.8%)
False		
K8. When is the proper time to put on gloves?		
Before assembling supplies		
Before performing venipuncture	16(18.4%)	71(81.6%)
K9. While taking blood sample, the needle should enter at an angle of		
$45\text{-}50^0$		
$35\text{-}40^0$		
$15\text{-}30^0$	61(70.1%)	26(29.9%)

Table-II. Knowledge of mix by inverting times for blood collection tubes.

K21. How many times the red cap (non-additive serum tube) should be inverted for mixing after blood collection?		
Mixing not required		
1-4		
more than 5	48(55.2%)	39(44.8%)
K22. How many times the yellow cap (tube with clot activator and gel plasma separator) should be inverted for mixing after blood collection?		
Mixing not required	83(95.4%)	4(4.6%)
1-4		
more than 5		
K23. How many times the Lavender cap (EDTA tube) should be inverted for mixing after blood collection?		
Mixing not required		
1-4		
more than 5	69(79.3%)	18(20.7%)
K24. How many times the green cap (Heparin tube) should be inverted for mixing after blood collection?		
Mixing not required		
1-4		
more than 5	80(92.0%)	7(8.0%)
K25. How many times the **blue** cap (1:9 Sodium citrate tube) should be inverted for mixing after blood collection?		
Mixing not required		
1-4	35(40.2%)	52(59.8%)
more than 5		
K26. How many times the **grey** cap (Glycolysis inhibitor tube) should be inverted for mixing after blood collection?		
Mixing not required		
1-4		
more than 5	73(83.9%)	14(16.1%)

Table-III: Practice questions responses.

Items	**n (%)**
Where do you dispose the needle after phlebotomy?	
Medical waste dustbin	15(17.2%)
Sharps container	72 (82.8%)
How many times have you taken a blood sample?	
>20 times	57(65.5%)
<20 times	30(34.5%)
How do you recap a needle?	
I don't recap	28(32.2%)
With single hand	47(54%)
With two hands	12(13.8%)
After taking the blood sample, how do you put the blood in the tube?	
By keeping the tube in a rack	18(20.7%)
By holding tube in one hand and putting the sample by the other	69(79.3%)
Do you touch the venipuncture site after disinfecting it?	
Never	69(79.3%)
Sometimes	13(14.9%)
Always	5(5.7%)
In admitted patients how do you take blood sample?	
From a new venipuncture	50(57.5%)
From existing peripheral venous access site	11(12.6%)
From first introducing an I.V device before connecting the cannula	26(29.9%)
Do you perform hand hygiene before putting on gloves?	
Yes	73(83.9%)
no	14(16.1%)
When taking sample for multiple tests for (a) Blood Culture (b) Red top (LFTs)	

and (c) lavender top (CP). Which tube do you inoculate on first, 2nd & 3rd number?	
A, B &C	45(51.7%)
B, A & C	41(47.1%)
C, B &A	1(1.1%)
Do you allow the antiseptic to dry before taking blood?	
Yes	66(75.9%)
no	21(24.1%)

Table IV. Attitude questions' responses

	Items	Yes%	No%
A1	It is OK NOT to perform hand hygiene if we are wearing sterile gloves before venipuncture.	37(42.5)	50(57.5)
A2	When in a hurry we may take a sample, even before an antiseptic is dry.	30(34.5)	57(65.5)
A3	One can learn venipuncture by practicing the techniques without going through a proper course.	30(34.5)	57(65.5)

Table-V: Table of significance.

	Attitude			Practice	
Knowledge	**Good**	**Moderate**	**Unacceptable**	**Acceptable**	**Unacceptable**
Good	12(21.4%)	3 (13.0%)	3 (37.5%)	3 (42.9%)	15 (18.8%)
Poor	44(74.6%)	20(87.0%)	5 (62.5%)	4 (57.1%)	65 (81.3%)
		p-value = 0.329		p-value = 0.152	

DISCUSSION

The knowledge of pre analytical process of laboratory tests plays a vital role in quality assurance of test results leading to diagnostic approach. In our country, the facility of trained phlebotomists is not available in most of the hospitals so blood sampling procedure is usually done by nursing staff, post graduate residents and house officers. The awareness of patient identification, proper vacutainers, tourniquet application, needle handling, order of draw and proper mixing of blood sample to avoid hemolysis is crucial for quality laboratory results. It is a bitter reality that most of the healthcare workers are unaware of proper protocol of blood sampling technique [8,9]. We have conducted this study to analyze the proficiency of nursing staff in our hospital to analyze their blood sampling technique to improve the quality of our laboratory test results.

In our study, we did a questionnaire-based survey involving 86 trained nurses, 39% having more than 5 years of work experience. Questions were designed to assess the participant's knowledge and practical approach regarding pre sampling, sampling and post sampling phase.

Before taking blood sample, confirmation of patient identity is as significant as the sampling technique. Almost all nurses (96.6%) were aware of the importance of proper identification however, only 54% selected the correct identifier that should be used for a fully conscious person. When we compared our results with other studies done around the globe, we found consistent results in other questionnaire-based studies in Pakistan and China, in which 64% and 58.8% nurses knew the correct procedure of patient identification respectively [8,10]. Research done in a tertiary care hospital in Turkey showed higher number of nurses (78%) who are unaware of proper identification procedure [4]. Two more studies performed in India and Croatia found 86.6% and 70% phlebotomists respectively who knew the proper identification process, which is much higher than our results [12,13].

In pre sampling phase, we asked questions to evaluate the knowledge of study participants about the correct site for venipuncture and correct method of sanitization and use of gloves before taking blood sample. We found that 72.4% participants knew the preferred vein for blood sampling i.e. median cubital vein.

When we compared our results with other studies, we came to know that knowledge of suitable site for blood collection is more or

less the same. As a study done in Sri Lanka in 2021 showed 83% participants with correct response [14]. Another study done at a tertiary care hospital's nurse in Turkey found 89% subjects with the correct answer[11] while in a study done in China, 87.2% of investigated nurses knew the preferred site [10].

While concerning proper method of sanitization which includes type of antiseptic used and use of gloves before taking blood sample, we came to know that 73.6% subjects knew the correct concentration of alcohol i.e. 70% should be used for sanitization. Almost all study subjects (90.8%) knew the use of gloves in phlebotomy. Same results were obtained in a study from India in which 88% participants were aware of proper use of gloves during phlebotomy and 84.6% answered correctly about the antiseptic [12]. Another questionnaire-based survey done in Turkey showed 82.5% subjects who knew about the correct use of antiseptic [15]. While another research done in the same country showed that majority (92%) wore clean gloves before phlebotomy procedure [11]. Our results were a little higher than a study done in China in 2018 in which 67% nurses knew the proper method of wearing gloves during phlebotomy.[10] Probably because that was a multicenter study and a little old too compared to ours.

In the sampling phase, we focused on the knowledge and skill of subjects about recommended procedure of tourniquet application, the method acquired after application of antiseptic at venipuncture site and precautions to avoid needle stick injuries. We were also concerned to check the awareness of our study participants about an important component of phlebotomy procedure, the correct order of draw.

In our study, majority of nurses, 90.8%, did not know the correct location for tourniquet application and 72.4% gave correct responses about tourniquet application time. When we compared our responses with other studies, we found that 62.4% nurses who participated in a study conducted in Sahiwal, Pakistan knew about the correct position of tourniquet application [8]. When we analyzed other studies done in Pakistan and in neighboring countries, our results regarding tourniquet application time are more or less the same i.e 74%, 78%, 65.6% and 84% respectively [12,10,8,11] (evaluation of phlebotomy in India, China (parent article), Pakistan and Turkey).

In our survey, we also checked the phlebotomist routine after applying antiseptic whether they give proper time of alcohol to dry before taking blood sample or if they touch the venipuncture site to feel the vein shaft after disinfecting the site. Only 19.5% participants knew the recommended time which should be given to dry the disinfectant, while on the other hand, 79% subjects answered that they never touch the venipuncture site after disinfectant application.

When we analyzed other studies for comparison, we came to know that a study done in a tertiary care hospital in Pakistan reported 52% phlebotomists who gave proper time (30 seconds) for drying of alcohol.[8] Another study carried in Lahore, which evaluated the pre analytical errors in post graduate trainees found 35.2% residents with knowledge of drying time of alcohol.[9] Another questionnaire based survey in India showed 88.6% participants with correct response.[12] These results are in contrast to our results that emphasizes on the importance of refresher training courses for nurses and phlebotomists in our set up.

When we assessed the knowledge of participants whether they retouch the cleaned site or not in other researches, we found that 91% subjects in a survey performed in India did not touch the phlebotomy site after disinfection [12]. Another study conducted in Sri Lanka with 100 participants, one third of study subjects admitted that they palpate the site after disinfection [14]. A study done in Izmir; Turkey showed that 96.4% subjects do not touch the sanitized site [15]. These results clearly show the high standard of training in this country.

Needle stick injury (NSI) is a significant risk factor to health care workers. Awareness of accidental prick should be the important component of nursing training program. Recapping of used needle after taking blood sample, transfer of blood sample in the

vacutainer tube and dealing with uncooperative patients without any support are important factors which may lead to needle stick injury [12]. A study done in Karachi showed 53.7% nurses who were exposed to NSI [16]. When evaluating this skill, we found only 20.7% nurses who used correct procedure of blood transfer to vacutainer tubes and half of the subjects (57.5%) knew the protocol if they get an accidental prick. A study in India declared 13.3% participants who got NSI [12]. In the study done in Karachi, Pakistan in 2023 found 59.7% nurses with low level of knowledge regarding NSI [16]. Research done in Turkey in 2020 showed that correct safety procedure for blood sampling was adopted only by 38% study participants [11].

The awareness of correct order of draw has an important role in minimizing the pre analytical errors. In our study, 47% phlebotomists were aware of correct order of draw. On comparison with other studies, we found similar results in a survey done in tertiary care hospital in Pakistan showing 49% correct responses [8]. Two other studies done in Asian countries showed dissimilar results with quite less number of nurses with correct knowledge about order of draw, 18% and 15.5% [12,10]. The study done in Turkey also found only 22% nurses with correct response [15]. This deficiency was also seen in studies on postgraduate trainees. Only 24% and 55% participants gave correct answer [17,19].

In post sampling phase, an important thing to avoid is hemolysis of blood sample. The common factors leading to hemolysis of blood sample are the use of needle with improper gauge, forceful pressing of plunger while transferring blood and insufficient knowledge of proper method of blood mixing. In our study, we checked the awareness regarding proper skill of inversion of tubes for mixing of blood sample and calculated the mean percentage of correct responses which came out to be 25.7%.

While a study conducted in Pakistan found only 8.8% nurses who knew about appropriate method of mixing of additive with blood sample [8]. Another study done in Turkey showed a mere 6% of nurses which were aware of correct mixing protocol [11]. However, an analysis done in Europe found 47% subjects with correct response [13]. Probably missed especially in hours of heavy workload.

The P-values being insignificant shows that in spite of knowledge non-adherence to the SOPs needs strict measures including inspection and clinical audits to make sure that the SOPs are followed. Further studies for the effect of strictness are needed for this purpose.

CONCLUSION

This study shows that significant number of nursing staff in our setup are not fully aware of basic protocols of venipuncture and their importance, which may lead to generation of not only erroneous lab reports but also agonizing the patients in case they have to revisit for repeat sampling. Formal education and training including summative assessments should be a part of curriculum of the nursing courses. Last but not the least it is the strictness on implementation which is most importantly required.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

GRANT SUPPORT & FINANCIAL DISCLOSURE

Declared none

AUTHORS CONTRIBUTION

Shehla Ambreen Alizai: Literature search, study design and concept, questionnaire design, data collection, drafting
Rabia Sadaf: Discussion writing
Maliha Atif: Introduction writing
Kanwal Shehzadi: Statistical analysis
Naima Noor and Muhammad Saeed: Data collection

REFERENCES

1. Crous L, Armstrong SJ. The bloody truth: Investigating nurse phlebotomy competencies at a private laboratory in Johannesburg, South Africa. Health SA Gesondheid. 2016; 21(1): 339-47. DOI: https://doi.org/10.1016/j.hsag.2016.06.002
2. Burchill CN, Seballos SS, Reineks EZ, Phelan MP. Emergency nurses' knowledge, attitudes, and practices related to blood sample hemolysis prevention: An exploratory descriptive study. J Emerg Nurs. 2021; 47(4): 590-8.

DOI: https://doi.org/10.1016/j.jen.2020.12.015
3. Batool H, Mumtaz A, Qadeer S, Bakht ZA. Impact of supervised phlebotomy training programme on performance skills of phlebotomy staff. Annals of King Edward Medical University. 2018 20; 24(2): 776-80. DOI:http://dx.doi.org/10.21649/journal.akemu/2018/24.2.54-58
4. Ersoy S, Ilanbey B. A single-center prospective study of the effects of different methods of phlebotomy in the emergency department on blood sample hemolysis rates. J Emerg Nurs. 2023; 49(1): 134-9. DOI: https://doi.org/10.1016/j.jen.2022.08.005
5. He M, Huang S, Xiong J, Xiao Q. Improving adherence to facility protocol and reducing blood culture contamination in an intensive care unit: A quality improvement project. Aust Crit Care. 2020; 33(6): 546-52. DOI: https://doi.org/10.1016/j.aucc.2020.03.002
6. Cai Q, Zhou Y, Yang D. Nurses' knowledge on phlebotomy in tertiary hospitals in China: A cross-sectional multicentric survey. Biochem Med. 2018; 28(1): 42-54. DOI: https://doi.org/10.11613/bm.2018.010703
7. Jain S, Chraiti MN, Pittet D, Mclaws ML. Blood collection guidelines for inpatients and outpatients, home-based care and long-term care facilities. J Hosp Infection. 2020; 104(4): 600-2. DOI: https://doi.org/10.1016/j.jhin.2019.10.017
8. Muzzamil F, Rafiq M, Siddiqui ZK, Hamza M, Arooj A, Lail RA. Awareness of pre-analytical errors amongst healthcare workers of DHQ teaching hospital, Sahiwal, Pakistan. Rawal Med J. 2022; 47(2): 426–30.
9. Waheed NA, Bajwa MS, Anwar S, Ali H. Pre-analytical phase awarness amongst doctors of a tertiary care hospital-A Cause for concern. Annals King Edward Medical University. 2019; 25: 58–64.
10. Unnithan A, Das S, Raju K. Evaluation of phlebotomy quality metrics: An effective tool for quality patient care. Adv Human Biol. 2023; 13(Suppl 1): S80-4. **DOI:** https://doi.org/ 10.4103/aihb.aihb_201_22
11. Sonmez C, Yıldız U, Akkaya N, Taneli F. Preanalytical phase errors: Experience of a central laboratory. Cureus. 2020; 12(3): 1–8. DOI: https://doi.org/10.7759%2Fcureus.7335
12. Unnithan A, Das S, Raju K. Evaluation of phlebotomy quality metrics: An effective tool for quality patient care. Adv Human Biol. 2023; 13(Suppl 1): S80-4. DOI: https://doi.org/ 10.4103/aihb.aihb_201_22
13. Banković Radovanović P. Quality improvement project: Reducing non-conformities of the samples for haemostasis testing in a secondary healthcare centre through the nurses' education in phlebotomy. Biochem Med. 2020; 30(2): 287-95. DOI: https://doi.org/10.11613/bm.2020.020708
14. Fernando ES, Kuruwitage GS, Rathnayake M, Das CV, Mahalingam N. Knowledge on practice of phlebotomy among nursing and biomedical students in Gampaha District, Sri Lanka. 2021; 2659.
15. Arslan FD, Karakoyun I, Basok BI, Aksit MZ, Celik E, Dogan K, *et al*. The effects of education and training given to phlebotomists for reducing preanalytical errors. J Med Biochem. 2018;37(2):172–80. DOI: https://doi.org/10.1515%2Fjomb-2017-0045
16. Ali M, Bibi A, Khan J, Arshad S, Durdana M, Musafir Y. Student nurses knowledge of needle stick injuries at a private institute, Karachi, Pakistan. J Heal Sci. 2023; 79–83. DOI: https://doi.org/10.54393/pjhs.v4i04.662
17. Kulkarni KK, Bhandari AP, Unni AK. Questionnaire-based study to assess knowledge of preanalytical phase of laboratory testing among trainee doctors in a tertiary care hospital medical college. J Lab Physicians. 2020; 12(03): 178-83. DOI: https://doi.org/10.1055/s-0040-1720945

Correlation of nucleated red blood cell counts by Sysmex XN-1000 with conventional microscopy

Nimrah Ishaque, Hijab Batool, Ashja Saleem, Muhammad Usman Siddique, Ayisha Imran, Nauman Aslam Malik

Chughtai Institute of Pathology, Lahore Pakistan

ABSTRACT

Objective: To correlate the results of manual microscopy with that of Sysmex XN-1000 analyzers and to determine its diagnostic accuracy, taking microscopy as gold standard.

Material and Methods: The study was conducted at Chughtai Institute of Pathology from July 1st 2022 - December 31st, 2022. Nucleated Red Blood Cells (nRBCs) were manually enumerated against 100 White Blood Cells (WBCs) on peripheral smears and 511 such positive cases were included. 500 samples negative for nRBCs by manual microscopy were also included. These positive and negative samples were run on two Sysmex XN-1000 analyzers. A result of 0.3 and above nRBCs/100 WBCs on Sysmex XN-1000 was considered positive and less than 0.3 as negative. Mean values of both manual and automated methods were correlated.

Results: Our study mentioned nRBCs ranging from 0 to 1330 nRBCs/100WBCs by manual count. Compared with Sysmex XN-1000 analysis, the Deming regression analysis showed the mean difference between the two methods was 0.055 (95%CI: −0.015 to +0.124) ($p>0.05$ no proportional bias between two methods). The Sysmex XN produced an area under the curve, by ROC analysis, at 0.894 ($P < .001$). The overall sensitivity was (90.56%) (CI 87.56-92.98%), with few false-negative results. The overall specificity is (88.30%) (CI 85.20%-90.96%) due to few false positivity. Positive predictive value and negative predictive values were 88.26% (CI 88.54-90.52%) and 90.6% (CI 87.99-92.69%) respectively. Accuracy was 89.42% (CI87.35-91.25%).

Conclusion: Sysmex XN1000 analyzer is a highly sensitive and accurate instrument which can replace nRBCs enumeration using manual counting by conventional microscopy.

Keywords: Nucleated red blood cells, Hematology analyzer, Sysmex XN 1000, Manual microscopy

This article can be cited as: Ishaque N, Batool H, Saleem A, Siddique MU, Imran A, Malik NA. Correlation of nucleated red blood cell count by Sysmex XN-1000 with conventional microscopy. Pak J Pathol. 2024; 35(1): 34-38.

DOI: https://doi.org/10.55629/pakjpathol.v35i1.787

INTRODUCTION

Peripheral blood examination in conjunction with a patient's clinical history and symptoms play a key role in diagnosing and treating a wide spectrum of hematological illnesses [1]. Nucleated red blood cells (nRBCs) are immature cells of erythropoietic lineage, normally present in bone marrow and peripheral blood of only newborns (until the 5th day of life) [2,3]. Their presence in the peripheral blood of older children and adults indicates a pathology.

It is associated with poor prognosis and early death [4]. Probable cause of their presence includes severe hypoxia, massive hemorrhage, hemolysis, hemoglobinopathies, marrow infiltration or any other stress to the bone marrow [5].

Until a few years ago, manual microscopy was the only reliable and gold standard method for differential blood cell counts. It was impossible for hematology analyzers to differentiate small lymphocytes from nucleated red blood cells. But manual microscopic counting of nRBCs, is a tedious and labor-intensive procedure with low precision, due to both inter and intra observer variation and efficacy [6]. The single method for manual count is enumeration of nucleated red blood cells

Correspondence: **Dr. Nimra Ishaque, Resident Pathologist, Chughtai Institute of Pathology Lahore Pakistan**

Email: nimrahishaque0411@gmail.com

Receiving Date: **13 Sep 2023** Revision Date: **26 Nov 2024** Acceptance Date: **01 Jan 2024**

against one hundred White Blood Cells (WBCs) on peripheral blood film under microscope. By this method it is not possible to count nRBCs below 1 nRBCs to 100 WBCs or 100/μL [7].

Latest hematology analyzers like Sysmex XN-Series have a specialized channel called WNR channel for nRBCs and WBCs differential [5]. This WNR channel uses a specific acidic reagent called Lysercell WNR which punctures the membranes of cells, causing hemolysis of reticulocytes and mature red cells, that do not have a nucleus. This process also differentially ruptures the cytoplasmic membrane of nRBCs and WBCs. WNR channel then uses a fluorescent dye, Fluorocell which enters the cells and stains their nucleic acids and organelles [8,9]. The XN-1000 analyzer measures the fluorescent cell intensities as forward-scattered plot (FSC) which represents cell volume/size and as the side fluorescent light (SFL), which represents the intracellular complexity. These plots are then expressed as a 2D scattergram. On this scatterplot, nRBCs have the lowest SFL intensity and basophils have the highest FSC and SFL intensities compared with those of other leucocytes [10]. In this way Sysmex distinguishes and enumerates nRBCs, differentiating them from leucocytes which are also nucleated.

nRBCs are the most common abnormal morphological finding encountered in a lab setting [11]. So manual counting of all these peripheral smears by a hematologist becomes a time-consuming process. Automated analyzers like Sysmex XN series (which have built-in nRBCs channel) can help lessen the burden on hematologists especially in a third world country, where the availability of such trained professionals is scarce. To lessen the manual review rate by using automated analyzer, we need to be certain that the analyzer detects nRBCs in all the samples and computes them accurately. Only one such study has been conducted in our population making it hard to permanently replace microscopy with automation [12]. Our study was designed to correlate the results of manual microscopy with that of Sysmex XN-1000 analyzers and determining its diagnostic accuracy, taking microscopy as gold standard.

MATERIAL AND METHODS

The study was conducted at Chughtai Institute of Pathology from July 1st, 2022, till December 31st, 2022, after obtaining approval from ethical and research committee of the institute. Sample size was calculated using OpenEpi, Version 3, open-source calculator using nRBCs as a reference parameter.

Peripheral blood EDTA samples for routine complete blood counts and hemoglobin electrophoresis, in which nRBCs were identified by manual microscopy were included in the study. 511 such positive samples were included in this study by consecutive convenient sampling. nRBCs were manually enumerated by two trained hematologists against 100 WBCs on peripheral smears stained with Wright-Giemsa stain. 500 nRBCs negative samples by manual microscopy were also included. After manual counting, these positive and negative samples were run on two Sysmex XN-1000 analyzers. Their results of nRBCs counts were noted. A result of 0.3 and above nRBCs/100 WBCs on Sysmex XN-1000 was considered positive and less than 0.3 nRBCs/100WBCs were documented as negative. Mean values of both manual and automated methods were correlated. Samples having storage changes, Red Blood Cell (RBC) agglutination, Total Leucocyte Count (TLC) of less than 1x 10^3μL and more than 100x10^3μL were also noted.

Correlation of nRBCs count by Sysmex XN-1000 and the manual count was carried out by the Deming regression analysis, Bland–Altman analysis, and Pearson correlation (r value). Receiver operating characteristic (ROC) analysis and the diagnostic accuracy were evaluated in terms of positive predictive value, negative predictive value, sensitivity, specificity and accuracy using the NRBC count per 100 WBCs. Samples having storage changes, RBC agglutination, low and high TLC are mentioned as percentages. The statistical analysis was performed using SPSS 23.0.

RESULTS

We included 1011 samples in our study out of which 511 were positive for nRBCs on manual count and 500 were negative. Our study mentioned NRBCs ranging from 0 to 1330 NRBCs/100WBCs by manual count. Compared with Sysmex XN-1000 analysis, the Deming regression analysis demonstrated a r value of 0.723 (95% CI 0.632 to 0.747) with the slope and intercept at +0.39 and +0.69, respectively (Figure-I) The mean difference between the two methods was 0.055 (95%CI: −0.015 to +0.124) (p>0.05 no proportional bias between two methods) (Figure-II).

Evaluation of clinical diagnostic performance of Sysmex XN series was carried out using the manual count as the comparative method. The Sysmex XN produced an area under the curve, by ROC analysis, at 0.894 (P < .001) (Figure-III). The overall sensitivity was (90.56%) (CI 87.56-92.98%), with few false-negative results. The overall specificity is (88.30%) (CI 85.20%-90.96%) due to few false positivity. Positive predictive value and negative predictive value were 88.26% (CI 88.54-90.52%) and 90.6% (CI 87.99-92.69%) respectively. Accuracy was 89.42% (CI87.35-91.25%) (Table-I). 3.13% of the samples had low TLC (<1x $10^{\wedge 3}\mu L$), 2.5% with high TLC (>100x $10^{\wedge 3}\mu L$), 3.5% storage changes and 0.98% showed RBC agglutination.

Table-I: Performance of nucleated red blood cells enumeration by Sysmex XN.

Statistics	Value	95% Confidence Interval
Sensitivity	90.56%	87.65% to 92.98%
Specificity	88.30%	85.20% to 90.96%
Positive predictive value	88.26%	85.54% to 90.52%
Negative predictive value	90.60%	87.99% to 92.69%
Accuracy	89.42%	87.35% to 91.25%

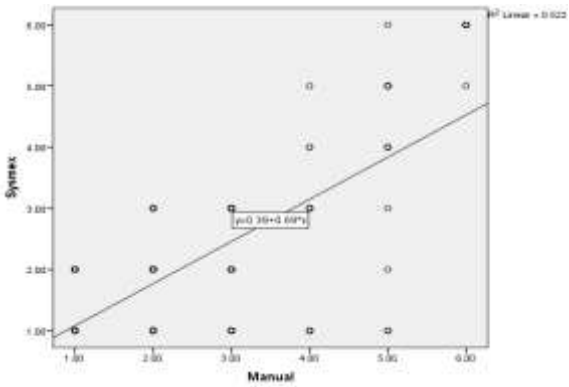

Figure-I: Correlation study of nRBCs count by both methods using the Deming regression analysis.

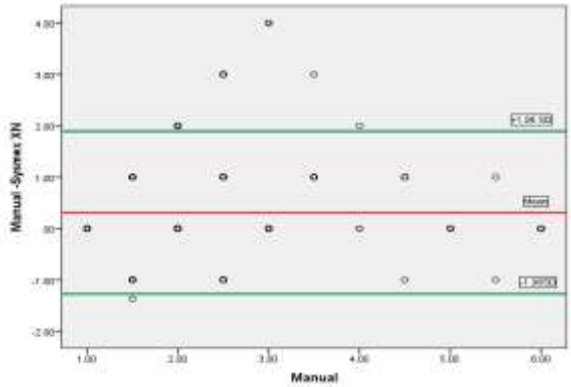

Figure-II: Difference estimation by Bland–Altman plot analysis between Sysmex XN vs the manual count.

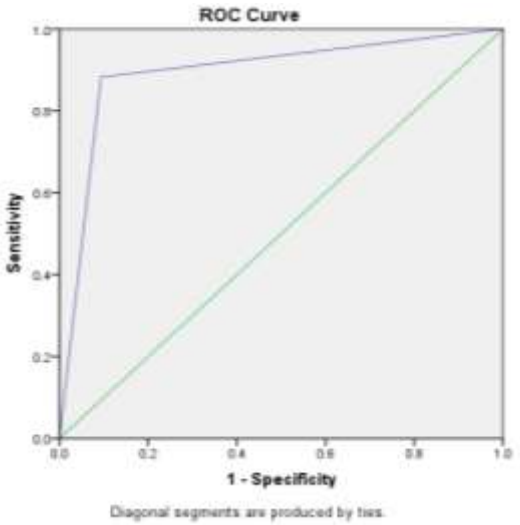

Figure-III: ROC analysis of the Sysmex XN performance for nRBCs detection

DISCUSSION

Nucleated red blood cells are an important prognostic indicator for many haematological and non-haematological diseases. They are especially important in patients with thalassemia, as the presence of nRBCs in their peripheral blood indicates ineffective erythropoiesis [13]. Furthermore, in critically ill patients nRBCs are now being used as early diagnostic and prognostic marker. [14] Hence, the

manual counting of these blood cells can be cumbersome and time consuming especially in a hospital setting, where the CBCs of patients are performed multiple times a day. For any laboratory setting, slide making just for the purpose of enumerating nRBCs is labor intensive and cost consuming [11,13]. Manual counts also have disadvantages of being imprecise at times with both inter and intra observer variation but until recently it has been the only gold standard method to report nRBCs [6].

Latest automated analyzers like Sysmex are fully equipped with advance technology to enumerate and report such parameters without any slide reviewing. They are preferable to manual count by being cost effective and convenient. Previous studies in other countries have been conducted to determine the precision and diagnostic accuracy of Sysmex XN series and they have been very encouraging [2,12]. Our study also shows that Sysmex XN-1000 analyzer with its WNR channel is highly sensitive (90.56%) and specific (88.30%) in enumerating nRBCs. Even in samples with a very low white cell count, storage changes and RBC agglutination it was able to accurately detect and count nRBCs. In such situations it is cumbersome to identify and count nRBCs for any hematologist. Our study shows that both methods give same range of enumeration of nRBCs. In the positive samples, those with nRBCs identified on manual microscopy, Sysmex XN 1000 showed positive predictive value of 88.26%. It was slightly lower than other studies due to some false negative values [2]. And in negative samples it showed negative predictive value of 90.6%. Overall, it showed accuracy of 89.42% which is remarkable. A previous study conducted in Pakistan showed that Sysmex analyzer measured nRBCs accurately up till 200 counts. Above that its measurement was not accurate [12]. This did not happen in our study. Sysmex's enumeration was comparable even in the samples having higher counts.

Of all the samples included in the study, 678 samples also had the request for Hemoglobin Electrophoresis. Most of the samples in which Sysmex XN-1000 was unable to detect nRBCs and gave the false count of 0.0 (n=60) (11.7% of total positive samples); while the major positive population with CBC showing nRBCs are of Beta-Thalassemia Major as shown by Hb-electrophoresis. Further studies should be conducted in determining the cause of such an anomaly in these patients, as Beta-thalassemia is very common in our population [15,16]. Apart from this Sysmex XN-1000 showed remarkable sensitivity and specificity to detect and compute nRBCs in peripheral blood samples included in our study. Its advance technology can replace manual microscopic counts and reduce the burden of any laboratory setting. Automated analyzers are convenient in terms of cost and labor [11].

In a country like ours where hematological diseases like Beta-Thalassemia are prevalent and resources and trained personnel are limited, cost effective parameters like nRBCs are integral in determining disease progression [4,16]. In such settings, automated instruments like Sysmex XN 1000 can alleviate the burden caused by slide making and reviewing based on nRBCs. It can also provide results that are easily reproducible and highly accurate. The implementation of automatic release of results by Sysmex will also allow to reduce turnaround time of CBC samples analysis of any laboratory [2,12]. It will also be helpful in hospital settings and laboratories in peripheral areas where the availability of trained hematologists is limited.

CONCLUSION

Sysmex XN1000 analyzer is a highly sensitive and accurate instrument, which can replace the gold standard method of nRBCs enumeration, which is manual counting by conventional microscopy. It will be more time efficient, reliable and automated reporting, taking out the manual bias and human inaccuracies.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

GRANT SUPPORT & FINANCIAL DISCLOSURE

Declared none

AUTHORS CONTRIBUTION

Nimrah Ishaque: Design, literature search, data collection, statistical analysis and article writing

Hijab Batool: Design, analysis and article writing.

Ashja Saleem and Muhammad Usman Siddique: Data collection, literature search and writing

Ayisha Imran: Drafted the study design and concept, analysis, and literature search

Nauman Aslam Malik: Overall supervision of the study, concept and proof reading

REFERENCES

1. Morton SU, Brettin K, Feldman HA, Leeman KT. Association of nucleated red blood cell count with mortality among neonatal intensive care unit patients. Pediatr Neonatol. 2020; 61(6): 592-7. DOI: https://doi.org/10.1016/j.pedneo.2020.07.009
2. Chinudomwong P, Khongjaroensakun N, Chatachote B, Chaothai N, Paisooksantivatana K. Improving the efficiency of the autoverification workflow for nucleated red blood cell reporting in the hematology laboratory. Int J Lab Hematol. 2021; 43(6): 1373-8. DOI: https://doi.org/10.1111/ijlh.13641
3. Orhon A, Topal H, Hakan N, Ozer EA. Cord blood nucleated red blood cell level: Is it a predictive marker for neonatal jaundice?. J Lab Med. 2018; 42(5): 171-5.
4. Narcı H, Oktay MM, Ayrık C, Çimen MB. Nucleated red blood cells as predictor of all-cause mortality in emergency department. Am J Emerg Med. 2021; 46: 335-8. DOI: https://doi.org/10.1016/j.ajem.2020.10.002
5. Wang J, Su Z, Zhao X, Jin X, Lu G, Yuan Y, Li T. Investigation of the possible causes of NRBC in ICU patients and the dynamic trend of NRBC count in survival and death patients or with different underlying diseases: A retrospective study. Evidence-Based Complement Alternat Med. 2023; 2023: 9076647 DOI: https://doi.org/10.1155/2023/9076647
6. Wang N, Jiang J, Wang JA, He Y, Wen N, Liu Y, *et al*. Detection of nucleated red blood cells using the Mindray BC-6800Plus hematology analyzer: a clinical performance evaluation. Ann Palliat Med. 2021;10(8):8808-17. DOI https://doi.org/10.21037/apm-21-1772
7. Constantino BT, Rivera GK. Cutoff value for correcting white blood cell count for nucleated red blood cells: what is it? Why is it important?. Lab Med. 2019 Oct 10;50(4):e82-90.
8. Kim HJ, Namgoong S, Park CJ. A Case of spuriously decreased white blood cell count on an automated Sysmex XN hematology analyzer: The difference between the WNR and WDF channels. Lab Med Online. 2021; 11(3): 191-3. DOI: http://dx.doi.org/10.47429/lmo.2021.11.3.191
9. Schapkaitz E, Raburabu S. Performance evaluation of the new measurement channels on the automated Sysmex XN-9000 hematology analyzer. Clin Biochem. 2018; 53: 132-8. DOI:https://doi.org/10.1016/j.clinbiochem.2018.01.014
10. Shehata AMF, Wanas SA, El-Hawy MA, Khalifa KA. Clinical significance of nucleated red blood cell count in pediatric patients with transfusion-dependent beta thalassemia. Menoufia Medical J. 2020; 33(3): 949. DOI: https://doi.org/ 10.4103/mmj.mmj_14_20
11. Houyhongthong V, Nunphuak W, Sripatumtong C, Parnsamut C, Ketloy C. Automated nucleated red blood cell count using the Mindray BC-6800 hematology analyzer. Int J Lab Hematol. 2018; 40(5): 611-6. DOI: https://doi.org/10.1111/ijlh.12876
12. Ahmed T, Mahmood A, Uddin N, Robert HM, Ashraf M, Swati UT. Performance evaluation of nucleated red blood cell (NRBC) count using a fully automated haematology analyzer versus manual counting. Pak Armed F Med J. 2021; 71(5): 1806-0. DOI: https://doi.org/10.51253/pafmj.v71i5.5706
13. Pikora K, Krętowska-Grunwald A, Krawczuk-Rybak M, Sawicka-Żukowska M. Diagnostic value and prognostic significance of nucleated red blood cells (NRBCs) in selected medical conditions. Cells. 2023; 12(14): 1817. DOI: https://doi.org/10.3390/cells12141817
14. Gullupınar B, Saglam C, Yavru EV, Aksoy EE, Koran S, Evrin T, *et al*. New hematological parameters as early diagnosis and prognostic markers in critically patients. Disaster Emerg Med J. 2022; 7(2): 100-6.
15. Khaliq S. Thalassemia in Pakistan. Hemoglobin. 2022; 46(1): 12-4. DOI:https://doi.org/10.1080/03630269.2022.2059670
16. Ehsan H, Wahab A, Anwer F, Iftikhar R, Yousaf MN. Prevalence of transfusion transmissible infections in beta-thalassemia major patients in Pakistan: A systematic review. Cureus. 2020 27; 12(8). DOI: https://doi.org/10.7759/cureus.10070

Pattern and burden of thrombocytopenia in chronic hepatitis C virus patients at a tertiary care hospital

Qurat Ul Ain Ayaz[1], Farhan Ali Khanzada[2], Anum Sharif[3], Sarah Farrukh[4], Raana Akhtar[5], Ambareen Hamid[6]

[1]Combined Military Hospital, Multan Pakistan
[2] Sheikh Zayed Medical College, Rahim Yar Khan Pakistan
[3]District Headquarter Hospital, Multan Pakistan
[4]Tehsil Headquarters Hospital Rachna Town Ferozewala, Sheikhupura Pakistan
[5]University College of Medicine and Dentistry, University of Lahore, Lahore Pakistan
[6]Kind Edward Medical University, Lahore Pakistan

ABSTRACT

Objective: To determine the frequency of thrombocytopenia in chronic Hepatitis C virus infection.

Material and Methods: This descriptive cross-sectional study was conducted at department of Hematology, King Edward Medical University, Lahore from February 2023 to October 2023. A total of 143 patients of either gender with chronic Hepatitis C virus infection were included in the study. For every patient, a fresh 3 ml blood sample was collected by syringe using aseptic technique in a vacutainer containing EDTA. A complete blood count was carried out using Automated Hematology Analyzer and peripheral blood smear was prepared using Wright Giemsa stain to establish whether thrombocytopenia was present or not. Thrombocytopenia was assessed by Neubauer chamber.

Results: Age range in this study was from 18 to 65 years with mean age of 44.132±7.34 years, mean duration of HCV 12.685±4.53 months and mean platelet count was 238076.923 ± 100165.08/µL. Thrombocytopenia was seen in 37.1% patients.

Conclusion: Our results show that chronic hepatitis C infection is related to high frequency of thrombocytopenia. The higher the duration of disease process, higher is the risk of thrombocytopenia in these patients. This study highlights that identification by screening, timely diagnosis and treatment of Hepatitis C infection can prevent thrombocytopenia and its complications. Serial hematological follow up can play a pivotal role in such patients.

Keywords: Chronic hepatitis, Hepatitis C virus, Thrombocytopenia

This article can be cited as: Ayaz QUA, Khanzada FA, Sharif A, Farrukh S, Akhtar R, Hamid A. Pattern and burden of thrombocytopenia in chronic hepatitis C virus patients at a tertiary care hospital. Pak J Pathol. 2024; 35(1): 39-43.

DOI: https://doi/org/10.55629/pakjpathol.v35i1.796

INTRODUCTION

Hepatitis C denotes inflammation of the liver caused by the Hepatitis C Virus (HCV) [3]. HCV infection is a global health concern, affecting over 170 million individuals, which accounts for roughly 3% of the world's population [9,10]. As of an estimate conducted in January 2021, 9,746,000 Pakistanis are HCV positive which corresponds to a prevalence of 4.3% [11]. A systematic review estimated the average prevalence of thrombocytopenia in chronic HCV infection to be nearly 24% [12].

Thrombocytopenia is a concerning outcome of Hepatitis C virus infection[4]. Thrombocytopenia is characterized by a platelet count lower than 150,000/mm^3. In individuals with chronic hepatitis, it is commonly observed, especially among HCV antibodies positive individuals, who are 2.6 times more likely to have a low platelet count compared to HCV-antibody negative individuals [1]. This drop in platelet count can often be the sole indication of viral hepatitis, leading to consideration of it as a potential cause in the differential diagnosis of primary immune thrombocytopenia (ITP) [2]. The underlying causes are varied, involving aspects like autoimmunity, direct bone marrow suppression, hypersplenism, diminished

Correspondence: **Dr. Qurat Ul Ain Ayaz, Combined Military Hospital, Multan Pakistan**

Email: rose2013bwp@gmail.com

Receiving Date: **03 Nov 2023**
Revision Date: **13 Feb 2024**
Acceptance Date: **20 Mar 2024**

thrombopoietin production, and adverse effects of therapy [5]. Beyond eliciting an autoimmune response that generates antiplatelet antibodies, the virus also directly suppresses the bone marrow, resulting in thrombocytopenia [6-8].

Determining the frequency of thrombocytopenia in chronic hepatitis C patients can prove to be helpful for clinicians. It may lead to a change in routine diagnostics to include investigations for thrombocytopenia as a part of the basic workup, modifying the treatment and identifying high-risk patients for developing complications such as variceal bleeds. The aim of this study is to provide an awareness on a scientific basis to healthcare providers, stake holders and policy makers for defining better treatment plans for high-risk patients suffering from HCV.

MATERIAL AND METHODS

This Descriptive cross-sectional study was conducted at department of Hematology, King Edward Medical University, Lahore. This study was conducted form February 2023 to October 2023 using non-probability purposive sampling technique. The calculated sample size was 143 keeping 95% confidence level and 5% margin of error and taking expected frequency of thrombocytopenia was 24% [12] .

Patients of age 18 to 65 years of either gender with chronic Hepatitis C virus infection confirmed on PCR (qualitative). Patients with decompensated liver disease, on treatment for Liver disease, autoimmune, drug induced, infections other than hepatitis C, HIV, leukemia, lymphomas, some type of anemia, Chemotherapy drugs, heavy alcohol consumption, pregnancy, bacterial infections, thrombotic thrombocytopenic purpura and hemolytic uremic syndrome were excluded from our study.

Informed written consent was taken from the participants with the assurance of confidentiality to include their data in study. The patients included in the study were screened for HCV infection. For every patient a fresh 3 ml blood sample was collected by a syringe using aseptic technique in a vacutainer containing EDTA. A complete blood count was carried out using Automated Hematology Analyzer (Sysmex KX-21) and peripheral blood smear was prepared using Wright Giemsa stain to establish thrombocytopenia (Thrombocytopenia defined as a platelet count of <150,000/µL assessed by Neubauer chamber).

Data was entered and analysed using Statistical Package for the social sciences (SPSS) version 20.00 by the principal investigator. Quantitative variables including age of the patient, duration of HCV and platelet count was presented as mean ± standard deviation. Qualitative variables including gender, socioeconomic status and thrombocytopenia were presented in the form of frequency and percentages. Data was stratified for age, gender, duration of HCV and socioeconomic status. Chi square test was applied taking p value ≤0.05 as significant.

RESULTS

In our study, 143 patients of HCV infection with thrombocytopenia were enrolled. Enrolled patients age ranged from 18 to 65 years with mean age of 44.132 ± 7.34 years. Mean duration of HCV infection was 12.685 ± 4.53 months and mean platelet count was 238076.923 ± 100165.08 / µL.

Out of 143, 105 (73.4%) patients were males and 38 (26.6%) were females. The demographic variables of this study include Gender, Age, and Duration of HCV and Socioeconomic status. Out of these 143 patients, 37 male and 16 female patients had thrombocytopenia. Twelve patients among age 18-40 years and 41 patients with age 41-60 years showed thrombocytopenia. Eight patients with duration of 6-12 months and 45 patients with >12 months were positive for thrombocytopenia. Six patients belonged to low, 41 patients with middle and 6 patients with high socioeconomic status.

Thrombocytopenia was seen in 37% patients and stratification of thrombocytopenia among males was (35.2%) and in females was (42.1%), age group stratification showed that 31.6% of patients belonged to age group 18-40

years and 39% of patients belonged to age group 41-65years with thrombocytopenia.

No significant association of thrombocytopenia was observed with respect to age, gender or socioeconomic status (p-value >0.05) as denoted in Table-I, Table-II and Table-IV. However, thrombocytopenia was observed to be significantly associated with duration of HCV infection (p-value 0.000), with higher cases occurring with HCV infection of more than 12 months duration as demonstrated in Table-III.

Table-I: Comparison of thrombocytopenia with respect to age:

Age (years)	Thrombocytopenia		p-value
	Yes	No	
18-40	12 (31.6%)	26 (68.4%)	0.414
41-65	41 (39%)	64 (61%)	
Total	**53 (37.1%)**	**90 (62.9%)**	

Table-II: Comparison of thrombocytopenia with respect to Gender:

Gender	Thrombocytopenia		p-value
	Yes	No	
Male	37 (35.2%)	68 (64.8%)	0.453
Female	16 (42.1%)	22 (57.9%)	
Total	**53(37.1%)**	**90 (62.9%)**	

Table-III: Comparison of thrombocytopenia with respect to Duration of HCV.

Duration of HCV (months)	Thrombocytopenia		p-value
	Yes	No	
6-12	8 (10%)	72 (90%)	0.000
>12	45 (71.4%)	18 (28.6%)	
Total	**53 (37.1%)**	**90 (62.9%)**	

Table-IV: Comparison of thrombocytopenia with respect to Socioeconomic Status.

Socioeconomic Status	Thrombocytopenia		p-value
	Yes	No	
Low	6 (28.6%)	51 (71.7%)	0.641
Middle	41 (38%)	67 (62%)	
High	6 (42.9%)	8 (57.1%)	
Total	**53 (37.1%)**	**90 (62.9%)**	

DISCUSSION

Thrombocytopenia is a common hematological abnormality seen in patients with chronic hepatitis C infection [13]. The prevalence of thrombocytopenia in HCV infection can vary widely depending on factors such as the stage of liver disease, genotype of the virus, and presence of co-existing conditions. Thrombocytopenia in HCV infection can have clinical implications, especially in advanced stages of liver disease. It can contribute to an increased risk of bleeding, particularly in individuals with cirrhosis or advanced fibrosis. Certain factors might increase the likelihood of thrombocytopenia in HCV infection, including advanced age, male gender, duration of HCV infection, co-infection with other viruses (such as HIV) and heavy alcohol consumption [14]. The treatment of thrombocytopenia in HCV infection involves managing the underlying liver disease. Antiviral therapy aimed at eradicating HCV can improve liver function and potentially lead to an improvement in platelet counts. Additionally, in cases where thrombocytopenia is severe, healthcare providers might consider interventions to increase platelet counts, such as platelet transfusions or medications that stimulate platelet production [15].

In our study, 143 patients of HCV infection with thrombocytopenia were enrolled. Enrolled patients age ranged in this study was from 18 to 65 years. A regional study conducted by Sarwar *et al* [16] also reported an age range of 21 – 63 years in patients with hepatitis C virus infection. Another study by Raziq *et al* [17] reported a mean age of 44 ± 12.38 years of patients with hepatitis C virus infection. Nawaz *et al* [18] from Karachi has also reported 47.25 ± 11.52 years mean age of the patients with hepatitis C virus infection, similar to our results. Rahman *et al* [19] has also reported similar trends of ages in HCV patients.

Of all the HCV patients, 73.4% were male and 26.6% were female. A study by Sarwar *et al*[16] also reported 61% male and 39 % female patients with hepatitis C virus infection which is comparable to our demographics. Raziq *et al* [17] has also reported that 62% patients with hepatitis C virus infection were male and 38% were female patients. Nawaz *et al* [18] from Karachi also reported 54% male patients with hepatitis C virus infection. Rahman *et al* [19] has also reported 60% of male patients suffering from HCV.

Mean duration of HCV infection was observed to be 12.6 months with a mean platelet count of 238076.923 ± 100165.08/ µL. Sarwar *et al* [16] also reported 206.87 ± 83.88 (10^3/µl l) mean platelet levels of patients with hepatitis C virus infection, similar to our results. Nawaz *et al* [18] from Karachi has reported 3.06 ± 1.78 years mean duration of illness among patients with hepatitis C virus infection, contrary to our results.

Thrombocytopenia was seen in 37% patients in our study. Sarwar *et al* [16] also reported 28% thrombocytopenia among patients with hepatitis C virus infection, similar to our results. Raziq *et al* [17] has also reported 32 % frequency of thrombocytopenia in patients with hepatitis C virus infection. Nawaz *et al* [18] from Karachi has also reported 53% thrombocytopenia among patients with hepatitis C virus infection, similar to our results. Rahman *et al* [19] has also reported 22 thrombocytopenia in HCV patients, similar to our results.

Stratification of thrombocytopenia among males was (35.2%) and in females was (42.1%), age group stratification showed that 31.6% of patients belonged to age group 18-40 years and 39% of patients belonged to age group 41-65years with thrombocytopenia. Among these thrombocytopenic patients 14.7% belonged to low socioeconomic status, 75.5% and 9.8% belonged to middle and high socioeconomic status respectively.

We analyzed our results to find an association between thrombocytopenia and multiple factors such age, gender, socioeconomic status and disease duration. The purpose of finding a significant association was to define predictability of developing thrombocytopenia in patients of HCV with regards to these factors. We did not find any significant association of thrombocytopenia with patient´s age, gender or socioeconomic status. Raziq *et al* [17] has also reported that there was no significant association of thrombocytopenia with regards to gender and age i.e. p-value of 0.331 and 0.932 respectively, similar to our results. Nawaz *et al* [18] from Karachi has also reported that there was no association of thrombocytopenia with regards to gender and age. Rahman *et al* [19] has also reported that there was no significant association of thrombocytopenia with regards to gender, age, socioeconomic status and duration of HCV, however our study found significant association with prolonged duration of illness which is self-explanatory as longer the duration of illness, more detrimental effects of the disease occur leading to effects such as thrombocytopenia.

CONCLUSION

Our results show that chronic hepatitis C infection is related to high frequency of thrombocytopenia. The higher the duration of disease process, higher is the risk of thrombocytopenia in these patients. This study highlights that identification by screening, timely diagnosis and treatment of Hepatitis C infection can prevent thrombocytopenia and its complications. Serial hematological follow up can play a pivotal role in such patients.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

GRANT SUPPORT & FINANCIAL DISCLOSURE

Declared none

AUTHORS CONTRIBUTION

Qurat Ul Ain Ayaz: Study concept, literature search, data analysis, questionnaire design, final approval to be published

Farhan Ali Khanzada: Data analysis, data interpretation, Drafting

Anum Sharif: Literature search, data interpretation, data collection

Sarah Farrukh: Conception / design of the work data interpretation, data analysis**,** final approval to be published

Raana Akhtar: Literature search, data interpretation, data collection

Ambareen Hamid: Study concept, literature search, data analysis, questionnaire design

REFERENCES

1. Rawi S, Wu GY. Pathogenesis of thrombocytopenia in chronic HCV infection: A Review. J Clin Transl Hepatol. 2020 28;8(2):184-91.

DOI: https://doi.org/10.14218/JCTH.2020.00007

2. Tajiri K, Okada K, Ito H, Kawai K, Kashii Y, Tokimitsu Y, *et al*. Long-term changes in thrombocytopenia and leucopenia after HCV eradication with direct-acting antivirals. BMC Gastroenterol. 2023; 23(1): 182. DOI: https://doi.org/10.1186/s12876-023-02829-w
3. Omer S, Iftime A, Constantinescu I, Dina I. Low-cost predictors for liver function and clinical outcomes after sustained virological response in patients with HCV-Related cirrhosis and thrombocytopenia. Medicina (Kaunas). 2023; 59(1): 146. DOI: https://doi.org/10.3390/medicina59010146
4. Manns MP, Buti M, Gane E, Pawlotsky JM, Razavi H, Terrault N, *et al*. Hepatitis C virus infection. Nat Rev Dis Primers. 2017 Mar 2; 3: 17006. DOI: https://doi.org/10.1038/nrdp.2017.6
5. Isfordink CJ, Maan R, de Man RA, van Erpecum KJ, van der Meer AJ. Should we continue surveillance for hepatocellular carcinoma and gastroesophageal varices in patients with cirrhosis and cured HCV infection? Eur J Intern Med. 2021; 94:6-14. DOI: https://doi.org/10.1016/j.ejim.2021.08.023
6. Isfordink CJ, van Erpecum KJ, Fischer K, van der Valk PR, van Vulpen LFD, Schutgens REG, *et al*. Liver-related complications before and after successful treatment of chronic hepatitis C virus infection in people with inherited bleeding disorders. Haemophilia. 2023; 29(1): 106-114. DOI: https://doi.org/10.1111/hae.14668
7. Ayoub HH, Mahmud S, Chemaitelly H, Abu-Raddad LJ. Treatment as prevention for hepatitis C virus in the Middle East and North Africa: A modeling study. Front Public Health. 2023; 11: 1187786. DOI: https://doi.org/10.3389/fpubh.2023.1187786
8. Choi GH, Jang ES, Kim YS, Lee YJ, Kim IH, Cho SB, *et al*. Hepatocellular carcinoma, decompensation, and mortality based on hepatitis C treatment: A prospective cohort study. World J Gastroenterol. 2022; 28(30): 4182-4200. DOI: https://doi.org/10.3748/wjg.v28.i30.4182
9. Abdelhamed W, El-Kassas M. Hepatocellular carcinoma and hepatitis C virus treatments: The bold and the beautiful. J Viral Hepat. 2023; 30(2): 148-59. DOI: https://doi.org/10.1111/jvh.13778
10. Roudot-Thoraval F. Epidemiology of hepatitis C virus infection. Clin Res Hepatol Gastroenterol. 2021; 45(3): 101596. DOI: https://doi.org/10.1016/j.clinre.2020.101596
11. Mooneyhan E, Qureshi H, Mahmood H, Tariq M, Maqbool NA, Anwar M, *et al*. Hepatitis C prevalence and elimination planning in Pakistan, a bottom-up approach accounting for provincial variation. J Viral Hepatitis. 2023; 30(4): 345-54. DOI: https://doi.org/10.1111/jvh.13802
12. Dahal S, Upadhyay S, Banjade R, Dhakal P, Khanal N, Bhatt VR. Thrombocytopenia in patients with chronic hepatitis C virus infection. Mediterr J Hematol Infect Dis. 2017; 9(1): e2017019. DOI: https://doi.org/10.4084%2FMJHID.2017.019
13. Chen SH, Tsai SC, Lu HC. Platelets as a Gauge of liver disease kinetics? Int J Mol Sci. 2022; 23(19): 11460. DOI: https://doi.org/10.3390/ijms231911460
14. Huang CE, Chang JJ, Wu YY, Huang SH, Chen WM, Hsu CC, *et al*. Different impacts of common risk factors associated with thrombocytopenia in patients with hepatitis B virus and hepatitis C virus infection. Biomed J. 2022; 45(5): 788-97. DOI: https://doi.org/10.1016/j.bj.2021.09.001
15. Huang CE, Chen YY, Chang JJ, Wu YY, Chen WM, Wang YH, *et al*. The impact of human platelet antigen allele on antiplatelet antibodies and cryoglobulins in patients with primary immune thrombocytopenia and hepatitis c virus-associated immune thrombocytopenia. Mediterr J Hematol Infect Dis. 2023; 15(1): e2023030. DOI: https://doi.org/10.4084/MJHID.2023.030
16. Sarwar K, Tipu I, Zahoor S. HCV induced thrombocytopenia in Punjab, Pakistan. BioSci Rev. 2020; 2(1): 19-27. DOI: http://dx.doi.org/10.32350//BSR.0201.03
17. Raziq MA, Hussain B, Wahhaj FQ. A Study of thrombocytopenia in cases of hepatitis C infection presenting at tertiary care hospital. Pak J Med Health Sci. 2021; 15(5): 926-8. DOI: https://doi.org/10.53350/pjmhs21155926
18. Nawaz Z, Aurangzeb M, Imran K, Rasheed T, Alam MT, Jaffery MF, *et al*. Frequency of thrombocytopenia; patients with chronic hepatitis C infection. Professional Med J. 2014; 21(4): 684-90. DOI: https://doi.org/10.4084%2FMJHID.2017.019
19. Rahman S, Rahman S, Khan S, Ahmad N, Rahman N. Frequency of thrombocytopenia in hepatitis C patients. J Med Sci. 2019; 27:(3) 190-3.

Spectrum of hematological disorders on bone marrow examination in a tertiary care Centre

Hina Bilal[1], Sundas Ali[2], Maha Tariq Kiani[2]

[1]Federal Medical College, Islamabad Pakistan
[2]Pakistan Institute of Medical Sciences, Islamabad Pakistan

ABSTRACT

Objective: The aims of this study were to understand the range of different blood-related disorders diagnosed through bone marrow examination and to explore the connection between the age groups and genders of our patients with the subtypes of acute leukemia.

Material and Methods: This was a cross-sectional study carried over a period of one year from January 2022 to December 2022, in the department of Pathology, Pakistan Institute of Medical Sciences (PIMS), Islamabad. Analysis of bone marrow was conducted on patients referred with suspected blood-related disorders, and a total of 254 cases were incorporated into the study. We divided age into groups as 0-12 years (paediatric age group) and >12 years (adults) for comparison with subtypes of acute leukemias. Pearson chi-square test was applied. P value of <0.05 was considered statistically significant.

Results: The age range was from 05 months - 91 years with male: female ratio of 1.2:1. The prevalent non-malignant conditions identified during bone marrow examination were peripheral destruction/consumption of platelets (24.8%) followed by anemias (22%) and infection- related changes (9.4%). Among hematological malignancies, acute leukemias were the most frequent (22.8%), with chronic leukemias following at 7.1%. We also observed that Acute Lymphoblastic Leukemia was more common in Paediatric age group, while Acute Myeloid Leukemia was more frequent type of acute leukemia in adults (p <0.001).

Conclusion: The examination of bone marrow specimens is a key procedure for swiftly obtaining a confirmatory diagnosis for numerous hematological disorders, including hematologic malignancies.

Keywords: Anemia, Bone Marrow Trephine Biopsy, Bone marrow aspiration, Leukemia, Megaloblastic anemia

This article can be cited as: Bilal H, Ali S, Kiani MT. Spectrum of hematological disorders on bone marrow examination in a tertiary care Centre. Pak J Pathol. 2024; 35(1): 44-48.

DOI: https://doi.org/10.55629/pakjpathol.v35i1.791

INTRODUCTION

Blood disorders encompass a wide spectrum of conditions, spanning from common ailments like iron deficiency anemia to more complex and malignant diseases such as leukemias, myelomas, and lymphomas [1]. The prevalence of hematological malignancies varies globally, with higher rates in better resourced countries and lower rates in resource constraint regions, often due to under-diagnosis and limited registration resources in developing nations. These malignancies account for approximately 6.5% of all cancers worldwide and stand as the fourth most commonly identified cancer in economically advanced nations for both men and women.

Hematological malignancies can be generally categorized into two groups based on their cell lineage: myeloid and lymphoid. Common subgroups within lymphoid neoplasms include Acute Lymphoblastic Leukemia, Chronic Lymphocytic Leukemia, Hodgkin and Non-Hodgkin's lymphoma, Multiple Myeloma, Waldenstrom macroglobulinemia. Myeloid neoplasms predominantly comprise Myeloproliferative Neoplasm, Myelodysplastic Syndrome, and Acute Myeloid Leukemia [2].

Correspondence: **Dr. Sundas Ali, Medical Officer/ Haematologist, Pakistan Institute of Medical Sciences, Islamabad Pakistan**

Email: **sundasali243@gmail.com**

Receiving Date: **14 Oct 2023**
Revision Date: **10 Nov 2023**
Acceptance Date: **04 Jan 2024**

A fundamental diagnostic tool for both blood-related (hematological) and non-blood-related (non-hematological) disorders is examination of bone marrow biopsies. [3]. Although it involves some invasion, patients typically handle this procedure well and provides rapid insights into many cases, aiding in timely diagnosis. This procedure proves cost-effective and invaluable in the field of hematology, facilitating the identification of both malignant and non-malignant hematological disorders [4].

Bone marrow aspiration and bone marrow biopsy complement each other and are often performed simultaneously for diagnostic purposes [5]. Bone marrow aspirate smears offer cytological insights into hemopoietic cells, while bone marrow biopsy assesses cellularity, infiltration patterns and fibrosis [4]. It plays a pivotal role in diagnosing conditions like Granulomatous diseases, myelodysplastic syndromes, myeloproliferative disorders, non-Hodgkin's lymphomas, aplastic Anemia, and metastatic tumours eliciting a fibrotic response [5]. This study aimed to explore the diverse spectrum of hematological disorders through bone marrow analysis and observe the association of age group and gender of patients with subtypes of acute leukemia. There is huge patient turn over in our setup and patients from different parts of Punjab and North visit our facility. The range of hematological disorders differs across regions. Therefore, the purpose of this study is to diagnose and offer valuable information about various blood-related disorders to clinicians for improved management of these cases.

MATERIAL AND METHODS

It was a cross-sectional study done in the Pathology department of Pakistan Institute of Medical Sciences (P.I.M.S) for a period of one year. The record of patients who were sent to Pathology department of PIMS for bone marrow examination, from January 2022 to December 2022 was scrutinized. Bone marrow examination of 600 patients was done in the pathology department in the year 2022. Follow up cases of hematological and non-hematological malignancies and cases for staging of other malignancies were excluded from the study. A total of 254 remaining cases were included in the study. All data was entered and analyzed by using SPSS version 16. The categorical variables like gender and diagnosis were measured as frequency and percentages. The quantitative numerical variables like age were measured as mean and standard deviations. Association of age-groups and gender with subtypes of acute leukemias was analysed. We divided age into groups as 0-12 years (paediatric age group) and >12 years (adults)for comparison with subtypes of acute leukemias. Pearson chi-square test was applied. P value of <0.05 was considered statistically significant.

RESULTS

Mean age of patients in this study was 23 ± 22.9 years. Most of the patients were of age group 0-10 years (46.9%), 13% patients were of age group 11-20 years while 9.4% of patients were of age group 41-50 years. When the cases were analysed, 63 (24.8%) patients had diagnosis of excessive peripheral platelet destruction/consumption and 56 (22%) of patients presented with Anemias. Out of total, 58 (22.8%) patients presented with acute leukemia while 18 (7.1%) presented with chronic leukemia (Table-I). Of 56 cases of Anemias, 21(37.5%) cases presented with megaloblastic Anemia, 13(23.2%) presented with mixed deficiency Anemia, 10 (17.8%) presented with iron deficiency Anemia, 8 (14.2%) presented with aplastic Anemia and 4 (7.1%) presented with haemolytic Anemia.Of the 58 cases of acute leukemias, 37(63.8%) patients had Acute lymphoblastic leukemia and 21 (36.2%) had Acute myeloid leukemia. So, over all non-malignant haematological disorders were observed more frequently in current study. Figure-I shows the Giemsa-stained bone marrow microscopic images of the most common cases in our study. We analysed association of age-groups and gender with acute leukemia subtypes. There was statistically significant association between age-groups and subtypes of acute leukemia (p value <0.001). (Table-III).

Table-I: Depiction of hematological disorders diagnosed on bone marrow examination (n=254)

Hematological Disorders	Frequency	Percent
Excessive peripheral destruction /consumption of platelets	63	24.8
Acute leukemia	58	22.8
Anemias	56	22
Infection related changes	24	9.4
Chronic leukemias	18	7.1
Multiple myeloma	14	5.5
Storage diseases	8	3.1
Visceral Leishmaniasis	5	2
Pure red cell aplasia	3	1.2
Burkitts lymphoma	3	1.2
Chediak-Higashi Syndrome	1	0.4
Chronic granulomatous disease	1	0.4
Total	**254**	**100**

Table-II: Association of age-groups and gender with subtypes of Acute leukemia (n= 58).

		ALL	AML	P-value
Age range	**0-12 years**	25	1	
	>12 years	12	20	.000
Total		37	21	
Gender	**Male**	22	10	
	Female	15	11	.384
Total		37	21	

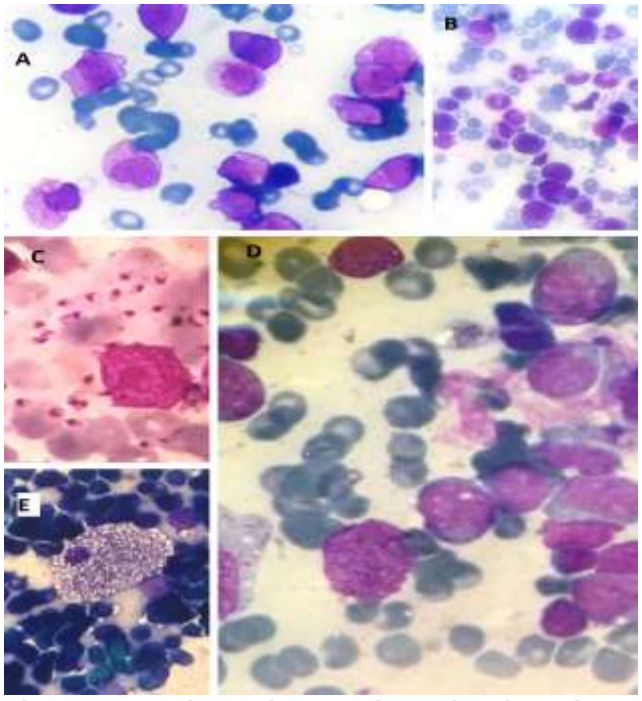

Figure 1: Photomicrographs showing bone marrow aspirate smears A) Acute Promyelocytic Leukemia B) Megaloblastic anemia C) Visceral Leishmaniasis D) Acute Myeloid Leukemia E) Niemann Pick Disease (Giemsa 1000X).

DISCUSSION

Over the past several years, the analysis of samples from bone marrow has gained widespread significance in the accurate diagnosis and staging of different blood diseases. Extracting bone marrow samples and their subsequent analysis can be instrumental in confirming presumed blood related disorders. In grown-ups, a complete bone marrow examination, incorporating both aspiration and trephine biopsy, is necessary for a detailed evaluation of the marrow's structure. It's worth noting that the arrangement of blood related and non-blood related diseases varies between developing and developed regions [6].

The analysis of both Bone Marrow Aspiration and Bone Marrow Trephine Biopsy has become a routine procedure for assessing a range of conditions, including low blood counts, hematologic malignancies, non-malignant conditions, and spread of distant cancers. Biopsy of the bone marrow plays a fundamental role in the investigation of disorders related to blood and might be the sole approach through which a precise diagnosis can be achieved. Notably, both aspiration and trephine biopsies are straightforward and secure methods that could be easily conducted on an outpatient basis [7]. This diagnostic approach can either confirm a clinically suspected disease or unveil previously unrecognized diagnoses [8]. Furthermore, Immunohistochemistry can be applied on trephine biopsy sections for reaching a definitive diagnosis especially in cases of bone marrow infiltration.

The mean age of participants in our investigation was 23 years with a standard deviation of ±22.9. A substantial proportion of patients fell into the 0-10 years age group (46.9%). On the flip side, a different study conducted by M. Atchyuta and colleagues documented the highest percentage of instances (19%) in the 31-40 age bracket, with the next highest in the 51-60 age range.[9]. This discrepancy may be attributed to a higher rate of paediatric patient referrals to the Pathology Department for bone marrow examinations. Out of 254 patients, 141 (55.5%) were males and 113 (44.5%) were females, resulting in a male-

to-female ratio of 1.2:1, consistent with the previously mentioned study, which reported a similar ratio of 1.1:1 [9].

In our research, the prevailing non-cancerousdisorder detected was peripheral destruction/consumption of platelets (ITP). The diagnosis of ITP depends on clinical suspicion and a standard peripheral smear, except for low platelet count. Bone marrow examination is conducted to rule out conditions such as leukemia, myelodysplastic syndrome, or aplastic anemia [10]. Anemias ranked as the second most common non-malignant disorders identified through bone marrow biopsy, with 56 (22%) patients presenting with various forms of anemia. Of 56 cases of Anemias, 21 cases presented with megaloblastic Anemia, 13 presented with mixed deficiency Anemia, 10 presented with iron deficiency Anemia, 8 presented with aplastic Anemia and 4 presented with haemolytic Anemia. Serum B12 and folate levels were not done in patients diagnosed as megaloblastic or mixed deficiency anemia either due to recent transfusions or financial constraints of patients. This pattern of anemia subtypes aligns with other studies [1,3], which also reported megaloblastic anemia as the most prevalent form, with mixed deficiency anemia and aplastic anemia coming next. In another research study, nutritional anemias, including megaloblastic, mixed deficiency (comprising both microcytic and macrocytic types), and iron deficiency, collectively constituted 32.5% of cases within the non-hematological category. Among these nutritional anemias, megaloblastic anemia emerged as the most prevalent non-malignant hematological disorder, accounting for 24.92% of cases, followed by mixed deficiency anemia at 4.71%. Iron deficiency anemia was the least frequently encountered, representing only 3.53% of cases [11]. The reason for iron deficiency anemia being the least common type of nutritional anemia detected on bone marrow biopsy is that the serum markers such as Iron, Ferritin, TIBC are available for its diagnosis easily. Ideally bone marrow biopsy should only be done if patient is not responding to iron therapy.

Regarding malignant disorders, our study found that acute leukemias were the most common, constituting 58 (22.8%) cases. Among these, acute lymphoblastic leukemia accounted for 63.8%, while acute myeloid leukemia constituted 36.2%. The diagnosis of acute leukemias on bone marrow biopsy in our study was supplemented by Flow Cytometry either on peripheral blood sample or bone marrow aspirate sample. In contrast to our findings, a study by Iqbal *et al*. reported a different distribution, with acute myelogenous leukemia diagnosed in 23.08% of patients and acute lymphoblastic leukemia in 23.08% [12]. This difference could be attributed to variations in age distribution, with ALL being more prevalent in children and AML in adults. Our study included a higher number of paediatric patients. In another study, the most prevalent malignant diagnoses, listed in descending order of frequency, were as follows: leukemia (20%), multiple myeloma (13%), and lymphoma (11%). Notably, acute leukemia constituted a significant portion, accounting for 46% (11 out of 24 cases) of all diagnosed leukemia cases when compared to chronic leukemia [13]. Our own findings echoed this trend, revealing a higher prevalence of acute leukemia cases in contrast to chronic leukemias and lymphomas. Our research indicated a higher occurrence of ALL in the pediatric age group, consistent with Ahmad *et al* s study, which found that ALL is more frequent and prevalent among those under 10 years old (31.25%, n=125). ALL stands out as the most prevalent malignancy in children, constituting 25% of all childhood cancers and about 75% of all childhood leukemia cases [14]. Conversely, conditions such as storage diseases, Visceral Leishmaniasis, Pure red cell aplasia, Chediak-Higashi syndrome, and Chronic granulomatous disease were less frequently observed.

Recognizing the constraints of our study is crucial, especially given the relatively modest sample size. To obtain more definitive findings, larger multi-centric studies conducted in local settings are recommended.

CONCLUSION

Conducting bone marrow aspiration and trephine biopsy is a crucial procedure appropriate for diagnosing various blood-related diseases in an outpatient setting. This research highlighted the significance of bone marrow examination in the diagnosis of hematological diseases. Bone marrow examination in our setup is used more frequently for diagnosis in pediatric population. We noted a diverse range of conditions, ranging from non-malignant to malignant blood-related disorders. The prevalent non-hematological issues identified in this study included excessive peripheral platelet destruction/consumption (ITP) and various types of anemias. The most frequent malignant disorders observed were acute leukemias.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

GRANT SUPPORT & FINANCIAL DISCLOSURE

Declared none

AUTHORS CONTRIBUTION

Hina Bilal: Concept, literature search, data collection, data analysis, data interpretation, drafting

Sundas Ali: Literature search, data collection, drafting, review

Maha Tariq Kiani: Data collection, drafting, review

REFERENCES

1. Qureshi H, Farooq N, Amjad M, Arif S, UllahIhsan. Pattern of hematological disorders on bone marrow aspirate examination. J Med Sci. 2019; 27 (2): 130-4.
2. Khan S, Mir A, Khattak BR, Khan SN, Hameed M, *et al*. Pattern of adulthood hematological malignancies in Khyber Pakhtunkhwa. J Blood Disord Transfus. 2019; 10: 1000424. DOI: https://doi.org/10.4172/2155-9864.1000424
3. Khan SP, Geelani S, Akhter S, Bhat S, Hussain S, Manzoor F, *et al*. Bone marrow aspiration in haematological disorders: Study at a tertiary care centre. Int J Res Med Sci. 2018; 6: 2361-4. DOI:https://doi.org/10.18203/2320-6012.ijrms20182817
4. Bansal M, Kumar N, Sharma HB, Sharma R. Pattern of hematological diseases diagnosed by bone marrow examination in a tertiary care hospital. Int J Acad Med Pharm. 2022; 4 (3); 240-3. DOI:https://doi.org/10.47009/jamp.2022.4.3.55
5. Bashir N, Musharaf B, Reshi R, Jeelani T, Rafiq D, Angmo D. Bone marrow profile in hematological disorders: An experience from a tertiary care centre. Int J Adv Med. 2018; 5:608-13. DOI:https://doi.org/10.18203/2349-3933.ijam20182111
6. Fareed N, Mehmood T, Quraishy MS, Fatima G. Pattern of hematological disorders on bone marrow examination: A tertiary care hospital experience. Hematol TransfusInt J. 2021; 9(6):117-20. DOI: https://doi.org/10.15406/htij.2021.09.00267
7. Mohammad NH, Hussein F, Ahmad I. The role of bone marrow aspiration and bone marrow biopsy in diagnosis of bone marrow metastases. IJBC 2017; 9(3): 80-83.
8. Piplani G, Teerthanath S. Study of spectrum of lesions in bone marrow aspiration and trephine biopsy from a tertiary care centre in South India. Int J Pediatr Res. 2022; 8: 138. DOI:https://doi.org/doi.org/10.23937/2469-5807/1510138
9. Atchyuta M, Premalatha P, Renuka IV, Krishnamashary PAV, Tejeswini V. Spectrum of hematological diseases diagnosed by bone marrow examination in a tertiary care hospital. Indian J Pathol Oncol. 2019; 6(2): 185-9. DOI: https://doi.org/10.18231/j.ijpo.2019.036
10. Purohit A, Aggarwal M, Singh PK, Mahapatra M, Seth T, Tyagi S, *et al*. Re-evaluation of need for bone marrow examination in patients with isolated thrombocytopenia contributors. Indian J Hematol Blood Transfus. 2016; 32(2): 193-6. DOI: https://doi.org/10.1007/s12288-015-0533-2
11. Rahim F, Ahmad I, Islam S, Hussain M, Khattak TAK, Bano Q. Spectrum of hematological disorders in children observed in 424 consecutive bone marrow aspirations/ biopsies. Pak J Med Sci. 2005; 21(4): 433-6
12. Iqbal K, Hussain Z, Malik SN, Rahman AK, Riaz M, Qureshi H, *et al*. Frequency of hematological malignancies based on bone-marrow aspiration and trephine biopsy in patients presenting to tertiary care hospital. Pak J Med Health Sci. 2022; 16(12): 403-5.
13. Tshabalala WS, Pillay S, Wilson DPK. Diagnostic outcomes of bone marrow aspirate and trephine biopsies performed at a hospital in KwaZulu-Natal, South Africa. Afr J Lab Med. 2020; 9(1):1028. DOI: https://doi.org/10.4102/ajlm.v9i1.1028
14. Ahmad S, Shah KA, Hussain H, Haq AU, Ullah A, Khan A, *et al*. Prevalence of acute and chronic forms of leukemia in various regions of Khyber Pakhtunkhwa, Pakistan: needs much more to be done!. Bangladesh J Med Sci. 2019; 18(2): 222. DOI: https://doi.org/10.3329/BJMS.V18I2.40689

GUIDLINES FOR AUTHORS

Pakistan Journal of Pathology (PAK J PATHOL) is the official journal of Pakistan association of Pathologists. Acceptance criteria for all papers and reviews are based on the quality and originality of the research and its clinical and scientific significance to our readership.

1. Editorial Policy

The Journal will publish research material of interest to the researchers, scientists and medical practitioners. The Journal publishes peer-reviewed original papers, reviews, case reports, and editorials concerned with clinical practice and research in the fields of pathology. Manuscripts should follow the style of the Vancouver agreement detailed in the International Committee of Medical Journal Editors'. In preparing manuscripts, authors should follow the "Uniform Requirements for Manuscript Submitted to Biomedical Journals updated October 2008, available at www.icmje.org" and specific author instructions detailed below.

2. Manuscript Submission

(1) Go to the Pak J Pathol official website: **https://www.pakjpath.com/**

(2) Click **'make a submission'** tab on Pak J Pathol website.

(3) Please read checklist and make sure that no point in the checklist is missing

(4) Click **'register'** if you are submitting to Pak J Pathol for the first time. If you are already registered with Pak J Pathol then click 'login'.

(5) A new page will open once you click 'register'. Enter the required information in the given fields. Pakistan Journal of Pathology requests all its authors **to register themselves with Pak J Pathol as reviewer** by clicking 'Yes, request the reviewer role' available at the bottom of 'registration' page. After clicking on 'register' a new page will open.

(6) Please click **'make a new submission'** on this page

(7) A new page will open, with 5 tabs on the top.

(i) Start,

(ii) Upload submission,

(iii) Enter metadata,

(iv) Confirmation,

(v) Next steps. You will be in 'start' tab.

(8) In 'Section' dropdown list, select the appropriate option. Please make sure that all the 'submission requirements' are fulfilled. Tick the checkbox against each requirement. **Note:** if any of the requirement is not fulfilled, and you put a tick mark against it, you still will be able to proceed and complete your submission process but the submission will be declined automatically by the system. In 'comments for the editor' area you can write your comments, however, it is optional. Under 'Acknowledge the copyright statement' tick the checkboxes against both the options. Click 'save and continue'

(9) Now you will be in 'upload submission' tab. A dialogue box will open and here you can upload all your files **(article text, title page, ERC/IRB approval letter, undertaking, processing fee)** one by one. Click 'save and continue'.

(10) Now you will be in 'enter metadata' tab. You may leave 'prefix' field blank. Write title of the article in 'title' field. Write running/short title in 'subtitle' field (you may leave it blank). Copy/paste abstract in abstract area. In 'list of contributors' sections, click 'add contributor' (if you have other authors with you). A new dialogue box will open. Please fill in details of each contributor (author). Please do give **affiliation** of each contributor. Additional details about the contributor can be given in the text box below. For 'contributor's role' please click 'author'. Please tick the check box 'Principal contact for editorial correspondence' for corresponding author only. Lower checkbox 'Include this contributor in browse lists?' will remain ticked for all the authors. Click 'save' and repeat the same process for all the authors one by one. Please **add all the authors** as per the pre-decided

sequence. Please fill the 'languages', 'subjects', 'discipline' and 'key words' fields appropriately. These fields are essential. Each key word **MUST be added SEPARATELY** one by one instead of 'copy/paste' all the key words together. Please click 'save and continue'.

(11) You are now in 'confirmation' tab. Please click 'finish submission'. Congratulations, you have successfully completed your submission to Pak J Pathol.

(12) You can track the status/progress of your article through editorial process any time by logging into your account.

3. Undertaking

The undertaking must be signed by all authors and must contain following statements

(1) The material submitted for publication is, original and has not been submitted for publication elsewhere.

(2) Co-authors qualify authorship and share the responsibility of contents in manuscript.

(3) The research has been approved by ethical committee, and there is no conflict of interest.

4. Format requirements of original article

A. Title page

The title page of the manuscript should include:

(1) Title: Should be concise and self-descriptive.

(2) Authors: First, middle and last name of each author in sequence of authorship merit.

(3) Affiliation for each author, with the name of department and institution.

(4) Corresponding address, cell number, and e mail address of any of first three authors.

(5) A short running head or foot line of no more than 40 characters.

B. Abstract: Abstract should not be of more than 250 words, it must be structured in headings as objective, material & methods, results, and conclusion. Also include 3-5 key words relating to the article subject as abbreviated in the Index -Medicus.

C. Material and Methods: Describe the type, duration & place of study. Summarize the demographic data of the subjects. Provide brief descriptions for methods along-with references. Identify all drugs and chemicals used, including generic name, dose, and route of administration. Reports of randomized clinical trials should present information on all major study elements and protocols. Describe statistical methods used, and specify computer program employed for this purpose.

D. Results: Present results in logical sequence in the text, tables, and illustrations. Do not repeat text data in the tables or illustrations.

E. Discussion: Justify result findings sequentially one by one. While justifying the findings, cite references that support or negate the findings of your study in a logical way. Compare your findings with other similar studies carried out locally and internationally. In this section limitation of study, recommendations, and conclusions drawn must be clearly mentioned.

F. References: References should be cited in consecutive numerical order at first mentioned in the text and designated by the reference number in parenthesis. The references must be written in Vancouver style. A few examples are given below:

(1) For journal articles, list the first six authors only and add, *et al* for the others. For example: Guilarde AO, Turchi MD, Siqueira JB Jr, Mochan DP, Tersi NL, Mouchan L, *et al*. Dengue hemorrhagic fever in adults: clinical outcome related to the serotypes. J Infect Dis. 2012; 197: 817-24.

(2) For books and book chapters, follow as: DeGroot IJ. Evaluation of adrenal function and adrenal disease. In: DeGroot L, Stanbury J B, eds. The thyroid and its diseases. 5th ed. New York: Wiley, 1985, pp 199-258.

(3) Publications in press follow as: Benner AI. Molecular mechanisms of alcohol in addiction. N Engl J Med. In press 1999.

(4) Journal article in electronic form, follow as: Cosby AI. Factors in the - emergence of communicable diseases. Emerg Infect Dis [serial online] 1995 Jan-Mar [cited 2000 July 5], Computerized Educational Systems, 2000.

(5) Monograph in electronic form, follow as**:** CDI, clinical dermatology illustrated [monographs on CD-ROM] Reeves JRT, Maibach H. CMEA Multimedia group, producers 2nd ed. Version 2.0. San Diego: CMEA, 1995.

(6) Conference proceedings follow as: Kimura J, Shibasaki H, editors. Recent advances in clinical neurophysiology. Proceedings of the 10th International Congress of EKG and Clinical Neurophysiology, 2000 Oct 19-25, Kyoto, Japan. Amsterdam: Elsevier, 1996.

(7) Dissertation, follow as: Webster SJ. Post-hospital general health care the elderly's access and utilization [Thesis]. St. Louis (MO): Washington Univ., 1995.

(8) Volume with supplement, follow as: Lolti HM, Chong AR. Risk assessment of Codmium carcinogenicity and occupational skin cancer. Global Health Perspect, 1999; 102 Suppl 1:274-94.

G. **Tables:** 3–5 figures and or tables are allowed (each table, complete with legends and footnotes, should be merged in the manuscript). Tables and figures should be embedded and not supplied separately. Table/figure should be written with a capital T and capital F with a hyphen between the word and number e.g., Table-1.
Note: Kindly design table/graph on MS Word/MS Excel. Do not attach table/graph in the form of pictures

H. **Illustrations and legends:** Illustrations should clarify and augment the text. Submit two complete sets of glossy illustrations. No hand drawn art will be accepted. Each illustration must be numbered and cited in consecutive order in the text. Legends for illustrations should be concise and should not repeat the text. Legends should be typed double-spaced. Each figure should be cited in consecutive numerical order in the text. Give the figures a number following the word Figure. Use letters to designate parts of illustrations (e.g, A, B, C) and describe each part clearly in the legend. Any letter designations or arrows appearing on the illustration should be identified and described fully.

I. **Letter from Institutional Review Board / Ethical Review Committee:** Authors are required to send approval letter from Institutional Review Board / Ethical Review Committee along with Original articles and Case reports.

J. **Permission Letter from Med Dte GHQ (Only for Army Serving Doctors)**
Med Dte GHQ permission letter is mandatory for all army serving doctors at the time of article/ case report submission.

K. **Processing / publication fee**
The processing fee of **Rs. 3000/-** (non-refundable) is to be paid at the time of submission of the article through demand/ bank draft payable in the favor of **Commandant AFIP** account. It is further intimated that authors` will have to pay **Rs. 5,000/-** as publication charges/ fee, if the article is accepted for publication. The publication charges for case report and short communication will be half of the above charges. Research Protocol will be published with the same publication charges as that of case report. (Payable before issuance of acceptance letter)

L. **Subscription Fee**

- Single Issue: Rs. 500/-
- Annual Subscription Rs. 2000/-

UNDERTAKING & COPYRIGHT AGREEMENT

Pakistan Journal of Pathology (Pak J Pathol)

All manuscripts must be submitted along with **"Undertaking & Copyright Agreement"** form, as given below, and signed by the Principal author and co-author(s).

It is certified that the following Editorial/ Original/ Review article titled "_____
_____
_____"
has been submitted to Pakistan Journal of Pathology (PAK J PATHOL) for publication. The undersigned stipulate that manuscript has been seen and approved by all authors involved and is neither published nor being considered for publication elsewhere. All copyrights ownership for the article are transferred to the Pak J Pathol.

Name	Institute / Affiliation	Qualification	Contact no	Email	Signature

Authors' Contributions:

Please insert below the detailed contributions made by each author. Examples may include literature search, study design and concept, questionnaire design, data collection, data analysis, data interpretation, drafting, etc.

The current ICMJE authorship criteria, of which all four criteria must be met for authorship, are as follows:

1. Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work;
2. Drafting the work or revising it critically for important intellectual content;
3. Final approval of the version to be published;

Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

INFORMATION FOR SUBSCRIBERS

SUBSCRIPTION RATES

Pakistan Journal of Pathology is published quarterly by AFIP on behalf of Pakistan Association of Pathologists (PAP).

Annual Subscription

Annual Subscription : Rs. 2000 (Post-paid)

Single copy : Rs. 500 (Post-paid)

Please make demand/ bank draft payable in the favor of **Commandant AFIP** account.

EDITORIAL SUBSCRIPTION AND ADVERTISING INQUIRIES SHOULD BE ADDRESSED TO:

EDITORIAL OFFICE:

Pakistan Journal of Pathology
Armed Forces Institute of Pathology (AFIP)
Rawalpindi – Pakistan
Phone: 0344-55164440

Email: pakjpathol@hotmail.com
Website: www.pakjpath.com

The editorial committee acknowledge the assistance of Computer Expert / Civil Clerk **Muhammad Baqir Zar** for manuscript typing composing and graphic analysis of this Journal.