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Aims & Scope

The Journal aims to publish research in all fields of clinical, diagnostic, experimental & preventive areas related to medical sciences to disseminate scholastic work among clinicians and scientists around the globe.

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Research article

Molecular Epidemiology of *Mycobacterium tuberculosis* in Di- vision Mirpur, Azad Jammu & Kashmir

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Abstract: The causative agent for TB is Mycobacterium tuberculosis a member of Mycobaterium Tuberculosis Complex. It is non-motile, slow growing, acid fast and rod in shape (bacilli). According to WHO in 2013, 9 million people contracted while 1.5 million died due to this disease, 550,000 children got TB while 80,000 were died who were HIV-negative. Furthermore, approximately 0.5 million people developed multidrug resistant TB (MDR-TB) and they needed comparatively longer and costly treatment. Pakistan has high incidence and prevalence of TB. The total 580 patients were examined during the study, out which of 332 were males while 248 were females. Only 17 percent were admitted (indoor patient department) in different hospitals of the division while 83 percent were from outdoor patient department of various settings. Pulmonary tuberculosis was detected in 97 patients while extra pulmonary tuberculosis was noticed in 5 individuals. All samples were examined by Direct Microscopy (88%) followed by Fluorescent Microscopy and found presence of Acid Fast Bacilli in 91 cases, Light microscopy missed 3 positive cases. Of the 109 specimens 68 grew during seven weeks of incubation while 20 grew in four weeks and 21 grew in second week of incubation. GeneXpert detected 102 samples as positive for Mycobacterium tuberculosis. Rifampicin (RIF) a first line treatment drug was detected as resistant in 2 (1.96%) patients thus, multi-drug resistant (MDR) was found in two cases, which was associated with Pro-E gene mutation.

Keywords: Tuberculosis (TB), GeneXpert, Fluorescent microscopy, Mirpur

Introduction

The causative agent for TB is Mycobacterium tuberculosis which is a member of Mycobacterium Tuberculosis Complex (MTC). Mycobacterium tuberculosis is a non-motile, with a very slow growth rate, acid fast and rod in shape (bacilli) belongs to the Mycobacterium genus and differs substantially from other bacteria due to the exceptionally thick cell wall and high genomic guanine-cytosine content. On DNA level species of MTC are alike to each other but different on the basis of phenotype, host tropism and disease causing ability (1,2,3). In 2013, 9 million people contracted while 1.5 million died due to this disease. According to World Health Organization (WHO), estimation 550,000 children got TB while 80,000 were died who were HIV-negative in the same year. Furthermore, approximately 0.5 million people developed multidrug resistant TB (MDR-TB) and they needed comparatively longer and costly treatment. With high occurrence of TB and perhaps getting high number of MDR and XDR. Pakistan is among the top five in the world (4), with overall rate of 85–100/100,000 but in Northern area of Pakistan, the rate is even higher where the rate has been reported to be 554/100,000.

For the first time, twenty diverse enzymes having cytochrome P450 or CYP were programmed for genome sequencing for H37Rv strain of M. tuberculosis (6). The important functions of these enzymes were described by huge number of P450s. The main function of P450 was recognized, to metabolize cho- lest-4-en-3-one (ie CYP125A1 and CYP142A1), chain lipids branched (ie CYP124A1), cyclic dipeptides oxidative couture (ie CYP121A1) sterol demethylation (ie CYP51B1) and menaquinone hydroxylation (ie CYP128A1) respectively in the host (7,8,9,10,11,12,13,14). In organisms that do not have true nucleus in it, M. tuberculosis P450 was the first categorized on the basis of structure and chemical as CYP51B1 and the principal associate of CYP51 gene (15). For the diagnosis of active TB after physician's thought radiological e.g; X-rays followed by different laboratory confirmation including culture initially smear microscopy of sputum is needed. Rapid diagnosis and related treatment is required to control the TB and drug resistance especially in resource constrains and prevalent entities but through these techniques it's impossible to assume the drug resistance in TB causing bacteria. Although the occurrence of TB and death rate reduced but MDR strains of TB bacteria are responsible for 480,000 cases of MDR in 2014 which is a great threat for patients as well as for TB control programs. Maximum frequency of MDR-TB is in European countries located on east.

Materials and Methods

The study was conducted at the state TB reference laboratory situated within the premises of Divisional Head Quarter Teaching Hospital, Mirpur, Azad Jammu & Kashmir (AJ&K). The laboratory was established by the Ministry of National Health Services, Regulation & Coordination while functioning under the supervision of Health Department of government of AJ&K. The laboratory has separate reception, an office of the manager and separate room for every procedure. The said setting has state of the art equipment/instrument including PCR-based fully automatic GeneXpert, bio-safety cabinets (BSCs), autoclave, incubator, fume hood, conventional microscope, fluorescence microscope and all personnel protective equipments (PPEs). Research was conducted between september 2019 to march 2020. The patients referred by their physicians or coming directly to the centre of Mirpur division and present any of the sign and symptom enrolled; the presence of symptoms like persistent cough >7 days, low or high grade fever, gradually or suddenly loss of weight, night sweating and tiredness. Two sputum samples were taken from adult individuals while gastric wash was taken from infants and young children who were suspecting pulmonary tuberculosis. Patients who were suspecting for extra pulmonary tuberculosis, all specimens except blood e.g; biopsy, aspiration of other body fluid were obtained. The culture for mycobacterium tuberculosis was performed by using Lowenstein-Jensen culture medium and incubated at 37^0C for six weeks in specially designed incubator. Molecular Analysis of Detection of TB by GeneXpert GeneXpert which is most precise and accurate tool for the molecular detection of Mycobacterium tuberculosis was also done on all 580 samples. GeneXpert works on Polymerase Chain Reaction (PCR) principal basis detect Mycobacterium and it's resistant against anti-tuberculosis drugs.

All suspected patients with TB aged new born and above residing in division Mirpur, AJ&K during the research period and informed consent were included in the study while the patients disinclination to consent were excluded. The patients coming for follow-up were excluded and those with <1.5 ml sputum samples or salivary samples were also excluded from the study. The Study was approved by local Ethics committee.

Figure 1. Geographical map of Azad Jammu and Kashmir (AJ&K)

Results

A total of 580 patients were examined during the study out of which 332 were males while 248 were females (Figure 1). Only 17 percent were admitted in different hospitals of the division while 83% were from outdoor patient department of various settings. The age distribution of the patients is presented in Figure 2; where 9 patients were under one year of age while 105 patients between 1 to 20 years of age. Pulmonary and Extra Pulmonary Tuberculosis were detected in 97 and 5 patients reseptively (Figure 3). Out of 580 patients 89 were positive for AFB with diverse loud of AFBs which mean, in some samples the loud of AFBs was scanty while others shows 1+, 2+, 3+ and 4+ respectively. The males were predominant over female. In 69 males tuberculosis was detected while 33 female were also victim by this disease

Detection of TB by Fluorescent Microscopy

All 580 samples were than confirmed by Fluorescent Microscopy and found presence of Acid Fast Bacilli in 91 cases which means Light microscopy missed 3 positive cases (Figure 4). The quantity of Acid Fast Bacilli was variable, e.g; scanty, 1+, 2+, 3+ and 4+. 491 samples were negative for Acid Fast Bacilli after examining Z.N stained smear under Fluorescent microscope.

Detection of M. Tuberculosis by Culture Culture

It was done on all patients and 109 showed growth of Mycobacterium tuberculosis while 471 did not grow (Figure 5). Of the 109 specimens 68 grew during seven weeks of incubation while 20 grew in four week and 21 grew in second week of incubation. In Mycobacterium tuberculosis culture positive category the ratio of males 61% (n=67) was greater in comparison with females 39% (n=42).

GeneXpert

GeneXpert detected 102 samples as positive for Mycobacterium tuberculosis. Rifampicin (RIF) which is one of the drugs from first line treatment was detected as resistant in 2 (1.96%) patients so, multi-drug resistant (MDR) was found in two cases. The mutation was noticed in Pro-E gene which was responsible for Rifampicin resistance. There was no drug resistance seen in 99 patients who were positive for tuberculosis by GeneXpert. In one sample status was shown as In-determined (IND) as GeneXpert cut off value is > 131/ml of sample. The

bacteria other than Mycobacterium tuberculosis from Mycobacterium Complex (MTC) were also found in 3 patients.

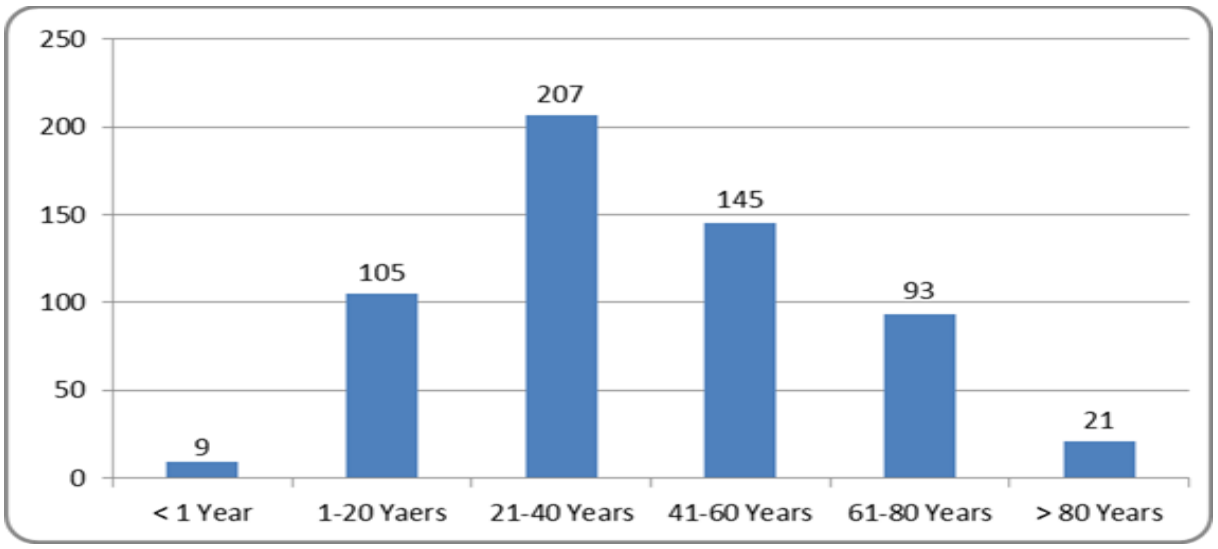

Figure 2: Age distribution of study population

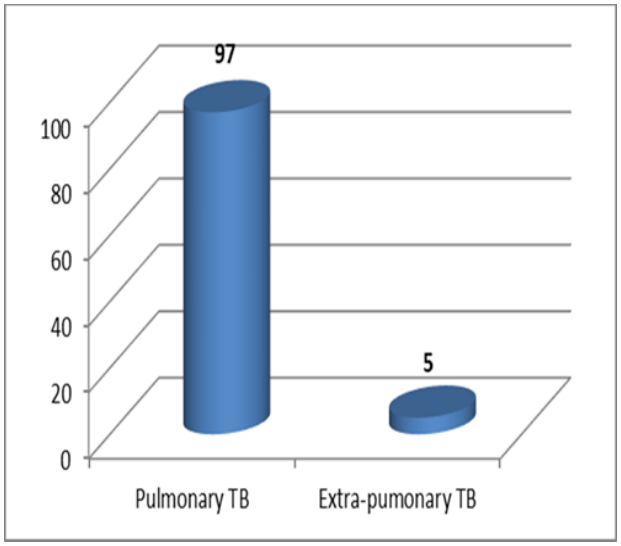

Figure 3: Pulmonary vs Extra-pulmonary TB breakout

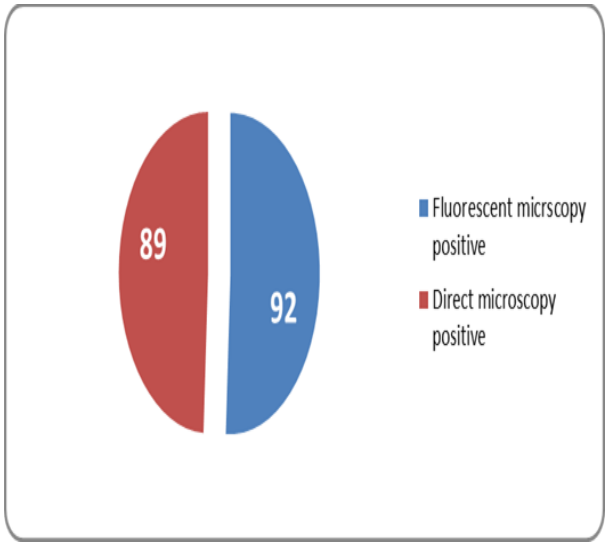

Figure 4: Light versus Fluorescent Microscopy in detection mycobacterium Tuberculosis

DISCUSSION:

The study presented, a detailed picture of TB, in the division Mirpur, Azad Jammu & Kashmir, Pakistan by combining PCR based GeneXpert, biochemical analysis and classical epidemiological methods. In the researches from around the globe and in our country the prevalence of drug-resistant tuberculosis was different. According to our study out of 580 clinical samples (both pulmonary and extra-pulmonary) 102 (17.58%) were positive for Mycobacterium tuberculosis and the prevalence of Rifampicin resistance to tuberculosis was only 1.96% from all pulmonary samples as well as extra-pulmonary samples.

In India 9% were Rifampicin (single drug-resistant) as reported in different studies. Surajit Lahiri et al. reported in 2011– 2012, Rifampicin mono-resistance in 4.69% while M. Giridhar Kumar et al. shows 0% Rifampicin

resistance in Mycobacterium tuberculosis in 2010–2012 chiefly from South India. High prevalence of multi-drug resistant tuberculosis were reported in many studies from different region of India mainly in patients first-time treated with deterioration, treatment after evasion and treatment after failure. The prevalence of drug-resistance in our study is low as compared to studies mentioned above. 20 – 60 years of age male patients were mostly infected with Pulmonary Mycobacterium tuberculosis while in female the burden was found in 20 – 40 years of age group. Extra pulmonary tuberculosis was found in age group of 20 – 40 years in both male as well as in females. While in Rifampicin resistant, males were main victim within the age of 20 – 60 having pulmonary tuberculosis. And in case of Rifampicin resistance, majority of pulmonary cases found in males and females were also from the age group of 20 - 40 years.

The development of resistance to anti-TB drugs used to treat tuberculosis (TB), and chiefly multidrug-resistant TB (MDR-TB), has become a momentous public health threat in a number of nations and is a problem to actual TB control. Many researches for the surveillance of drug resistance conducted in India indicated relatively low rate of multi-drug resistance but, this interprets into a huge total number of drug resistant TB cases and as hitherto the management of patients with MDR-TB is insufficient. Within the Revised National Tuberculosis Control Program (RNTCP) specific measures are being taken to address the multi-drug resistant-TB problem through suitable management of individuals and policies to preclude the spread and dissemination of multi-drug resistant-TB. Antimicrobial drug resistance in TB has clinical, microbial and programmatic reasons. In case of microbiological perspective, the drug resistant is caused by the genetic alteration which stops the drug to effects the organism and show ineffectiveness against the altered bacilli. The second major cause of drug-resistance is the administration of insufficient and poorly treatment regimen which makes strain dominant in patient infected with tuberculosis. Therefore, the poor treatment, poor antimicrobial therapy is totally man-made phenomenon in the development of multi-drug resistance tuberculosis.

CONCLUSION:

The specimen for the diagnosis of Tuberculosis should be tested by all techniques including; direct and fluorescent microscopy, culture and GeneXpert technology. Microscopy by both technique has low sensitivity and specificity. GeneXpert technology is much better in terms of sensitivity and specify when compare with microscopy while till date culture is most predominant in the account of sensitivity but need longer time due to slow growth of Mycobacterium tuberculosis.

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Research article

Phytochemical analysis of some medicinal plants

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Abstract: The chemical compounds that formed in plants during normal metabolic process are termed as phytochemicals. The composition of phytochemicals is usually complex in nature and differs among stages of development and plant origin. Phytochemicals used higher plants as warehouse for them which are useful in pharmaceutical industry. Some useful medicinal properties are associated with different parts of plants that result from interaction of secondary metabolic products. The aim of this study was to determine the presence of different phytochemicals in 04 different medicinal plants including Smilax china, Tribulus terristris, Glycyrrhiza glabra and Curcuma amada. This study was conducted in Karachi for determination of phytochemical activity of said plants by using plant extracts. Results showed that saponins were only absent in Curcuma amada while in all the other 3 they were present. Reducing sugar was only present in Smilax china and Glycyrrhiza glabra and tannins were in Smilax china, Tribulus terristris and Glycyrrhiza glabra. Anthocyanins were only positive in Glycyrrhiza glabra. It was concluded that Glycyrrhiza glabra had all 04 targeted phytochemicals (i.e. saponin, reducing sugar, tannins and anthocyanins) while Tribulus terrestris had only saponins and tannins in it. Only saponin, reducing sugar and tannins were present in Smilax china and none of the targeted phytochemical was present in the extract of Curcuma amada.

Keywords: Phytochemical properties, medicinal plants, Smilax china, Tribulus terristris, Glycyrrhiz aglabra, Curcuma amada

Introduction

Phytochemicals are the compounds that are formed in plants during normal metabolic process. The composition of phytochemicals is usually complex in nature and differs among stages of development and plant origin. There are varieties of functions of plants that are associated with these secondary metabolic products, some of them include medicinal effects[1]. The concept of development of semi synthetic and synthetic analogues of plant compound for medicinal use was brought in 20th century[2]. These analogues associated with the maximal therapeutic effects as a result phytochemicals attracted increasing research focus for therapeutic care and food industries[3].

In identifying the sources of industrially and therapeutically important compounds, the screening of phytochemicals in dietary plants is very important[4]. For identification of secondary metabolites in plants, it is imperative to take some crucial steps[5]. There are various types of phytochemicals that found in different types of plants. Each of these has its own unique property and function too. These functions include nutritional benefits, physiological functions, phytotoxicity, Anti-nutritional property, Pro-oxidants, Anti-oxidants, Anti-carcinogenic, Analgesic, Anti-inflammatory properties & other

therapeutic effects[9]. Phytochemicals are commercially used in Rotenone, nicotine and pyrethrins and pesticides. Tannins contain astringent activity and antimicrobial agents (isquinones) such as hypericin[11]

Phytochemicals should be evaluated from different extracts of plants. There is a significant method for extraction of phytochemicals, also some traditional extracting procedures are present and there are certain novel extraction methods. As maceration, Soxhlet and percolation methods are mostly used in screening of phytochemical studies. They also include some forward procedures like microwave assisted (MAE), ultrasound-assisted extraction (UAE), accelerated solvent extraction and supercritical fluid extraction (SFE)[14].

The study was aimed to evaluate presence of targeted phytochemicals i.e. saponins, carbohydrates, tannins and anthocynins in some medicinal plants i.e. Smilax china, Glycyrrhiza glabra, Curcuma amada and Tribulus terristris. As we all know, phytochemicals have many significant therapeutic effects, so the evaluation of the presence of these therapeutic effects in targeted plants is the core purpose of this study.

Materials and Methods

This analysis was conducted in the city Karachi for the determination of phytochemical activity of some medicinal plants. From the given plants, extracts were taken, and following test were performed for the indication of their presence. For extraction, we took 04 medicinal plants i.e. Glycyrrhiza glabra, Smilax china, Curcuma amada and Tribulus terrestris. These plants were bought from local market and extracts were prepared in an aqueous environment. Reagents/ chemicals and solutions used in the tests include NaOH solution, Methyl orange (indicator), Fehling's solution A and B & Ferric chloride and aqueous water.

METHODS:

TEST FOR SAPONIN: Took 1 ml of aqueous extract, dissolved in distilled water in test tubes, following by shaking the solution vigorously. Observed for the froth which indicate presence of saponins in the extract.

TEST FOR CARBOHYDRATES:

For reducing sugars:
At the first step 2ml of the extract was taken, added 1 ml of water followed by 20 drops of Fehling's solution A + Fehling 'solution B in test tube. Presences of brick red color indicated presence of reducing sugar in the given extracts.

TEST FOR TANNINS
1 ml of extract was taken then added 1 ml of distilled water followed by addition of 2 -3 drops of ferric chloride in diluted form. The change of color from green to blue green was observed. If green to blue green color change appears, it indicates presence of cathechic tannins, while if blue black color appear this indicates that Gallic tannins are present.

TEST FOR ANTHOCYANINS

NaOH 50 ml was taken in a burette, then took a beaker containing few ml of aqueous solution of the extract and titered it, the color change was then observed. If the color changes to red color, it indicates the pH is less than 3, if the color changes to blue color, this indicates the pH is between 4 and 6.

Results

Phytochemical screening of the given plants showed that saponins were only absent in Curcuma amada while in all the other 03 they were present. Reducing sugar was only present in Smilax china and Glycyrrhiza glabra while the tannins were in Smilax china, Tribulus terristris and Glycyrrhiza glabra. The results of Anthocyanins are were only positive in Glycyrrhiz aglabra.

Table 1: Phytochemical analysis in Plant extracts

S. No.	Phytochemicals	Smilax china	Tribulus terristris	Glycyrrhiza glabra	Curcuma amada
1	Saponins	+	+	+	-
2	Reducing sugar	+	-	+	-
3	Tannins	+	+	+	-
4	Anthocyanins	-	-	+	-

DISCUSSION:

Phytochemicals have a number of significant therapeutic effects like Allyl sulfides, Carotenoids, Flavonoids and polyphenols have anti- oxidant activity and reduces the risk of cancer. These are mainly found in onions, lleks, garlic, fruits and vegetables. Similarly Indoles, protease inhibitors and terpenes stimulate the enzymes and reduce the risk of breast cancer by making estrogen less effective. Some phytochemicals that found in soy and cranberry have hormonal actions and physicals actions too like isoflavones and proanthocyanindins respectively. They reduce osteoporosis and menopausal symptoms by working same as estrogen and having anti-adhesion property too. Phytochemicals like saponins and capsaicin interfere with DNA replication and have anti carcinogenic property while Alicin have anti- bacterial effects. All they are found in beans hot peppers and garlic respectively.

In order to identify the sources of industrially and therapeutically important compounds, the screening of phytochemicals in dietary plants is very important. For identification of secondary metabolites in plants, it is imperative to take some crucial steps29.

In our daily life we eat nutrients that contain phytochemicals, but there are some refined foods like liquor or sugar. Few dietary nourishment for example vegetables, fruits, bean, whole grains and herb contain numerous phytochemicals. There are a number of methods for extraction of phytochemicals some of them are traditional extracting procedures in addition to a number of novel extraction methods. As maceration, Soxhlet and percolation methods are mostly used in screening of phytochemicals studies. They also include some forward procedures like microwave assisted (MAE), ultrasound-assisted extraction (UAE), accelerated solvent extraction and supercritical fluid extraction (SFE)30.

In this study we mainly focused on 04 phytochemicals including Saponin that are glucosides with foaming property. Saponins are phytochemicals found in beans, herb and plants and having anti-cancerous effects. The next are reducing sugars, they have activity of reducing agent as they have free aldehyde group or a free ketone group. Along with monosaccharides there are reducing sugar with some disaccharides, oligosaccharides and polysaccharides. They are involved in reproduction, help to boost immune system, blood clotting and development of disease. They are energy transporters. Tannins are also called tannoids and tannic acid. They are amorphous substance having the color of pale yellow to light brow. Physically they are in the form of powders, spongy volume. They are nayurally present in the plants. They are widely used in many purpose including dyeing of cloths and fabrics, making of ink, it has great importance in medicinal application. The last ones are anthocyanins, they are extracted by many edible plants and have anti- cancer, anti- diabetic, anti-inflammatory, anti-obesity and anti- microbial effects. They are used for the prevention of cardiovascular disease as well 31.

CONCLUSION:

It is concluded that the Glycyrrhiza glabra had all 04 targeted phytochemicals (i.e. saponin, reducing sugar, tannins and anthocyanins) while Tribulus terrestris had only saponins and tannins in it. Only saponin, reducing sugar and tannins were present in Smilax china and none of the targeted phytochemical was present in the extract of Curcuma amada.

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Research article

Additional cytogenetic abnormalities in resource constraint countries; an additional burden on Chronic Myeloid Leukemia patients ?

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Abstract: The objective of this study was to analyze the additional cytogenetic abnormalities at baseline in chronic myeloid (CML) leukemia patients, compare its characteristics with patients having normal karyotype and to identify the rationale of performing cytogenetics in treatment naive CML patients. A case control study was conducted, 18 cases and 36 controls were recruited from 2010-2018. Controls were diagnosed CML patients without additional cytogenetic abnormalities. SPSS was used to analyze the data, chi-square and independent sample t- test were applied to observe the association. Kaplan -Meier was used to observe the survival outcomes. At follow up, after the initiation of treatment, there was no differences in cases and controls with respect to the hemoglobin, total leucocyte and platelet count. Molecular response at 06 month was similar between two groups while at 12 months there was a significant difference, where controls were found to have higher response rate. Survival outcomes were also found comparable in cases and controls. Our findings reflect negligible difference in clinical and molecular responses between cases and controls in CML patients. Thus, performing cytogenetics at baseline might not be helpful to predict progression of disease and treatment outcome.

Keywords: Additional cytogenetic abnormalities, case-control, Chronic myeloid leukemia, Pakistan

Introduction

Chronic Myeloid Leukemia (CML) is a myeloproliferative neoplasm distinguished by the existence of Philadelphia chromosome (Ph) resulting from a translocation between chromosome 9 and 22, i.e. t(9;22)(q34;q11) [1]. The translocation causes the formation of a chimeric oncogene, breakpoint cluster region-Abelson leukemia virus oncogene (BCR-ABL) encoding the p210BCR-ABL[2]. Regardless of the distinguished cytogenetic and molecular features harbored by CML; the patients have a diverse clinical presentation, treatment responses, and survival [3]. Moreover, the heterogeneous characteristics of the disease are also evident at cytogenetic and molecular levels [4,5]. Depending upon the different breakpoints of the BCR gene, most CML cases possess a fusion oncogene comprising either the b3a2 or b2a2 transcripts[5]. Moreover, 5% - 10% of patients have variant translocations in which at least a third chromosome is involved in the rearrangement[6] addressed as additional cytogenetic abnormalities (ACA).

The presence of these abnormalities is responsible for the prediction of adverse prognosis, disease progression, poor overall survival, and treatment outcome with conventional therapy being reported widely in the blast and accelerated phase as compared to the chronic phase[7]. The most common ACAs include trisomy[8], a second Ph chromosome, isochromosome (17)(q10), 1der(22) which are considered as "major route changes," . However, the infrequent chromosomal aberrations such as trisomy 21, t(3;12), t(4;6), t(2;16), and t(1;21) are designated as minor ACAs[8]. The major route abnormalities have auxiliary negative prognosis as compared to minor route abnormalities[9] and previous studies have reported a negative prognostic impact on treatment response and survival of patients particularly in patients who are treated by first-line tyrosine kinase inhibitors (TKI). However, the findings are conflicting and ambiguous [7,8,10] and it might be due to the heterogeneous collection of cytogenetic abnormalities[14]. However, according to the ELN recommendation 2013, it was proposed that it is not essential to perform cytogenetics at baseline. On contrary, it is also emphasized to conduct cytogenetic analysis until the achievement of complete cytogenetic response (CCyR) and major molecular response(MMR)[11,12]. European society of medical oncology (ESMO) guidelines also suggest performing the cytogenetics at 3 and 6 months and every 06 months subsequently until the achievement of complete cytogenetic response[13].

Majority of studies in the literature have discussed significance of assessment of cytogenetics during the treatment to rule out progression of the disease and to initiate a dose adjustment of TKI. The existing guidelines reflect the international literature while local data is diminished and in fact none of the guidelines has been developed for developing countries so far. In this context, we aimed to conduct a case control study in which we recruited patients with additional cytogenetic abnormalities as cases and compared the clinical and molecular responses with control group in order to identify the rationale of conducting cytogenetic at baseline which is costly and putting more burden on patients when it is a prerequisite to perform BCR-ABL by PCR at baseline as per the ELN recommendations.

Materials and Methods

This study was approved by Institutional Review Board (IRB) in March 2018 and the data was recruited from the patients who visited the National Institute of Blood Diseases and Bone Marrow Transplant Karachi Pakistan during May 2010- September 2018. In this case control study, 18 CML cases with ACA were recruited retrospectively and for each case, 02 age, sex, and Sokal score matched controls without ACA were enrolled. Baseline cytogenetic analysis was performed overnight, 24-hrs unstimulated, and 72-hrs stimulated bone marrow cultures using standard procedures. The GTG (G-bands via trypsin using Giemsa) banding technique was applied, karyotypes were described according to the International System for Human Cytogenetic Nomenclature (ISCN) 2013, karyogram was made using Metasystem®. BCR-ABL1 by real-time quantitative PCR (RT-qPCR) was done by QIAGEN kits on Rotor-Gene Q 5plex HRM instrument with 72-tubes rotor, performed on peripheral blood and bone marrow. Response to treatment was assessed according to ELN recommendations 201312. Informed consent was obtained from all the participants included in the study. Statistical Package for Social Sciences (SPSS) version 23.0 was used to analyze the data. Descriptive and inferential statistics including chi-square and independent t-test was applied to observe the association. P-value ≤ 0.05 was considered significant. Statistically significant differences in complete hematological response (CHR) at 03 months and molecular response at 06, 12, and at the end of study between cases and control were assessed by chi-square test. Differences in hemoglobin (Hb), total leucocyte count (TLC), and platelet counts at baseline and at the end of the study between cases and controls were assessed by independent t-test. Progression-Free Survival (PFS) and Overall Survival (OS) were estimated by the Kaplan–Meier method. Progression-free survival was calculated from the first dose of TKI to the first documentation of disease progression into accelerated or blast phase and OS was calculated from the first dose of TKI to the date of death or last follow-up.

Results

Fifty-four participants were included in the study. Of these, 18 were diagnosed cases of CML having ACA, and 36 were taken as matched controls. Baseline hemoglobin (P-value 0.293), TLC (P-value 0.607), and platelet counts (P-value 0.698) were the same in both groups and were found to be non-significant. The complete hematological response was assessed at 03 months post-treatment and it was found that control group was greater in number achieving CHR than cases (P-value 0.016). However, at the study's end, the normalization of Hb (P-value 0.076), TLC (P-value 0.292), and platelet counts (P-value 0.655) were same in both groups.Fifteen cases were evaluable for molecular response and survival outcome analysis for which 30 best matched controls were selected. It was found that achievement of molecular response at 06 months was similar in both groups. However, at 12 months controls were greater in number than cases in molecular response achievement. At the study's end as per ELN recommendations, both the groups had a similar molecular responses. (Table 01). Estimated PFS and OS for cases and controls were 80% and 96 % and 87% and 90% respectively. (Table 02, Figure 01 & 02). Treatment response and survival of ACA cases along with compare and contrast with international studies is depicted in Table 03. [1,8,7,14-19]

Table : 01 Molecular Response Between Cases And Controls

	Molecular Response Achieve (n)	Molecular Response Not achieved (n)	P-Value
at 06 months			
Cases	06	09	0.399
Control	16	14	
12 months			
Cases	8	07	0.032
Control	25	05	
at the study's end			
Cases	08	07	0.111
Control	23	07	

Table : 02 Progression Free Survival and Overall Survival of Cases and Controls

	Groups	Total number of cases and control	Number of Events	PFS /OS (%)	95% Confidence Interval		Log Rank (Mantel-Cox) P-value
					Lower Bound	Upper Bound	
Progression Free Survival (PFS)	Cases	15	03	80	906.224	1443.376	0.066
	Control	30	01	96	3305.826	3772.174	
Overall Survival (OS)	Cases	15	02	87	1029.933	1489.622	0.597
	Control	30	03	90	2912.171	3685.132	

Table: 03 Treatment response and survival of ACA cases: Compare and contrast with international studies[1,7,8,14-19].

Author, Country	Year of publish	Hematological Response	Molecular Response	Progression Free Survival	Overall Survival
Present Study Anwar et al.	2019	3months: significant Overall: non significant	Overall: Non significant 12 months: significant	Non significant	Non significant
Chandran et al India[14]	2019	Non significant	Non significant	Non significant	Non significant
Safaei et al Iran[15]	2018	Not assessed	significant	Not assessed	significant
Millot, et al France [16]	2017	Not assessed	Non significant	Non significant	Non significant
Alhuraiji et al USA [7]	2017	Non significant	Non significant	Non significant	Non significant
Savasoglu et al Turkey [17]	2016	Not assessed	Non significant	Not assessed	Non significant
Crisan et al Romania[18]	2015	Not assessed	10/11 achieved CCyR	Non significant	Non significant
Aissata et al Cote d'Ivoire[19]	2013	59% patients achieved CHR	Major Cytogenetic response in 52% patients , MMR in 3% patients	Not assessed	Not assessed
Luatti et al Italy [8]	2012	Non significant	12 Non significant Overall significant	Non significant	Non significant
Hsiao et al Taiwan[1]	2011	Non significant †	Not assessed	significant	Non significant †

Non significant: results in patients with and without ACAs were same, † evaluated in patients in chronic phase only

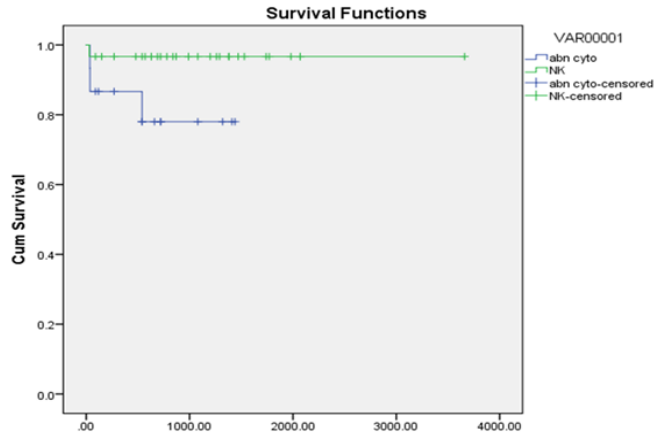

Figure: 01 Progression Free Survival in Cases and Controls
NK= Normal Karyotypr
abn cyto= Abnormal cytogenetics

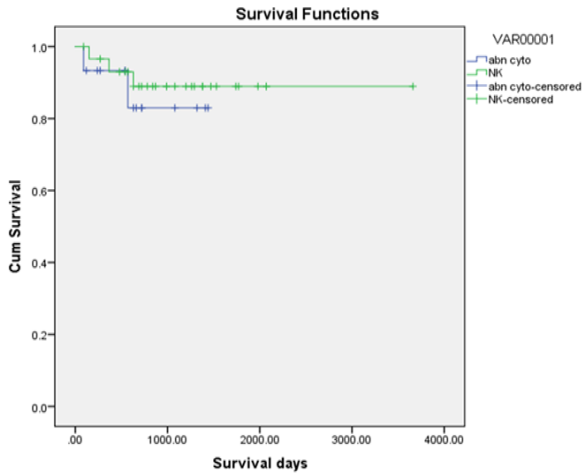

Figure: 02 Overall survival in Cases and Controls
NK= Normal Karyotype
abn cyto= Abnormal cytogenetics

DISCUSSION:

Additional chromosomal abnormalities in CML at baseline and during treatment is a well-known phenomenon. There are some suggested mechanisms but the exact pathogenesis and underlying biology remain unclear and the adverse impact conferred by its presence has been controversial in the literature19,20. In our study we investigated the difference in post-treatment hematological and molecular response in patients with and without ACA in order to omit cytogenetic analysis at baseline at least in old age and non-affordable patients. ELN recommendations consider the presence of ACA at diagnosis as a warning feature requiring close monitoring12 particularly the major-route abnormalities16 and this is in concordance with previous studies reporting low response rate and overall inferior survival of patients compared to those without ACA7,8, 10. On the contrary some studies reported that ACA at diagnosis could not be considered an adverse prognostic factor in the chronic phase under first-line TKI treatment and the type of abnormality at baseline have a minimal role on the outcome, although the highest risk abnormalities (i.e., abnormalities in chromosomes 3 and i17q) are rarely if ever detected at diagnosis7, 16.

In our study when we assessed hematological response, the normalization of Hb, TLC, and platelets were identical in cases and controls but majority of patients in control group achieved CHR at 03 months as compared to cases. However, Alhuraji et al found similar median time of achievement of CHR in patients with and without ACAs7. Other regional and international studies also did not find significant difference in CHR between cases and controls as depicted in Table :021, 7,8, 14. One of the reasons in our cases for not achieving early CHR could be the TLC outlier readings of 02 of the patients. One of them was non compliant and other patients had complex karyotype at baseline as some studies have reported an adverse outcome of patients having complex karyotype9. In molecular response assessment it was found that patients who were having ACA had similar overall response as that of control in the study. At 06 months, the attainment of response was similar between cases and controls whereas another study reported a higher response rate at 06 months in patients without ACA7. Also the reverse pattern of response was observed at 12 months and we found higher rate of response at 12 months in controls whereas the study by Alhuraji et al7 reported no significant difference between the two groups. Studies were done in France, USA, Romania, Turkey, Italy, and India also reported non significant difference in molecular response amongst patients with and without ACA7,8,14,16-18. Molecular response at the end of the study was also similar in patients with and without ACA in our study which is in concordance with the findings reported in literature7. Overall, there was no difference in OS and PFS in the current study between the cases and controls and it is also in concordance with other

studies7,8,14,16-18 . It is reported in the literature that major route abnormalities are associated with early progression to advanced phase and a German study stated that PFS and OS were shorter than in patients with standard t (9; 22)9, 18. However, in our study patients who were progressed to advanced phase had minor, major, and complex ACA. In our cases, out of 15, 02 patients died and they were detected with minor and complex ACA at diagnosis. Hence the association of having major route abnormality and mortality that has been discussed in literature is unlike in our study and it is supported by another study done in Turkey in which they did not find statistical correlation between patients with major and complex ACA and without ACA17. In this study they also investigated the association of BCR-ABL kinase domain (KD) mutations and ACA and didn't find a significant association17.

In our study, we didn't identify ACA in patients during treatment. One of the studies emphasized that the detection of ACAs during the TKI treatment is a warning sign of disease progression and intermittent cytogenetic monitoring is mandatory as it is considered to be a form of treatment failure18. However some other studies did not describe the significant contribution of cytogenetic analysis in patients with molecular failures and also its impact on the course of the disease12.

Cytogenetics as compared to RT-qPCR have low sensitivity due to the low number of analyzed metaphases, the need for a bone marrow aspiration makes it a painful procedure for the patients11,20 particularly in the old age group. The option to exclude routine cytogenetic monitoring may not only prevent uncertain classifications complicating the analysis of response assessment11 but may also reduce the additional burden on non affordable patients in terms of cost when it is a prerequisite to get BCR-ABL done for disease diagnosis and monitoring. Thus it is still a matter of debate and we cannot predict the treatment outcome solely on the basis of presence of ACA and it could be questionable whether cytogenetic evaluation at diagnosis carries value If regular molecular monitoring is available. Cytogenetic assessment could however be worth performing in instances where a molecular warning or failure response is obtained.

Limitations of our study were that we didn't look for TKI mutation analysis and its correlation with ACA, hence the outcome in these patients could not be predicted in our study, also the sample size is less to conclude such findings assertively although we reduced this by adding controls studies with larger sample size are needed.

CONCLUSION:

Patients with additional cytogenetic abnormalities in our cohort showed similar hematological and molecular responses as controls. In developing countries with limited resources, financial constraints and scarce medical facilities, omission of cytogenetic analysis at baseline when molecular level tests are available may be considered except in instance where molecular warning or failure occurred. However, further prospective studies with a large sample size are needed in this regard.

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Research article

Frequency of Causes of Spurious Platelets Count on Routine Complete Blood Count by an Automated Hematology Cell Analyser

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Abstract: The present study was aimed to find out causes of platelet count flagging marked on Complete Blood Count strip. This Cross-sectional study was conducted at Hematology Department, Children Hospital and ICH, Lahore. A total of 350 blood samples were processed for the study and among them a total of 86 samples of blood in EDTA vial were studied with specific tag on platelet count on CBC strip. A specific designed Performa was used for data collection. All the collected data was entered to SPSS 22 (statistical package for social sciences) for analysis. Out of 86 samples 64 samples (74.4%) showed spurious platelet count due to EDTA. Pseudo thrombocytopenia was observed in 9 samples (10.5%). Abnormal CBC 4.7 %, overfilling of sample 4.7%, acute burn anemia with frequency of 3.5%, iron deficiency anemia and clotted samples have 2.3% frequency separately. While 3.5% samples were included in miscellaneous list. We concluded that EDTA dependent pseudo-thrombocytopenia is the most prevalent. Other technical errors (pre-analytical, analytical) can be reduced by proper training and education of laboratory staff. It is very important to rule out the correct cause of spurious platelet count on CBC as otherwise it will lead to unnecessary medication and investigations for patient.

Keywords: Pseudo-thrombocytopenia, EDTA, frequency, spurious platelet count.

Introduction

Automated hematology analyzers are widely used in clinical practice and research settings to characterize blood cells for disease detection and monitoring of blood cell counts. Sophisticated

analyzers can measure cell mor- phology and can also detect small cell populations to diagnose rare blood conditions1. Its widespread use plays a major role in improvement of cellular hematology as it gives quick and accurate results2. When platelet count on the CBC strip goes out of range, the automated analyzer have ability to tag them on the strip. This tagging can be of any type according to the software of automated hematology cell analyzer2,3. Automated cell analyzer may tag them with a flag, a dot or mark it on histogram etc.

All the hematology instruments use software generated flagging to identify abnormal platelet distributions that may indicate presence of platelet specific interference or the presence of platelet clumping. The reporting hematologist must review the blood smear before sending out the complete blood cell count reports when the flag of platelet clumping occurred. Hematologist have to observe the platelet in direct blood smear for approximate quantity and shape or detected by hematology analyzer. In both systems, the collected blood is diluted and counted by passing blood through an electric counter4.

Platelet tagging can be due to some pathological reasons but mostly platelet tags are due to spurious count of platelets which lead to pseudo thrombocytopenia or pseudo thrombocytosis. In these cases, following are the main reasons (5-11)

1. Platelet clumping due to EDTA anticoagulant.
2. Adherence of platelet to mature polymorphonuclear neutrophils
3. Clotting within the sample.
4. Fragmented nucleated cells.
5. Lipids (sample taken after meals, lipid drips)
6. Micro-organisms

Pseudo thrombocytopenia and pseudo thrombocytosis are very common in routine complete blood counts, which can have deleterious effects on patients, resulting in getting even platelet transfusion if it is not clearly checked. To analyze the cause of platelet flagging is very vital in dealing with automated hematology analyzer. It is an important aspect of quality assurance related to complete blood count automated analyzer. The objective of this study was to find out the causes of platelet count flagging marked on Complete Blood Count strip.

Materials and Methods

It is a cross-sectional study conducted in Hematology & Transfusion medicine Department, The chil- dren's hospital & ICH Lahore from July 2018 to Jan 2019. Data was collected with the help of designed proforma. A sample size of 350 blood samples was collected. Sample was collected by aseptic technique. 5 cc of blood was drawn in sterile syringe and promptly poured in EDTA containing anticoagulant vial. This anticoagulated blood was received in hematology department and processed within 2-3 hours. Those samples giving flag on platelet counts were further investigated by manual checking of

samples and peripheral films to find out causes of platelet flagging. Samples were processed first by gently mixing of the vial, and then the blood was brought near the sucking tip of the Hematology analyzer Celltac MEK-6450. The machine processes, counts the cells according to their size and granular morphology. Data was presented in tables, graphs or pie diagrams and analyzed by using Statistical Package for the Social Sciences (SPSS) software computer program version 20.

Results

Out of total 350 samples that were analyzed, 86 samples showed platelet flags. All the 86 samples were considered for analysis making the response rate 100%. Regarding the type of tag on CBC strip and the platelet count the frequency of "*" (Flag) type tag is 82 (95.3%) (Table 1). The most comment platelet count range was 150-400 × 10^9/L (Table 2, Figure 1). The Distribution of causes of Spurious Platelet counts are shown in Figure 2. EDTA was the cause in 74.4% samples. EDTA was the most common and highest causes of platelet count tag on CBC in range of 150-400x10^9. When platelet count is <150x10^9 then again EDTA the common cause. When platelet count is >400 x 10^9 i.e., the Upper limit PU, the most common cause of spurious count is multiple transfusions (Figure 3). The most common platelet appearance on the slide was platelet clumps (39, 45.3%) followed by Platelet satellitism around WBC (9, 10.5%) (Table 3).

Table 1. Type of tag on complete blood count strip

Tag type	Frequency	Percent
*	82	95.3
PU*	2	2.3
AG*	2	2.3
Total	86	100.0

*Flag; PU=Platelet Upper Limit; AG= Aggregates

Table 2. Platelet count of the samples enrolled in the study

	Frequency	**Percent**
150-400 × 10^9	66	76.7
<150 × 10^9	8	9.3
>400 × 10^9	12	14.0
Total	86	100.0

Table 3. Platelet appearance on Slide and their frequency and percentages

Platelet Appearance	Frequency	Percent
Platelet Clumps	39	45.3
Platelet satellitism around WBC	9	10.5
Platelet neutrophil clumps	7	8.1
Coagulation within sample	6	7.0
RBC fragments	5	5.8
Platelet Aggregates	4	4.7
Anistocytosis	4	4.7
Normal platelets	4	4.7
Small aggregates	3	3.5
Miscellaneous	3	3.5
Large platelets	2	2.3
Total	86	100

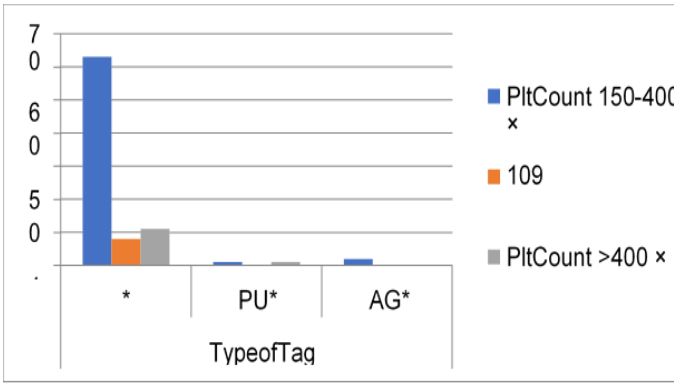

Figure 1. Comparing platelet count and type of tag

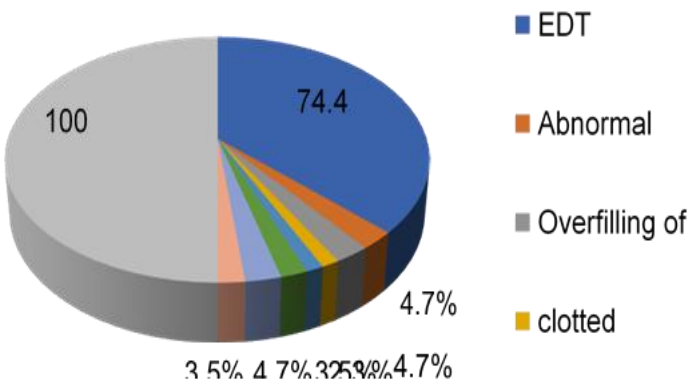

Figure 2. Distribution of causes of spurious platelet

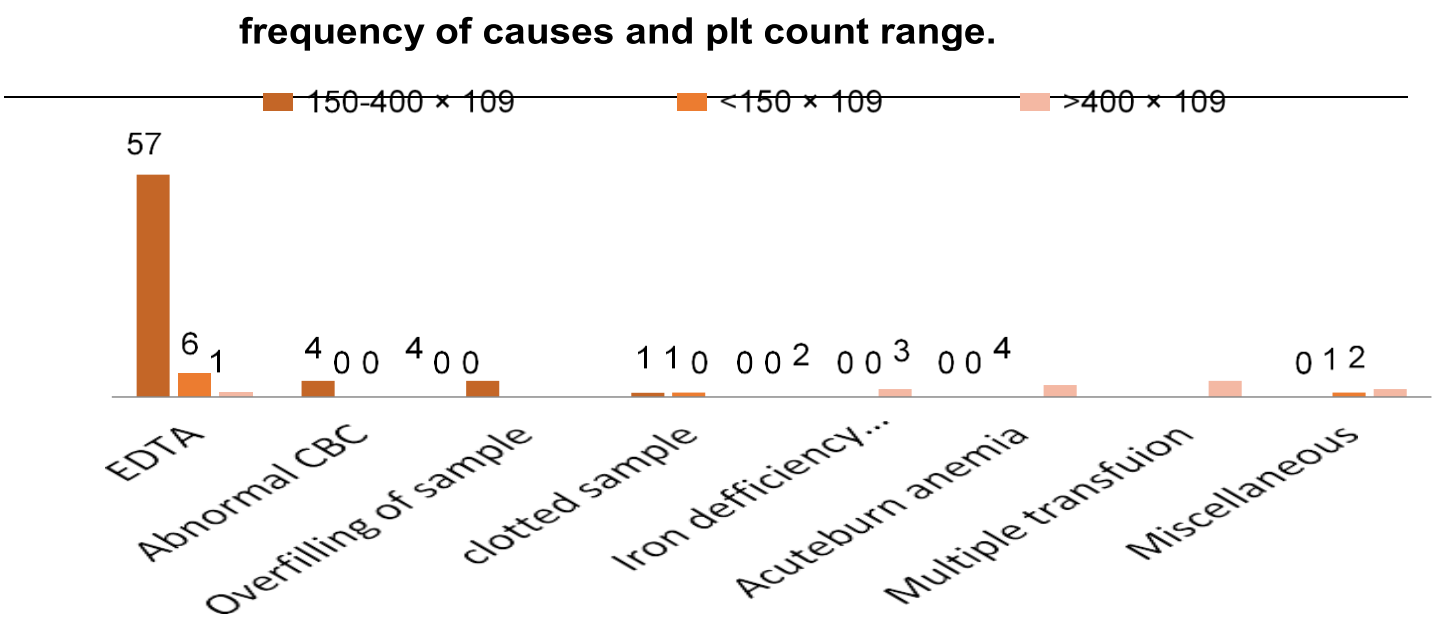

Figure 3. Comparing Platelet Counts and Causes of Platelet Flags

DISCUSSION:

Automated hematology analyzers have great contribution in hematology department as they can cope up with high workload. However Pseudo-thrombocytopenia remains a challenge in hematology laboratory. The pre-analytical problem that platelets tend to easily aggregate in vitro, giving rise to lower platelet counts, has been known since EDTA and automated platelet counting procedures were introduced in the hematology laboratory. The present study was aimed to know the frequency of causes of spurious platelet counts on CBC by automated hematology cell analyzer. Out of 86 samples 82 (96%) were tag by "*" 2 (2%) with "PU*" and the remaining 2 (2%) with "AG*". Samples were divided into three categories on the basis of platelet count range. Firstly 150-400* 109 i.e., normal range, secondly platelet count<150 * 109 (below lower limit) and thirdly platelet count >400 * 109 (above upper limit).

Eighty-six samples were considered in the current study. Different causes of spurious platelet counts and these causes were based upon the platelet appearance on smear slide as mentioned in table 3, the most common cause with highest percentage was EDTA. Out of 86 samples 64 samples (74.4%) were giving spurious platelet count due to EDTA. EDTA is the highest frequency cause of spurious platelet counts on CBC. As regard with the previous studies EDTA dependent pseudo thrombocytopenia is a common laboratory phenomenon with most prevalence rate (25%) according to a study conducted by peter Keller in 2014. In some situations, platelet satellitism around WBC was also the cause of pseudo thrombocytopenia. In the current study this cause was observed in 9 samples (10.5%). This observation was the same as presented by Dr. Nicola bizzaroet al., (1991) which showed platelet satellitisms to polymorphonuclears. Seven samples gave pseudo thrombocytopenia due to platelet neutrophils clumps (8%). Other causes are minor but also of great importance. As causes for

spurious counts vary from sample to sample. Abnormal CBC 4.7%, overfilling of sample 4.7%, acute burn anemia with frequency of 3.5%, iron deficiency anemia and clotted samples have 2.3% frequency separately. So, with all this frequency distribution of causes, it is clear that the mostly spurious counts of platelets is because EDTA anti-coagulant.Spurious platelet counts may be induced in other situations as well and for one mechanism it can be different, ranges between the true and spurious counts, from the patient to patient. It is also pertinent to note that the tags in the results are generated because of other unusual findings in other parameters of the CBC as well.

Peter keller et.al in 2014 presented a research report named as "A case of EDTA-dependent pseudo-thrombocytopenia: simple recognition of an underdiagnosed and misleading phenomenon" in which they stated that EDTA-dependent pseudo-thrombocytopenia (EDTA-PTCP) may be a usual laboratory finding which can range from 0.1-2% in case of hospitalized subjects. This is commonly unnoticed because the blood smear films are not usually assessed neither the histograms in the analyzers are correctly interpreted by the lab staff. Jianhua Lin et.al in 2014 also found that the incidence of EDTA – PTCP was 0.09% in the duration ranges from 02 weeks to 6 months and found that it was time dependent which can be occurred as early as 10 minutes after sample collection. Dr. Nicola Bizzaro et.al in April 1991 conducted a study on "platelet Satellitisms to polymorphonuclears, cytochemical, immunological and ultrastructural characterization of eight cases" and concluded that Satellitisms of platelets to polymorphonuclears was observed in 08 of the study subjects. They reported that this occurred only in the blood anticoagulated at room temperature with EDTA. However function of the platelets and the neutrophils remained normal both in vivo and in vitro. A report was presented in (2015) named as "EDTA dependent pseudo thrombocytopenia'' in this report it is stated that since 1973, EDTA dependent PTCP is approximately 0.1-2%in hospitalized patients. The primary antibodies involved in this mechanism are immunoglobulins IgG, IgM, IgA and combinations of them are also observed. In a study by the van der Meer and colleagues the incidence pseudo thrombocytosis was found in 43 patients out of 169 patients with acute leukemia and among them 07 were categorized as patients with major bleeding risk because of actually low platelet counts. Due to time and resource limitation, the study do not elaborate the procedures to reduce these spurious results. As this study is conducted on limited sample size. It should be conducted on vast level and solutions to minimize these spurious results should also be studied deeply for the betterment and reliability of CBC results by automated hematology analyzer.

Conclusion

The present study concluded that EDTA dependent pseudo thrombocytopenia is the most prevalent. Other technical errors (pre-analytical, analytical) can be reduced by proper training and education of laboratory staff. It is important to rule out the correct cause of spurious platelet count on CBC. Otherwise, it will lead to unnecessary medication and procedures for patient's treatment.

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Conflict of interests: None

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Editorial

What does Breast Cancer Screening mean from economy point of view- a Pakistani Perspective?

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Breast cancer is known for centuries, where oldest evidence reported from Edwin Smith Papyrus who called it as having no cure. However that is still true in some way when it comes to advance disease, however for early breast cancer its not true. The credit for cure goes to screening techniques followed by appropriate treatment options with availability of modern drugs. Breast cancer screening has been in practice for decades and reportedly reduces breast cancer mortality. In developed countries where there are national breast cancer screening programs widely practiced breast cancer survival in screened population has reached to almost 100%.

Developing countries have seen considerable rise in breast cancer incidence in the recent past and suspected to rise even more in the upcoming years. Apparently national screening programs pose a great economic bur den on any country. The screening tools such as mammogram machines, training of the human resource the start then recurring cost of X-ray films if digital mammograms are not being used. Therefore many developing countries do not consider it cost effective including Pakistan.

Now let's take a look at the upcoming burden of breast cancer as predicted by GLOBOCAN 2020 (figure 1). The reported rate of breast cancer in 2020 in Pakistan was Approximately 26000, this rate will approach to 50000 by 2040[1]. When we say 50000 per annum means 137 women per day will be coming with breast can- cer. As reported earlier that a great majority of breast cancer in Pakistan comes with advance stage. Thus the burden of treatment on one hand and poor survival is a challenge of the other hand. The benefit of Breast cancer screening are multifaceted:

1. As screen detected cancer are very small, can be successfully treated by surgery only minimal or even without any further adjuvant therapy, thus saving cost on expensive chemotherapy, immunotherapy and hormonal therapy.
2. As a less aggressive treatment is given thus less hospitalization and early discharge with less morbidity, reducing the cost of hospitalization and additional expenditure of prolonged hospital stay.
3. Early detection improves survival producing positive impact on the family and saving them from stress they can potentially go through in case of breast cancer mortality.

There are direct and indirect economical benefits are chemotherapy. Lets take it in the cost comparison. Each patient going through breast cancer treatment government even through sehat

card pays 2.0 million rupees per patient. Thus for 50000 patients the amount will be 100 Billions per year, with the mortality rate of more than 26000 per annum. On the other hand providing screening service at selected points or each BHU (taken that there are approximately 5300 BHUs in the country) will cost less than one year cost of the treatment for all these women.

In conclusion it is utmost important to develop strategies at provincial and national level to start mass screening following International guidelines followed by audits and extensive research programs to make national guidelines in order to improve survival outcome and reduce economic burden.

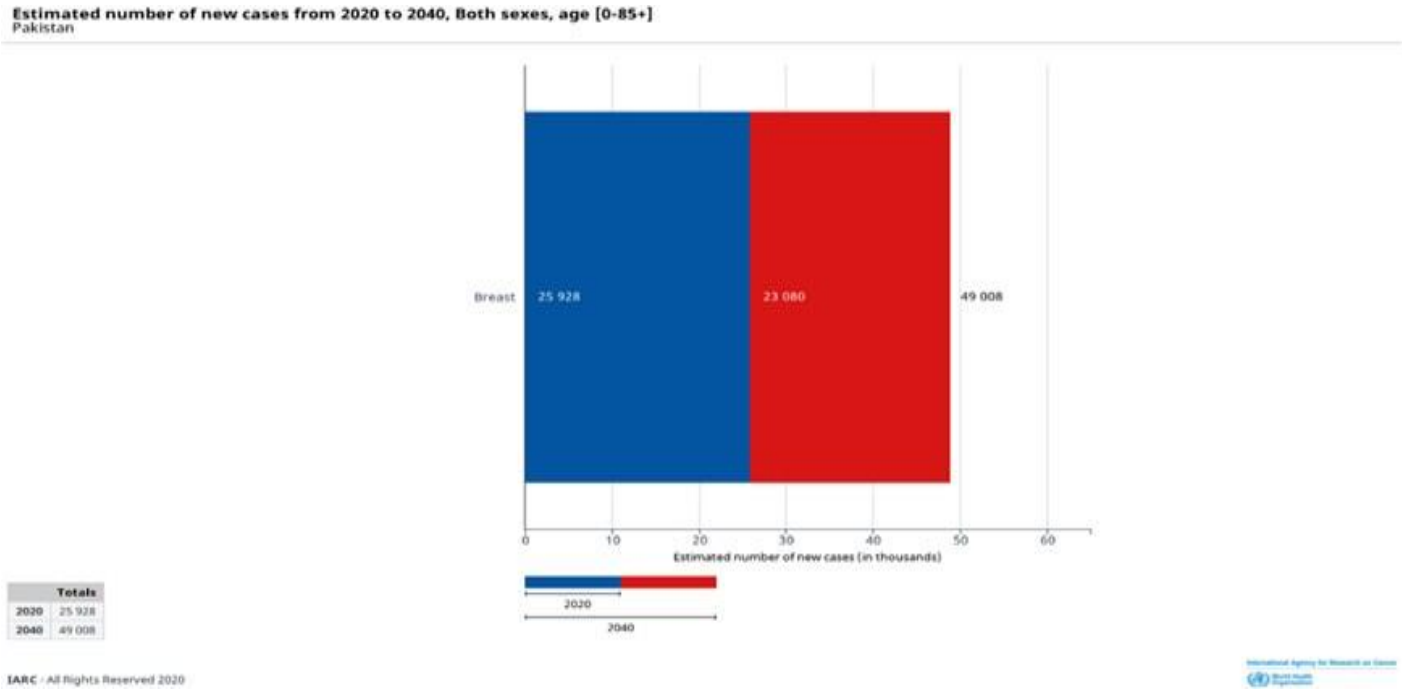

Figure 1. Globocan 2020: cancer statistics data of Breast cancer in Pakistani women and prediction -2040 References:

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