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Microbiology and Pathology

Glycosylated Haemoglobin (HbA1c)

University Sewage Oxidation Pond

Highlights

Metabolic Pathways Drug Targets

Sensitivity Pattern Enterobacteriaceae

Discovering Thoughts, Inventing Future

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Glycosylated Hemoglobin (HbA1c): An Indispensible Tool in the Management of Diabetes Mellitus

By Sujan Narayan Agrawal

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The authenticity and use of HbA1c in the management of diabetes are established by many landmark trials and studies like the Diabetes Control and Complication Trial, (DCCT), UK Prospective Diabetes Study (UKPDS), Standardization of Glycated Hemoglobin testing, National Glycohemoglobin Standardization Programme, etc. These studies have developed the methods of HbA1c estimation, the reference range for identification of pre-diabetics and diabetics, and range at which micro and macro-vascular complications occur. If the pitfall of HbA1c is kept in mind, it is a proven tool in the management and follow-up of Diabetes Mellitus.

Keywords: *diabetes mellitus, hba1c, DCCT, UKPDS, NGSP, IFCC, master equation, macro and micro vascular complications*

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Glycosylated Hemoglobin (HbA1c): An Indispensible Tool in the Management of Diabetes Mellitus

Sujan Narayan Agrawal

Abstract- Diabetes mellitus was known to mankind since antiquity although the name diabetes mellitus is introduced much later. The disease is characterized by elevated blood sugar and estimation of Glycosylated hemoglobin (HbA1c) remains one of the most important and indispensable tools of investigation besides fasting and postprandial blood sugar levels. The glycosylation is the nonenzymetic conjugation of a sugar to the amino group of proteins.

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Keywords: diabetes mellitus, hba1c, DCCT, UKPDS, NGSP, IFCC, master equation, macro and micro vascular complications.

I. Introduction

Diabetes mellitus was known to mankind since antiquity. Its mention is there as early as 1500 BC in Egyptian manuscripts, although the name diabetes mellitus was added later on. The term diabetes means to siphon (Greek) since the patient passes a large amount of urine and Mellitus means sweet like honey (Latin). [1]

The disease is characterized by elevated blood sugar and estimation of glycated Hb (HbA1c) remains one of the most important and indispensable investigations, besides estimation of blood sugar, fasting and postprandial. The blood glucose concentration depends upon the many factors like food intake, exercise, stress, etc, but the concentration of HbA1c in the blood reflects the average glucose over preceding 8-12 weeks. Thus estimation of HbA1c provides additional criteria to assess the control of Diabetes and is free from diurnal fluctuations which occur with blood glucose. The adaptation of HbA1c estimation has become an integral exercise in the management of diabetic patients because it offers Several advantages like patient need not be fasting, the sample can be collected at any time, it is stable and has got little biological variability. It also predicts the development of micro and macro vascular complications. [2], [3], [4]

a) The HbA1c

In normal adults, haemoglobin usually contains HbA (~97% of the total) (Table 1), HbA2 (~2.5%), and HbF (~0.5%).

HbA is made up of four polypeptide chains, two α- and two β- chains. The glycosylation is the non-enzymatic attachment of a sugar to the amino group of proteins. Analysis has revealed that HbA1c has a hexose attached covalently to the NH2-terminal valine residue of the β-chains of HbA. [5] The International Union of Pure and Applied Chemistry have defined HbA1c as the fraction of the β-chains of hemoglobin that has a stable hexose adduct on the NH2-terminal amino acid valine. [6]

b) The landmark trials/studies

1. The Diabetes Control and Complications Trial (DCCT) research group (1993) studied the effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. This study documented that, lowering blood glucose concentration as assessed by estimation of HbA1c resulted in the delayed onset and reduced rate of progression of microvascular complications in type 1 diabetics. It is a dominant predictor of retinopathy and cardiovascular complications. It unequivocally established the value of measuring HbA1c in patients of diabetes. [2]
2. UK Prospective Diabetes Study (UKPDS) in 1998. [3] This study was done to ensure that the results obtained by DCCT trials are comparable to with UKPDS trials. The estimation of HbA1c is done by ion-exchange, high performance liquid chromatography (HPLC) in DCCT Trials. Ten year follow-up demonstrated that the risk of myocardial infarction was significantly lower in patients who had lower HbA1c at the end of UKPDS trial. [7]
3. Standardization of glycated haemoglobin testing American Association for Clinical Chemistry (AACC)

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established a committee in 1993 to standardize Glycosylated haemoglobin testing. [8]

The NGSP (National Glycohemoglobin Standardization Programme) was created three years later to execute the protocol developed by AACC committee. The DCCT and UKPDS trials have established beyond doubt the direct relationship between HbA1c concentration and outcome risks in patients with diabetes. The concept is that all clinical laboratories should report an HbA1c value equivalent to that reported in the DCCT and UKPDS. [9] The goal of the NGSP is to standardize glycated hemoglobin test results to those of the DCCT and UKPDS.

4. International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) in 1995. The primary objective of this committee was to develop a true reference method for HbA1c. [10] Instead of standardizing to a comparison method, the primary objective of the IFCC committee was to develop a true reference method for HbA1c.The NGSP and IFCC networks have complementary roles in the HbA1c standardization process. The two networks work together to form a solid basis for the establishment of a reliable and accurate HbA1c measurement platform.
5. The master equation a linear relationship can be derived between HbA1c results of IFCC reference method and NGSP network. [11] The calculated regression equation is:-

$$NGSP=0.09148(IFCC)+2.152.$$

This equation is called "master equation" and permits conversion between the two sets of values. But, the HbA1c values measured by the IFCC method are significantly lower than the NGSP values, and moreover, the difference is not constant.

The three preeminent clinical diabetic organizations, the American Diabetic Association (ADA), the European Association for the Study of Diabetes (EASD), and the International Diabetes Federation (IDF), attempted to resolve the dispute by agreeing to consider reporting HbA1c with estimated average glucose (ear). [12], [13] a linear relationship was established between HbA1c and mean blood glucose. [14]

c) Reporting of HbA1c

Another significant development was how to report HbA1c.In 2007 IFCC adapted reporting by International System of Units (SI units) rather than the percentage. This type of reporting avoids the confusion of using percentage (as the unit), which is having different reference interval. Thus the IFCC values are now expressed as millimoles of HbA1c per mole of HbA1c. [15], [16]

In 2004, a working group was constituted with representatives from ADA, ESAD, and IDF with a view to harmonizing HbA1c reporting. This group was termed as ADA/ESAD/IDF working group of the HbA1c assay. The consensus statement published in 2007 reiterates that the HbA1c should be reported worldwide in IFCC units (mmol/mol) and derived NGSP units (%) using IFCC-NGSP master equation. [17]

Reporting of HbA1c

HbA1c estimation is recommended by International guidelines as a preferred measure when evaluating the overall control of diabetes and patient's risk for complications. [18], [19] HbA1c is recognized as a reliable marker for the overall glucose exposure and its direct consequences. In setting a target level of HbA1c in an individual patient, due consideration should be given to patient's health, the risk of hypoglycemia, co-morbidities, and his/her specific health issues. The IDF (International Diabetes Federation) and the American College of Endocrinology (ACE) recommend HbA1c values below 6.5% while ADA (American Diabetes Association) recommends control below 7.0% for most patients. [20] Thus the estimation of HbA1c has become a key measure for diagnosing, screening and monitoring diabetes, and is an indispensable tool.

i. *Criteria for Identification of Prediabetic*

A Prediabetic is defined as, a person, having impaired fasting glucose and impaired glucose tolerance, based on 2-hour OGTT (oral glucose tolerance test) and FPG (Fasting Plasma Glucose). The patients having HbA1c values 6.00% to 6.49% are considered at high risk for developing diabetes as advised by ADA and WHO. [21], [22], [23]

Table 1: Criteria for identification of Prediabetic. [23]

Measurement	ADA, 2015 criteria	WHO, 2006/2011 criteria
Haemoglobin A1c	5.7%–6.4% (39–46 mmol/mol)	6.0%–6.5% (42 mmol/mol)
Fasting plasma glucose	100-125 mg/dL 5.6-6.9 mmol/L	110-125 mg/dL 6.1-6.9 mmol/L
2 hour post-prandiol plasma glucose	140-199 mg/dL 7.8-11.0 mmol/L	140-200 mg/dL 7.8-11.1 mmol/L

ADA = American Diabetes Association; WHO = World Health Organization

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ii. *Criteria for diagnosis of Diabetes*

The 1997 Expert Committee on the Diagnosis and Classification of Diabetes Mellitus encouraged that that it is based on the glycaemic level at which microvascular complications develops. [24] Evaluating HbA1c in 0.5% increments, investigators found that the incidence of diabetic retinopathy rose above baseline at HbA1c of 6.5%, the now accepted diagnostic value.The committee established that there is increased risk of diabetic retinopathy at fasting plasma glucose level greater than or equal to 126mg/dl (7.0mmol/L). It was done to define a cut-off point of HbA1c for diagnostic purposes. In a DETECT-2 trial, the investigators found microvascular complications above 6.5% hbA1c. [25] Diabetes can be diagnosed by using venous plasma sample for HbA1c and fasting plasma glucose, 2-hour oral glucose tolerance test (OGTT) ads to the diagnostic accuracy. OGTT also identifies the patients with impaired glucose tolerance; this along with impaired fasting glucose is a marker for impaired beta cell function with future progression to frank diabetes.

Random plasma glucose >200mg/dL (11.1 mmol/L) and symptoms of hyperglycemia like polyurea, polydipsia, blurred vision and weight loss also confirm the diagnosis (of Diabetes Mellitus). At any given time fulfillment of any two of the three criteria clinches to the diagnosis. [23]

Table 2: Criteria for the diagnosis of Diabetes. [23]

Measurement	ADA 2015 diagnostic value
Haemoglobin A1c	> 6.5 %(48 mmol/mol)
Fasting plasma glucose	> 126 mg/dL (7.0 mmol/L)
2-hour postprandial plasma Glucose.	> 200mg/dL (11.1 mmol/L)

ADA= American Diabetic association

Table 3: HbA1c and corresponding estimated average glucose. [26]

HbA1c %	Mean plasma glucose (mg/dL)	IFCC Units (mmol/mol)
6	126	42
6.5	141	48
7	154	53
7.5	169	59
8	183	64
8.5	198	69
9	212	75
9.5	226	80
10	240	86
11	269	97
12	298	108

IFCC= International Federation of Clinical Chemistry and Laboratory Medicine

d) Pitfalls in HbA1c Measurements

i. *Falsely lowered HbA1c levels*

The condition that shortens the life-span of RBCs or causes a rapid turnover of it shortens the exposure of cells to as follows:

Acute or chronic blood loss due to any causes, hemolytic anemia, spherocytosis, splenomegaly, Hemoglobinopathies and associated anemia, chronic malaria, glucose-6-phosphate-dehydrogenase (G-6-Pd) deficiency, sickle cell anemia etc, since they change turnover of RBCs. Erythrocyte survival is also shortened in chronic hyperglycemic states. This decreased survival may underestimate the severity of hyperglycemia at higher HbA1c levels. [27], [28]

The end-stage renal disease generally has falsely low HbA1c levels. It is due to chronic anemia and reduced red cell survival. So in end-stage renal disease, the clinician should take into consideration this fact while evaluating glycaemic status. [29], [30]

Pregnancy: A1C may not be a true reflection of glycaemia during pregnancy primarily because of both the decreased life span of the red blood cell from about 120 days to about 90 days as well as increased erythropoietin production. So, it is advised not to use the criteria of HbA1c levels for the diagnosis of gestational diabetes. [31]

Miscellaneous: the vitamin supplements like Vitamin-E, Ribaverin, and interferon Alfa are associated with falsely lowered HbA1c levels.

ii. *Falsely elevated HbA1c levels*

Any condition that prolongs the lifespan of red blood cells or decreases its turnover may cause a false elevation of HbA1c levels. These conditions include Iron deficiency anemia, vitamin B-12, and folate deficiency anemia.[32] Uremia, hypertriglyceridemia (concentration > 1750mg/dL), severe hyperbilirubinemia can also show falsely elevated HbA1c levels [33-34] So in the presence

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one has to be cautious in its interpretation. Salicylates, opiates and lead poisoning may be responsible for the false elevation of HbA1c levels. [35] While making changes in therapy based on elevated HbA1c levels these conditions must be taken into consideration to avoid, inappropriate therapeutic regimen and hypoglycemia.

In situations where the HbA1c results do not give a true picture due to false elevation or false low results, it is desirable to use alternative indices. These include fructosamine, glycated albumin, 1, 5-Anhdroglucitol (1, 5-AG), and continuous glucose monitoring (CGM).

II. Conclusion

The measurement of plasma glucose is the most reliable mean to diagnose diabetes mellitus. HbA1c measurement remains the gold standard to monitor glycaemic control and also an important diagnostic criterion. The results should be interpreted keeping in mind that, the other clinical conditions and co-morbidities, may give falsely elevated or lowered levels of hbA1c. In such situations, the alternative measure of glycaemic control should be considered like fructosamine, glycated albumin, 1, 5-AG or continuous glucose monitoring. The importance of the estimation of HbA1c is that it is validated as a predictor of diabetes-related outcomes. The diagnostic HbA1c cut-off point of 6.5% is associated with retinopathy prevalence and is the diagnostic thresholds for Fasting plasma glucose and 2-hr post-prandial-glucose. The HbA1c range of 5.7-6.4% in addition to impaired fasting glucose (IFG), and IGT (impaired glucose tolerance) have been included in the "categories of increased risk for diabetes".

The study of HbA1c has answered a very basic question of glycaemic control after which complications are prone to occur. It has got certain inherent limitation, despite that estimation of HbA1c remains the gold standard in the management of Diabetes mellitus.

The key learning points

1. An HbA1c level ≥ 6.5% is the cut-off point, used for the diagnosis of diabetes mellitus.
2. The values of HbA1c in the range of 5.7% to 6.4%.are included in the "categories of increased risk for diabetes".
3. HbA1c is formed by the glycosylation of Haemoglobin, so anything which interferes with a lifespan of RBCs or process of glycosylation may give a false result.
4. The use of Hba1c levels for monitoring of diabetes control should take into consideration the other co-morbidities.
5. The combination of FGT and HbA1c significantly enhances the diagnostic accuracy of these tests.
6. The estimation of HbA1c has unique potential of assessing the retrospective glycaemic control as well as predicting a risk for retinopathies and cardiovascular risks.
7. Thus the HbA1c test continues as an important diagnostic and prognostic tool, for the better patient care and successful clinical outcome.

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Characterization and Antibiotics Sensitivity Pattern of Enterobacteriaceae in Obafemi Awolowo University Sewage Oxidation Pond

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Keywords: enterobacteriaceae, sewage oxidation pond, total bacteria count, antibiotic sensitivity, biochemical characterization.

GJMR-C Classification: NLMC Code: QW 4

CHARACTERIZATION AND ANTIBIOTICS SENSITIVITY PATTERNOFENTEROBACTERIACEAE INOBAFEMIAWOLOWOUNIVERSITYSEWAGEOXIDATIONPOND

Strictly as per the compliance and regulations of:

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Characterization and Antibiotics Sensitivity Pattern of Enterobacteriaceae in Obafemi Awolowo University Sewage Oxidation Pond

Sokan-Adeaga Micheal Ayodeji[α], Sokan – Adeaga Adewale Allen[σ]
& Sokan-Adeaga Eniola Deborah[ρ]

Abstract- Some members of the family Enterobacteriaceae were isolated from Obafemi Awolowo University sewage oxidation pond (target site) and characterize by various biochemical test (citrate test, Gram stain, catalase test, idole production, fermentation of sugar, starch hydrolysis, Nitrate reduction, oxidative-fermentative test, gelatin hydrolysis and Methyl Red Voges-Proskauer test); the antibiotic sensitivity pattern of the isolate were also carried out. All was done under an aseptic condition. The total bacterial count (TBC) obtained from the target site was higher than those obtained from the relative site (1: before the sewage enter) and (2: after leaving) the oxidation pond. The biochemical tests identified the following: Citrobacter diversus, Salmonella arizonae, Typical Salmonella, Escherichia coli and Providencia alcalifaciens, all belonging to the family Enterobacteriaceae. Investigation of the antibiotic sensitivity test revealed that Cefuroxime and Ampicillin were not effective against all the bacteria isolates. However they each exhibit a varying degree of resistance and susceptibility to Ciprofloxacin, Tetracycline, Norfloxacin, Amoxycillin, Ofloxacin, Chloramphenicol, Gentamycin and Nitrofurantoin. Hence the study confirms that Obafemi Awolowo University sewage oxidation pond has a very high number of Enterobacteriaceae which show relative resistance to various antibiotics.

Keywords: enterobacteriaceae, sewage oxidation pond, total bacteria count, antibiotic sensitivity, biochemical characterization.

I. Introduction

The increased use of waters by humans especially as a receptacle for the disposal of human waste, the effects of added organic matters and pathogens are of public health concern (Prescott et al., 2005; Al-Bahry et al., 2011). Contamination of drinking water by faecal waste has led to a major epidemic of disease caused by water-borne pathogens (Nester et al., 2001; Felfoldi et al., 2010). Sewage has been recognized over the years as a major source of water contamination. In the urban environment, sewage discharges are major component of water pollution and contributes to oxygen demand and nutrient loading thereby promoting growth of toxic algae and the aquatic plants resulting in destabilization of aquatic ecosystem (Olajire and Impekperia, 2000; Morrison et al., 2001). Untreated domestic sewage contains large quantity of pathogenic organisms which are released into water bodies. These pathogens include: viruses, bacteria, protozoa and parasitic worms which are causative agents of many communicable diseases such as typhoid fever, diarrhea, amoebic dysentery, cholera and infectious hepatitis (Farmer and Kelly, 1991; Chiu, 2004; Gillespie et al., 2011). The heterogeneous composition of sewage allows the development of diverse heterotrophic bacteria populations including the members of the "family Enterobacteriaceae" (Atlas and Bartha, 1997; Croxen and Finlay, 2010).

Enterobacteriaceae is a family of Gram-negative, facultatively anaerobic, non-spore-forming rods. Morphological and biochemical characteristics of this family include being motile, catalase positive, and oxidase negative; reduction of nitrate to nitrite; and acid production from glucose fermentation (Farmer and Kelly, 1991; Grimont and Grimont, 2006; Denton, 2007). However, there are also many exceptions. Currently, the family comprises 51 genera and 238 species. The number of species per genus ranges from 1 to 22. Twenty-two genera contain only one species, while seven genera have more than ten species (Brisse et al., 2006; Janda, 2006). Enterobacteriaceae is closest to Vibrionaceae and Pasteurellaceae as sister clades with all members except for the genera Arsenophonus and Thorsellia being clustered together in one clade (Borenshtein and Schauer, 2006). Of the 30 genera with two or more species, 21 are likely to be monophyletic based on clustering on 16 rDNA sequence and other data. However, seven genera are likely to be polyphyletic requiring further reclassification (Paradis et al., 2005; Pham et al., 2007, Auch, 2010). Enterobacteriaceae has been heavily sequenced from across the spectrum of the family diversity with 180 complete genomes covering 47 species and 21 genera. The genome size ranges from 422,434 bp, coding for just 362 ORFs, to 6,450,897 bp, coding for 5,909 ORFs

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(Hedegaard et al., 1999; Konstantinidis and Tiedje, 2005). Enterobacteriaceae is ubiquitous in nature. Many species can exist as free living in diverse ecological niches, both terrestrial and aquatic environments, and some are associated with animals, plants, or insects only. These groups of microbes are pathogens in human, animal and/or plant causing a range of infections (Gillespie et al., 2011). There are numerous applications using members of Enterobacteriaceae including biocontrol in agriculture, production of numerous recombinant proteins and nonprotein products, control of infection diseases, anticancer agents, biowaste recycling, and bioremediation (Dento, 2007). Genome-based phylogeny and genomics are expected to further delineate the members of Enterobacteriaceae and refine the classification of the genera and species within this family (Pham et al., 2007).

Due to its high sanitary efficiency, treatment of waste water by stabilization ponds is recommended for sensitive coastal areas; it is suitable for peri-urban settings and requires large surface area (Picot et al., 1992). Oxidation (stabilization) pond is a simple scientifically designed pond with 2-6 feet depth, in which algal-bacterial growth in situ helps in the reduction of biochemical oxygen demand of wastewater (Ghrabi et al., 1993). These ponds are effective, low-cost and simple technology for the treatment of wastewater before it is discharged to an aquatic ecosystem (Mahajan et al., 2010) and are commonly used in tropical countries to purify wastewater. The efficiency of the pond depends on climatological conditions like light, temperature, rain, wind and also the wastewater quality. Oxidation pond typically operate in an extended aeration mode with long detention and solids retention time (Sperling and Lemos, 2005) and is a widely adopted technique for the treatment of domestic and industrial wastes. It is one of the methods widely used in the tropical areas of the world for treating wastewater (Hosetti and Frost, 1995). Oxidation pond comprises different groups of organisms such as bacteria, algae, protozoa, fungi, viruses, rotifers, nematodes, insects and crustacean larvae etc. which coexist and compete with each other (Nair, 1997). The bacteria present in the pond respire aerobically and anaerobically by decomposing the biodegradable organic content of the waste and release carbon dioxide, ammonia and nitrates (Tharavathy and Hosetti, 2003). These compounds are utilized by the algae, which together with sunlight and photosynthetic process releases oxygen, enabling the bacteria to breakdown more waste and accomplish reduction in BOD levels (Tharavathy and Hosetti, 2003). Initial research on oxidation ponds (1946 to 1960) describes pond activity in terms of mutualistic behaviour of algae and protozoa through photosynthesis (Nair, 1997). According to the conditions of the oxidation pond aerobic, facultative and anaerobic bacteria grow and stabilize the organic substances present in the wastes through biological processes (Hosetti and Frost, 1995).

Contemporary populations of enteric bacteria, when compared with those from the pre-antibiotics era, display a higher tolerance in their nonspecific responses to several antibiotics (Houndt and Ochman, 2000). The increase in antimicrobial resistance, observed in a bacterial population, may result from the clonal selection of organisms that tolerate sublethal antimicrobial doses and that present greater fitness under conditions of selective pressure, or from the spreading of resistance genetic determinants through horizontal gene transfer. The most plausible hypothesis is that, in the natural environment, both mechanisms are responsible for the dynamics of the bacterial population. In different environments, bacteria are expected to experience distinct selective pressures for antibiotic resistance and, hence, distinct patterns of antibiotic resistance acquisition and evolution. Urban wastewater treatment plants represent important reservoirs of human and animal commensal bacteria in which antibiotic resistance determinants and/or organisms persist in the final effluent and are released to the environment (Reinthaler et al., 2003; Tennstedt et al., 2003).

Previous works done on community sewage oxidation pond in tropical countries had centered only on the physicochemical characterization and microbiological examination at the family level. There is hardly any detailed documentation in available literature on the characterization and antibiotics sensitivity of the family Enterobacteriaceae isolated from a sewage oxidation pond in Nigeria. Thus the main objective of this study was to isolate and characterize the five genera of Enterobacteriaceae isolated from Obafemi Awolowo University sewage oxidation pond; and also assess their antibiotic sensitivity pattern.

II. Experimental Procedures

a) Study Design

The study was experimental and laboratory based, involving morphological characteristics, bio-chemical characterization; and antibiotics sensitivity test of the bacterial isolates found in sewage samples.

b) Study Location

The study was carried out at Obafemi Awolowo University (O.A.U) Oxidation pond located at the outskirt of the O.A.U campus, Ile-Ife, Osun State, Nigeria.

c) Sample Collection

The sewage samples were collected with the aid of sterilized sampling bottles from three (3) sampling sites designated as follows:

Point A: Where the sewage enter into the oxidation pond.

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Point B: The Obafemi Awolowo University Oxidation Pond.

Point C: Meeting point (confluent) between the effluent of the oxidation pond and the stream that flows along Ede road.

A long rope was tied around the neck of each bottle, covered and sterilized by autoclaving at 121°C for 15 minutes. The bottles were allowed to gradually sinked into each sample collection point to collect each sewage sample. Each bottle were allowed to filled to the brim, bought out of the sewage sample collection point and immediately covered to prevent contamination from gaining entrance into the sewage sample. The sample bottles were labeled accordingly, transported to the laboratory and used immediately.

d) Isolation and Enumeration Procedure

i. *Serial Dilution of Sewage Sample and Plate Count*

Pour plate dilution technique described by Seeley and Van Demark (1981) was used to determine the total bacterial count (TBC). The stock samples to be examined were thoroughly mixed to ensure the uniform distribution of the microbes in the sample.

Point A: 1 in 100 dilution was used for sample from point A.

Point B: 1 in 1000 dilution was used for sample obtained from point B.

*Point C:*1 in 10 dilution was used for sample from point C.

After the samples have been serially diluted, 0.5ml of the different dilutions was aseptically transferred into each of the sterile – petridishes containing set, solidified Eosin Methylene Blue (EMB) Agar. The culture plates were inverted to avoid moisture droplets falling on the growth which could prevent formation of discrete colonies. The plate was the incubated at 37°C for 24 hours. Before preparing pure isolates, viable counts were carried out on each of the three different plates (i.e. plate A, B and C), where the number of bacteria per ml of the sample were obtained by multiplying the number of colonies on each of the plate by each plate dilution factor.

ii. *Media for Isolation of Bacteria Isolates*

The following media were used for the bacteria isolation viz:

- Eosin Methylene Blue (EMB) Agar
- Nutrient Agar
- Normal Saline

iii. *Isolation and Preparation of Stock Culture*

The bacterial colonies were noted and counted; and five different colonies were differently transferred into five different sterile Petri dishes each containing a sterile EMB Agar. The plates were then inverted and incubated at 37°C for another 24 hours.

Isolate from each of the five different plates were each transferred into five different tube containing a set nutrient agar in a slanting position. After which they were kept in the refrigerator from where they were picked for subsequent Biochemical Tests performed.

e) Morphological Characteristics (Identification Procedures)

For the morphological characteristics of the isolate (i.e. Enterobacteriaceae) only Gram Staining was carried out to know the shape of each of the five isolates since they are all Gram-negative based on the fact that they are all isolated from EMB Agar.

f) Biochemical Characterization of Bacterial Isolates

The following biochemical tests were carried out to characterize and identify the FIVE BACTERIA ISOLATES (Enterobacteriaceae) obtained.

i. *Catalase Test*

This test is carried out to find out the production of catalase by bacteria isolate. The hydrogen peroxide is usually toxic and is decomposed immediately by the enzyme catalase as soon as it is formed. A loopful of hydrogen peroxide was emulsified into the culture from the plate on a clean grease free slide. The occurrence of effervescence caused by the liberation of oxygen bubbles indicated a positive test (i.e. the presence of catalase in the culture under test).

This test was carried out to detect the production of the enzyme, catalase by an organism. The enzyme converts Hydrogen peroxide to Water and Oxygen as shown in the equation below:

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

ii. *Citrate Utilization Test*

The coliform bacteria may be differentiated by their ability to utilize citrate as a sole source of carbon. The culture was incubated at 37°C for 2 – 5 days and examined for change in colour of the bromothymol blue indicator. A change in colour of bromothymol blue indicator from green to blue indicates utilization of the citrate i.e. positive or otherwise negative.

g) Fermentation of Sugars

In this test, the prime concern is to determine what sugars are fermentable by the unknown. If the organism does ferment a particular sugar, acid will be produced and gas may be produced. The presence of the acid is detectable with a pH indicator. Gas production is revealed by the formation of a void in the inverted vial of the Durham tube. The fermentable sugars used are: Glucose, Mannitol, Maltose, Sucrose and Fructose.

Each tube of each fermentable sugar was inoculated with a loopful of the test organism from the 24-hour old peptone water culture and incubated at

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37°C for 7 days respectively. Observation were made daily for the production of acid and gas in each Durham.

h) Indole Test

This test is important in the differentiation of Coliforms and depends on the production of indole from trytophane by the bacterium. Tryptone water is used rather than from peptone water, but peptone water was used to prepare a 24-hour old broth culture.

Each tube containing sterile tryptone water was inoculated with a loopful of a broth culture of the organism. The tubes were incubated at 37°C for 5 days. After, incubation, 0.5ml Kovac's reagent was added to the content of each tube, shaken gently, and then allowed to stand. A deep red colour developed in the presence of indole, which separated out in the alcohol layer. This is a positive reaction, otherwise indicates negative result.

i. *Methyl Red Voges Praskauer (MRVP) Test*

This test help to help distinguished the Coli and Aerogenes bacteria from each other. The MRVP medium prepared and distributed into test tubes plugged with cotton wool. Each tube was aseptically inoculated with a loopful of 24 hour-old culture of the isolate and incubated at 37°C for 5 days. Uninoculated controls were also incubated. After incubation period, the content of each tube was aseptically divided into two portions labeled M and V respectively. To the portion labeled M, 5 drops of methyl red solutions was added. To the other portion labeled V, 0.5ml of 6% α-napthol added, followed by 0.5ml of KOH. The content of each test tube was mixed thoroughly. The test tubes were allowed to stand for 5 minutes and observation was made for the formation of colour.

A development of red colour in the M portion indicates a positive reaction while development of yellow colour in the M Portion indicates a negative reaction. To the V portion, the development of a red colouration constitutes a positive reaction otherwise negative reaction.

ii. *Oxidative-Fermentative Test*

Bacteria which attack carbohydrate either do so aerobically (i.e. OXIDATIVELY) or anaerobically (i.e. FERMENTATIVELY). The carbohydrate most frequently used is dextrose but lactose, sucrose or any other carbohydrate may also be used.

For each carbohydrates, two tubes of medium was stab inoculated with a 24 hours – old culture. The surface of the medium in one tube was covered with sterile paraffin and later covered with a sterile cotton wool, while the surface of the medium in the other tube was only covered with a sterile cotton wool, the tubes were then incubated at 37°C for up to 14-days and examined. A change in the colour of medium from green to yellow indicated acid production. While fermentative organisms produced acid in both tubes, oxidative organisms produced acid in the tube covered with sterile cotton wool only.

i) Nitrate Reduction Test

Many microorganisms are capable of reducing nitrate to nitrite or even further to hydroxylamine, ammonia or nitrogen. Thus an intermediary in the reaction is NITRITE and the first test applied was for its presence.

Each tube of nitrate medium was inoculated with a loopful of a 24hour-old peptone culture of the bacteria. Incubated at 37°C for 5-days and examined for the presence of gas in the inverted Durham tube. Tubes without gas were were test for the presence of nitrite using Gries –Ilosvay reagent. A red, pink or maroon colour indicated a positive reaction otherwise negative. The negative tubes were further treated for the presence of residual nitrate by the addition of zinc dust.

j) Hydrolysis of Gelatin

The medium was dissolved in distilled water and boiled to dissolved completely after which twenty millilitres (20ml) was poured into sterile petridishes and allowed to solidified. 24 hours old culture of isolate was streaked across the plate and incubated at 37°C for 5 days. After incubation, the plates were flooded with the reagent (mercuric chloride solution) and observation.

Unhydrolysed gelatin forms a white opaque precipitate with the reagent while hydrolysed gelatin appears therefore as a clear zone when flooded with the reagent.

k) Hydrolysis of Starch

Twenty millimetres of molten starch were poured into sterile petridishes and allowed to cool and set. The test organisms were each streaked across the surface of each of the plate containing the set starch agar. The plates were incubated at 37°C for 5 days. After incubation, gram's iodine was flooded on the plates.

i. *Antibiotics Sensitivity Test*

Medium: Sensitivity Test Agar (S.T.A)
Peptone water

ii. *Preparation*

48 grammes of S.T.A were dissolved in 1litre of distilled water. The mixture was warmed on a hot plate to dissolved. The medium was sterilized by autoclaving at 121°C for 15 minutes and allowed to cool. Twenty millilitres of S.T.A were dispensed into sterile petridishes and allowed to set.

iii. *Procedure*

A loopful obtained from a 24 hour-old culture of the organism was placed at one edge of the surface of the S.T.A. A sterile swab was used to spread the organism uniformly on the surface of the medium.

An antibiotics disc was aseptically removed with the aid of a sterile forceps and placed in the centre of the petridish, such that no air bubbles are trapped

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between the disc and the plate. The plates were then incubated at 37°C for 24 hours and the diameters of clear zones of inhibition around the discs were measured in millilitres with a transparent ruler. Zones with diameter of less than 2mm were recorded as resistant while, those with 2mm and greater were taken to be susceptible or sensitive.

III. Results

a) Features of Sewage Samples Examined

A total of three samples were taken from three different sampling sites designated as point: A, B and C. The features of each sample was noted and observed. The observed features are shown in Table 1.

Table 1: Features of sewage samples from the sample collection sites.

Samples	Features
Sample collected from point A	Colourless liquid solution with clear appearance having solid dispersed faecal particles.
Sample collected from point B	A tinge green colour liquid solution with colloidal appearance having lot of minute, scattered and large faecal particles. Algal growth gave it a tinge green appearance.
Sample collected from point C	Colourless liquid solution with clearer appearance.

b) Total Bacterial Counts in Sewage Samples

The result of the total bacteria counts in each sewage sample obtained/collected from the three different sampling sites is shown in Table 2.

Table 2: Total bacterial count in sewage samples

Dilution of Sample	Temperature of Incubation	Point of sample Collection	Total Bacteria Count in (CFU/ml)
10^{-2}	37°C	A: Point of entry of sewage.	1.40×10^{1}
10^{-4}	37°C	B: O.A.U oxidation pond	8.29×10^{2}
10^{-1}	37°C	C: Discharge of sewage effluent into stream along Ede road.	2.98×10^{2}

The sewage sample obtained from point B has the highest number of Bacterial population, followed by that from point C while that from point A has the least total bacterial count (TBC). Total bacterial population in descending order: point B > point C > point A.

c) Observed Growth of Bacterial Colonies on Eosin Methylene Blue (EMB) Agar

The cultural/morphological characteristics of the bacteria isolates obtained from point B (O.A.U oxidation pond) is depicted below in Table 3.

Table 3: Cultural/Morphological characteristics of the bacterial isolates from B

Characteristics	Test	Isolates				
		1	2	3	4	5
Cultural Agar Colonies	Medium Position Shape Elevation Size Margin Surface Pigment	EMB Distinct Circular Raised with convex level Small Entire Smooth and glistering Dark green	EMB Distinct Circular Raised with convex level Small Entire Smooth and glistering Green	EMB Distinct Circular Raised with convex level Small Entire Smooth and glistering Light green	EMB Distinct Circular Raised with convex level Small Entire Smooth and glistering Greenish metallic with dark colour	EMB Distinct Circular Raised with convex level Small Entire Smooth and glistering Red with dark centred
Cultural Agar Colonies	Medium Size Margin Surface Elevation Pigment	NA Small Entire Smooth Low convex level Cream colour	NA Small Entire Smooth Low convex level Cream colour	NA Small Entire Smooth Low convex level Cream colour	NA Small Entire Smooth Low convex level Cream colour	NA Small Entire Smooth Low convex level Cream colour
Morphological Test	Gram stain Cell form	- Rod (straight)	- Rod (straight)	- Rod (straight)	- Rod (straight)	- Rod (straight)

EMB: Eosin Methylene Blue Agar

NA: Nutrient Agar

Note: Negative

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Table 3 above shown all the five (5) isolates which appear pink, hence they are all Gram-negative (-) and also appeared rod in shape and are all straight. Hence all five isolates: 1,2,3,4 and 5 are Gram-negative and straight rods.

d) Biochemical Characterization

Results obtained from the biochemical characterization of bacteria isolates from the sewage sample collected from Obafemi Awolowo University oxidation pond (point B).

i. *Catalase Test*

Table 4 showed that all the five isolates were positive for catalase test. This implies that all isolates were able to synthesize the enzyme catalase.

ii. *Citrate Utilization Test*

All the isolates except isolate 4 (which is citrate negative) had the ability to utilize citrate as the sole source of carbon as the citrate broth of all the isolates (except isolate 4) changed from green to blue colour at the end of the incubation period (i.e. 5 days) as shown on Table 4.

iii. *Indole Production*

Isolates: 1, 4 and 5 were positive for indole production, while Isolates: 2 and 3 were negative for indole production. In these positive tubes, there were production of indole as there were changes in colour in these tubes from yellow to a deep red colour in the upper layer of these tubes from yellow to a deep red colour in the upper layer of these tubes, after the addition of Kovac's indole reagent after 5 days incubation period, as shown on Table 4.

iv. *Methyl Red and Voges Proskauers Tests (MRVP Test)*

Table 4 showed that all isolates were positive for methyl red test as the isolates changed the colour of their medium to red on addition of methyl red while all the isolates were negative for voges Proskauers Test as indicated by the cream colour obtained in all the tubes inoculated with the isolate each on addition of Barritt's reagent.

v. *Nitrate Reduction Test*

Table 4 shown Isolates: 1, 3, 4 and 5 reduced Nitrate to Nitrite while Isolate: 2 produced gas in the inverted durham tube which implied the complete reduction of nitrate to nitrogen gas production.

vi. *Hydrolysis of Gelatin*

Isolates: 1, 3, 4 and 5 were not able to hydrolyze gelatin on addition of mercuric chloride after the 5 days incubation period as white opaque precipitates were formed. While isolate: 2 was able to hydrolyzed gelatin on addition of mercuric chloride after 5 days incubation period as a clear zone was produced as depicted on Table 4.

vii. *Hydrolysis of Starch*

Isolates: 1 and 4 were able to hydrolyzed starch with the presence of a blue-black colouration which developed on flooding the plates with Gram's iodine while isolates: 2, 3 and 5 were not able to hydrolyze, as a clear zone were formed on flooding the plates with Gram's iodine after the 5 days incubation period Table 4.

Table 4: Biochemical Characterization of Bacteria Isolates obtained from the Obafemi Awolowo University oxidation pond

Biochemical Reaction	Isolates				
	1	2	3	4	5
Catalase Test	+ ve	+ ve	+ ve	+ ve	+ ve
Citrate Utilization Test	+ ve	+ ve	+ ve	- ve	+ ve
Indole Production	+ ve	- ve	- ve	+ ve	+ ve
Methyl Red	+ ve	+ ve	+ ve	+ ve	+ ve
Voges Proskauer	- ve	- ve	- ve	- ve	- ve
Hydrolysis of Gelatin	White opaque (- ve)	Clear zone (+ ve)	White opaque (- ve)	White opaque (- ve)	White opaque (- ve)
Nitrate Reduction Test	Nitrate to Nitrite	Complete reduction of nitrate (Gas produced in inverted durham tube).	Nitrate to Nitrite	Nitrate to Nitrite	Nitrate to Nitrite
Starch Hydrolysis	Blue-black (+ ve)	Clear zone (- ve)	Clear zone (- ve)	Blue-black (+ ve)	Clear zone (- ve)

The results on Table 5 shown that all the isolate were facultatively anaerobic as they each produced acid (i.e. colour change from green to yellow) in both the oxidative and fermentative tubes at the end of 14 days incubationperiod.

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Table 5: The oxidative – fermentative results of Bacterial isolates obtained from O.A.U oxidation pond

Isolates (Bacteria)	Oxidative medium	Fermentative medium	Conclusion
1	Changed from green to yellow with gas produced.	Changed from green to yellow with gas produced.	Facultative Anaerobes
2	Changed from green to yellow.	Changed from green to yellow with gas produced.	Facultative Anaerobes
3	Changed from green to yellow with gas produced.	Changed from green to yellow.	Facultative Anaerobes
4	Changed from green to yellow with gas produced.	Changed from green to yellow with gas produced.	Facultative Anaerobes
5	Changed from green to yellow with gas produced.	Changed from green to yellow.	Facultative Anaerobes

viii. *Fermentation of Sugars*

All the isolate fermented Glucose and Fructose with the production of acid and gas. Mannitol was fermented by all the isolates (except isolate 5) with the production of acid and gas. Maltose was fermented only by isolates: 1, 3 and 4 (but not by isolates: 2 and 5) with the production of acid and gas. Sucrose was only fermented with only the production of acid but without gas production by isolates: 1, 4 and 5 (but not by isolate: 2 and 3) as shown in Table 6.

Table 6: Fermentation of sugars by bacterial isolates obtained from O.A.U sewage oxidation pond

Carbon Source	Reaction Given by Bacterial Isolates				
	1	2	3	4	5
Glucose	AG	AG	AG	AG	AG
Mannitol	AG	AG	AG	AG	AG
Maltose	AG	NIL	AG	AG	NIL
Sucrose	A	NIL	NIL	A	A
Fructose	AG	AG	AG	AG	AG

Note: A: Acid Production
G: Gas Production
NIL: No Production of Acid and Gas

e) *Antibiotics Sensitivity Test*

Table 7 showed the antibiotics sensitivity pattern of the bacterial isolates as obtained on the PD 002 Gram-negative Discs.

Isolate 1 were sensitive to Ciprofloxacin, Tetracycline, Norfloxacin, Amoxycillin, Of loxacin and Gentamycin but resistant to Chloramphenicol, Cefuroxime, Ampicillin and Nitrofurantoin.

Isolate 2 were sensitive to Ciprofloxacin, Norfloxacin, Ofloxacin and Gentamycin but resistant to Tetracycline, Amoxycillin, Chloramphenicol, Cefuroxime, Ampicillin and Nitrofurantoin.

Isolate 3 were sensitive to Ciprofloxacin, Tetracycline, Norfloxacin and Ofloxacin but were resistant to Amoxycillin, Chloramphenicol, Cefuroxime, Ampicillin, Gentamycin and Nitrofurantoin.

Isolate 4 were sensitive to Ciprofloxacin, Tetracycline, Norfloxacin, Amoxycillin, Ofloxacin, Chloramphenicol, Gentamycin and Nitrofurantoin but were resistant to Cefuroxime and Ampicillin.

Isolate 5 were sensitive to Gentamycin but resistant to Ciprofloxacin, Tetracycline, Norfloxacin, Amoxycillin, Ofloxacin, Chloramphenicol, Cefuroxime, Ampicillin and Nitrofurantoin.

Table 7: Antibiotics Sensitivity Pattern of Bacterial Isolate from O.A.U Sewage Sample

Disc Code	Antibiotics	Concentration (μg)	Diameter of Zones of Inhibition of Isolates				
			1	2	3	4	5
PD 002 Gram-negative URINE LEVEL TYPE 2	CIP	5	18	22	20	18	0
	TE	50	18	0	20	18	0
	NB	10	15	12	6	18	0
	AX	20	4	0	0	19	0
	OF	5	25	23	23	19	0
	C	10	0	0	0	20	0
	CF	30	0	0	0	0	0
	AM	25	0	0	0	0	0
	GN	10	12	12	0	18	20
	N	100	0	0	0	17	0

Note: CIP – Ciprofloxacin
TE – Tetracycline
NB – Norfloxaci
AX – Amoxycillin
OF – Ofloxaci
C – Chloramphenico
CF – Cefuroxime
AM – Ampicillin
GN – Gentamycin
N – Nitrofurantoin

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IV. Discussion

The findings of this research work was able to established the presence of four genera and five species of the family Enterobacteriaceae in the sewage sample collected from the sewage oxidation pond of Obafemi Awolowo University (O.A.U), Ile-Ife, Nigeria. The name of the five species belonging to four different genera of the family Enterobacteriaceae were:

Isolate 1: Citrobacter diversus

Isolate 2: Salmonella arizonae

Isolate 3: Typical Salmonella

Isolate 4: Escherichia coli

Isolate 5: Providencia alcalifaciens

Several workers had previously reported that the species of Salmonella, Providencia, Escherichia, Citrobacter, Shigella, Leptospirilla and other genera of the family Enterobacteriaceae as the most pathogenic and most encountered organisms in sewage oxidation pond. They are the causative agents of such diseases as food poisoning, meningitis, bacillary dysentery, gastroenteritis, typhoid, enteric fevers (e.g typhoid fever), plague and hospital-acquired infection (Farmer and Kelly, 1991; Nataro and Keper, 1998; Felfoldi et al., 2010; Al-Hasan et al., 2011; Gillespie et al., 2011).

The results of the total Enterobacteriaceae count in sewage samples showed that the number of Enterobacteriaceae in point A is very low, which is probably due to the inability of the bacteria to reproduce or proliferate and dispersity of the bacteria because of the speed by which the sewage is flowing into the oxidation pond caused by the high pumping pressure that is being use to conveyed the sewage from the O.A.U Community through the sewers to the oxidation pond. The population of Enterobacteriaceae in point B is very high, this is probably due to the favourable environmental conditions such as the alkalinity of the environment, optimal growth temperature (20 – 38°C), high concentration of organic matter, mutual association between the pond algae and the bacteria and the stagnancy of the pond. The population of Enterobacteriaceae in point C is intermediate between the numbers of Enterobacteriaceae in point A and point B which is due to the poor infrastructure facilities in the pond and the poor disinfection of the effluent from the oxidation pond.

The results of the biochemical characterization of the Enterobacteriaceae isolated from Obafemi Awolowo University sewage oxidation pond showed that: Citrobacter diversus, Salmonella arizonae, Typical salmonella, Escherichia coli and Providencia alcalifaciens were all catalase positive, voges – praskauer negative, citrate positive except E.coli that was citrate negative, methyl red positive, indole positive except Salmonella arizonae and Typical Salmonella that were indole negative, thus corroborating the results of the work carried out by Balows (1991) and Brooks et al., (2003). The results also showed that all the Enterobacteriaceae isolated from Obafemi Awolowo University sewage oxidation pond were capable of nitrate reduction. Citrobacter diversus, and Typical Salmonella, E. coli and P. alcalifaciens were only capable of reducing nitrate in nitrite, while S.arizonae was capable of complete reduction of nitrate to nitrogen gas production. The results also showed that the isolated Enterobacteriaceae were facultative anaerobes. Several workers (Farmer and Kelly, 1991; Gillespie et al., 2011) also reported similar results for all Enterobacteriaceae.

The results and this investigation also showed that all the isolated Enterobacteriaceae fermented sugars such as glucose and fructose producing acid and gas, while all the isolated Enterobacteriaceae except P. alcalifaciens, fermented mannitol to produced acid and gas. Maltose was fermented with the production of acid and gas by all the isolated Enterobacteriaceae except S. arizonae and P. alcalifaciens. Sucrose was only fermented with the production of acid and no gas production by all the isolated Enterobacteriaceae except S. arizonae and Typical Salmonella, corroborating the results of the work carried out by Madigan et al., (1997); Brock and Madigan, (1998) and Madigan et al., (2008) on Enterobacteriaceae. The results of Gelatin hydrolysis showed that all the isolated Enterobacteriaceae except S. arizonae were Gelatin-negative, while the results of the starch hydrolysis showed that C. diversus and E.coli were capable of starch hydrolyses, while S. arizonae, Typical Salmonella and P. alcalifaciens were not able to hydrolyse starch.

Enterobacteriaceae form part of the normal flora of the intestinal tract of man and animals (Denton, 2007). The Enterobacteriaceae encountered in this study were tested against antibiotic disc to determine their relative susceptibility. The results of the antibiotic sensitivity test showed that Ciprofloxacin, Norfloxacin and Of loxacin were effective against all the Enterobacteriaceae isolates except Providencia alcalifaciens that proved resistant to the antibiotics. Gentamycin proved effective against all the Enterobacteriaceae isolates except Typical Salmonella that was resistant to the antibiotic. However, all the Enterobacteriaceae isolates were resistant to Cefuroxime and Ampicillin, while all the Enterobacteriaceae isolates except Escherichia coli proved resistant to Chloramphenicol, and Nitrofurantoin. Similar results were obtained for Enterobacteriaceae and some other bacteria by previous workers (Paterson, 2006; Pitout, 2008).

The relative resistance of C. diversus, S. arizonae, and Typical Salmonella, E. coli and P. alcalifaciens towards antibiotics treatment is of great

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public health concern. Previous reports have also indicated that some Coliforms bacteria isolated from raw sewage and sewage effluents exhibit resistance to a number of antibiotics and that the resistant strains were capable of transferring their resistance to susceptible C. diversus, S. arizonae, Typical Salmonella, E.coli and P. alcalifaciens (Houndt and Ochman, 2000). In a comparative study of three activated sludge treatment plants, Reinthaler et al. (2003) concluded that, although no significant increases in antibiotic resistance phenotypes were observed over the course of sewage treatment, this process may contribute to the dissemination of resistant bacteria to the environment. In addition, Tennstedt et al. (2003) reported the presence of antibiotic resistance determinants in self-transmissible genetic elements of bacteria residing in the activated sludge and final effluent released from a wastewater treatment plant.

The high rate of antibiotics resistant in isolates recovered from the sewage oxidation pond is of concern because it may suggest the ineffectiveness of these drugs in the treatment of infections caused by these organisms. The sewage entering the oxidation pond of Obafemi Awolowo University is contributed by some heterogeneous group of people including students, workers and farmers who live or work on the campus. Thus, one may suggest that the antibiotics pattern of the Enterobacteriaceae isolates obtained in this work is a reflection of the nature of the faecal materials from the population. There is high tendency that successive abuse of antibiotics by some, if not many of these peoples on many occasions must have contributed to the development of resistant features by the Enterobacteriaceae isolates obtained in this work. This suggests caution in the use of antibiotics in the treatment of infections caused by the isolated Enterobacteriaceae.

V. Conclusions

The aim of this research was to isolate, characterize and screen the antibiotics sensitivity pattern of the Enterobacteriaceae in the Obafemi Awolowo University (O.A.U) sewage oxidation pond. This study was able to identified five different species belonging to the family Enterobacteriaceae from the oxidation pond. The species were Citrobacter diversus, Salmonella arizonae, typical salmonella, Escherichia coli and Providencia alcalifaciens; and these organisms are usually associated with intestinal infections which may spread to other parts of the body. These species also showed relative resistance to antibiotics treatment, and thus pose serious public health challenge.

Since the effluents from this oxidation pond are discharge into a nearby stream which may be used by villagers living along the stream flow, the University authority should pay an immediate attention to the improvement of these ponds to safeguard the health of the villagers and other people who may have contact with the stream. Thus, the sewage oxidation pond should be properly manages and maintains for effective performance. The effluents from the oxidation pond should be disinfected properly by adequate chlorination before discharging it into the environment.

Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this research paper.

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Stem Cell Therapy in Multiple Myeloma

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Abstract- Multiple myeloma is a haematogical malignancy caused by abnormality of plasma cells characterized by (a) hypercalcemia, (b) renal insufficiency or failure, (c)bone pain and abnormal bone radiographs, (d) anemia and (e) a monoclonal protein in urine or serum or both. It begins in the form known as monoclonal gammopathy of undetermined significance (MGUS) and progresses to asymptomatic myeloma and then lastly to symptomatic myeloma. The three main domains in the understanding of Pathophysiology are cytokines and cell signaling, bone Marrow Microenvironment and cell Cycle. Bifunctional alkylating agents like Melphalan and cyclophosphamide are considered as standard therapy for multiple myeloma. Patients who are chosen for stem cell transplantation can be treated with Lenalidomide, an immunomodulatory agent and an aminosustituted variant of Thalidomide. It is given along with dexamethas one. In stem cell therapy, the patients receive stem cells intravenously similar to the blood transfusion and this phase takes 1-5 hours. After entering the blood stream through a process called engraftment, the stem cell produce new WBCs, RBCs and platelets. The immune rejection of donor cells by the host immune system is a major drawback of transplantation. Stem cell therapy in multiple myeloma is very effective and should be opted as one among the best for both older patients and patients younger than 65 years.

Keywords: autologous stem cell transplantation; multiple myeloma; stem cell therapy; transplant related mortality.

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STEMCELLTHERAPYINMULTIPLEMYELOMA

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Stem Cell Therapy in Multiple Myeloma

Roshna P P [α] & Sreejith K [σ]

Abstract- Multiple myeloma is a haematogical malignancy caused by abnormality of plasma cells characterized by (a) hypercalcemia, (b) renal insufficiency or failure, (c)bone pain and abnormal bone radiographs, (d) anemia and (e) a monoclonal protein in urine or serum or both. It begins in the form known as monoclonal gammopathy of undetermined significance (MGUS) and progresses to asymptomatic myeloma and then lastly to symptomatic myeloma. The three main domains in the understanding of Pathophysiology are cytokines and cell signaling, bone Marrow Microenvironment and cell Cycle. Bifunctional alkylating agents like Melphalan and cyclophosphamide are considered as standard therapy for multiple myeloma. Patients who are chosen for stem cell transplantation can be treated with Lenalidomide, an immunomodulatory agent and an aminosustituted variant of Thalidomide. It is given along with dexamethas one. In stem cell therapy, the patients receive stem cells intravenously similar to the blood transfusion and this phase takes 1-5 hours. After entering the blood stream through a process called engraftment, the stem cell produce new WBCs, RBCs and platelets. The immune rejection of donor cells by the host immune system is a major drawback of transplantation. Stem cell therapy in multiple myeloma is very effective and should be opted as one among the best for both older patients and patients younger than 65 years.

Keywords: autologous stem cell transplantation; multiple myeloma; stem cell therapy; transplant related mortality.

I. Introduction

Multiple myeloma is a haematogical malignancy caused by abnormality of plasma cells. It is made distinctive by mainly five features which includes (a) hypercalcemia, (b) renal insufficiency or failure, (c)bone pain and abnormal bone radiographs, (d) anemia and (e) a monoclonal protein in urine or serum or both.[1] It also leads to osteolytic bone lesions and immunodeficiency.[2] Multiple myeloma is incurable and it effects mainly elderly persons.[3] It comes out to be the second most common hematologic neoplastic abnormality and it depicts 10% of all hematological cancers and 1% of all other malignancies.[4]It diminishes the gross health related quality of life in patients especially pertaining to physical functioning.[5] Clinical manifestations of multiple myeloma includes bone pain, lytic bone lesions, thrombocytopenia, pathologic bone fracture, bleeding, fatigue, haste and easy bruis ability, recurrent infections, spinal cord compression, hyperviscosity and hypogamaglobulinemia.[1,2,4] Hypervis-cosity which is one of the major presentation here is caused by the presence of monoclonal Ig in the sera.[4] Etiological factors of multiple myeloma include radiation exposures, workplace exposures, lifestyle factors, precursor medical conditions etc.[1]Prolongation of the survival is the major treatment goal for multiple myeloma. The development and introduction of chemotherapy conjunct with the autologous stem cell transplantation (ASCT) elevated the life endurance rate further. Allogenic stem cell transplantation is the only existing curative treatment because of the antitumor immunity mediated by donor lymphocytes.[4]

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II. Path Physiology

In multiple myeloma, the bone healing is diminished because of the decreased osteoblastic activity and increased osteoclastic activity. The bone embedded growth factors enhances progression of tumor as bone is resorbed. It begins in the form known as monoclonal gammopathy of undetermined significance(MGUS) and progresses to asymptomatic myeloma and then lastly to symptomatic myeloma which results into osteolytic lesions and bone marrow infiltration.[6]Several intracellular and intercellular signaling stream, including RANK/RANKL/OPG, Notch, Wnt, and chemokines and interleukins are associated in the complex pathophysiological process.[8]A single molecular inadequacy cannot be accounted for explaining the pathogenesis of multiple myeloma. Defects of four main domains have been identified which are abnormalities of apoptotic mechanisms, signaling pathways, bone marrow micro environment and cell cycle.[1]

a) Cytokines and cell signaling

Interleukin (IL)-6 is one of the most significant survival and proliferation factors in myeloma is which is produced by the bone marrow stromal cells – macrophages, fibroblasts, osteoclasts, osteoblasts and monocytes. In almost cases myeloma cells and their cell lines are capable of producing IL-6 and its receptor, IL-6 receptor results in the stimulation of autocrine system. Messages are transmitted by IL-6 intracellularly through the signal-transducing protein gp130. This activates 2 pathways: the Ras-MAP kinase pathway (Hallek et al. 1998) and the JAK-STAT pathway. Through the latter pathway (JAK-2 and STAT3), the antiapoptotic proteins Mcl-1 and Bcl-XL are up-regulated and through the former pathway, transcription factors such as ELK-1, AP-1, and NF-IL-6 are up-regulated.[1]Notch signaling pathway resulting from four transmembrane receptors (Notch 1-4) is actively involved in multiple myeloma

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induced osteoclastogenesis by the production of osteoclastogenic factor RANKL by multiple myeloma cells.[8]

b) Bone Marrow Microenvironment

A synergistic relationship exists within the myeloma cells and the cells comprised in the bone marrow microenvironment which includes fibroblasts, osteoblasts, and osteoclasts. The IL-6 produced in large amount will result in the production of IL-1α, VEGF, and macrophage inflammatory protein-1β (MIP-1β) and activate osteoclasts. A cell adhesion molecule, CD56 (N-CAM) is expressed in most plasma cells and accounts for myeloma homing and cell adhesion to the marrow. Cell-cell liaison between marrow stromal cells and myeloma cells happens by the help of VCAM-$_1$ and $\alpha_4\beta_1$-integrin and increases the osteoclaststimulating action.[1]Enhanced expression of receptor activator of nuclear factor-kappa B and low level of its decoy receptor by osteoblast causes bone resorption and results in bone lesions.[7]

c) Cell Cycle

There are four regulatory signals mechanizing in the escalation of myeloma cells which are enhanced expression of cyclin D1, hypermethylation of the cyclin-dependent kinase (CDK) pathway, the ras oncogene mutations, and loss of p53 (Hallek et al. 1998). Majority of the data suggests hypermethylation of p15 or p16 is associated with disease progression. K- and N-ras mutations have been described in 25% to 100% of newly diagnosed patients and in one third of the patients causes cyclin D1 expression.[1]Cytogenetics reveal the patients with hyperdiploid multiple myeloma tend to have better forecast than hypodiploid patients.[7]

III. Conventional Treatment

The main treatment goal is to improve the quality of life of the patients and to prolong the duration of non progressive disease.[9] Patients should be refrained from the treatment until they reach the final stage that is symptomatic multiple myeloma. The diagnosis can be done on the bais of manifestation of monoclonal protein in serum or urine and an affirmation of end-organ damagewhich can be identified by assessing abnormalities like hypercalcemia, renal insufficiency, anemia, bone lesions with pathologic fractures. Patients with active multiple myeloma can be classified in two which is high-risk or standard-risk ailment and then subject to the appropriate treatment. Bifunctional alkylating agents like Melphalan and cyclophosphamide are considered as standard therapy for multiple myeloma.[1] Patients who are chosen for stem cell transplantation can be treated with Lenalidomide, an immunomodulatory agent and an aminosustituted variant of Thalidomide. It is given along with dexamethasone.[10]The next option for induction chemotherapy is Bortezomib which is a proteasome inhibitor Velcade. It is combined along with dexamethasone cyclophosphamide or Adriamycin to improve efficacy.[11]Patients are evaluated for response after every treatment cycle. At the time of relapse the autologous stem cell transplantation eligible patients who did not received it with first phase of the treatment should be treated with high dose chemotherapy and then only it should be followed by autologous stem cell transplantation.[12]

IV. Stem Cell Therapy

Stem cells are produced from bone marrow which contains several types of cells like t-cells, B-cells, interferons etc. Mechanism underlying in working of t-cells is that they adhere to cancerous cell surface bind to the cell membrane and effectively damage the cancerous cells through the process like apoptosis and phagocytosis. Hence stem cell therapy becomes crucial in future.[16]The patients receive stem cells intravenously similar to the blood transfusion and this phase takes 1-5 hours. After entering the blood stream through a process called engraftment, the stem cell produces new WBCs, RBCs and platelets. After transplantation engraftment occurs within 2-4 weeks.[18]

The procedure of stem cell transplantation includes conditioning, infusion and monitoring the patient postinfusion. Antibiotics are given orally as a prophylaxis and the main antibiotics given are penicillin and levofloxacin. Acyclovir and fluconazole are also given for prophylaxis. The breakthrough fever should be managed and maintained >38^{o}C, with vancomycin or cefepime. The standard drug is melphalan (200mg/m^2) for competent patients and melphalan (140mg/m^2) if the patient is weak or the serum creatinine is ≥2.0mg/Dl.[22]

a) Auotologous transplant

The treatment with high dose melphalan was complicated by prolonged myelosuppression, and bone marrow support was subsequently indulged. High-dose chemotherapy followed by autologous stem cell transplantation improves the overall response rate and survival. Autologous bone marrow transplantation is replaced by autologous peripheral blood stem cell transplantation because in latter, there is more rapidengraftment and less contamination with myeloma cells. The complete number of CD34+ cells/kg is the most practical and reliable method for identifying the adequacy in collection of stem cell. The mortality rate from autologous stem cell transplantation is currently less than 5%.[1]and it issafer and effective method for elderly and make the patients fit even in the time of novel agents.[14]

b) Transplantation timing

The transplantation timing is very important to be considered in both salvage and consolidation

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therapy.[1]The best timing to undertake Autologous stem cell transplantation is when the patients are having least residual disease and in patients who have not received numerous preliminary chemotherapy treatments.[21]The main advantage of early transplantation is that it circumvents inconvenience and the high cost of chemotherapeutic agents.[1]

i. *Aspect of Purging*

Tumor purging is the process of treating the residual ailments in the patients after they have been undergone with chemotherapy or transplantation. Immunocy to chemical assays and molecular techniques are used in detection and characterisation of tumor contamination.[23] Derivatives of cyclophosphamide or monoclonal antibodies can be used to purging with marrow and it has found to be reliable method. It has a disadvantage that it causes long lasting my elosuppression following after the transplantation.[1]

ii. *Allogenic transplant*

Allogenic transplantation abolishes the defect of contamination with the tumor cell in the stem cells that is inevitable with autologous stem cell transplantation. In those patients with a molecular complete remission, the relapse rate was only 16% in the allogeneic group and 41% in the autologous group. This shows that molecular complete responses are related with a longer relapse-free survival.[1] Reduced intensity allogenic transplantation strategy has been employed to reduce the transplant related mortality (TRM) while retaining the graft versus myeloma effects.[13]Survival status is very low in this case, so it is not deemed as a criterion for both newly diagnosed and relapsed multiple myeloma patients.[15]

c) Donor lymphocyte infusions

After the administration of donor peripheral blood mononuclear cells for relapse after allogeneic transplantation,a graft-versus-myeloma reaction has been found.[1] Donor lymphocyte infusions were used as treatment for multiple myeloma relapse and also as for relapse in multiple myeloma patients who are currently abiding allogenic hematopoietic stem cell transplantation. This strategy induces response rates of 40-52%.Its side effects include bone marrow aplasia, infectious complications, immune escape of plasmocytoma in extramedullary tissues.[17]

d) Non myeloablative Allogeneic Transplant

Allografts have been implicated with lowsurvival despite a significant decrease in the relapse rate and graft-versus-myeloma effects, in nearly all comparisons because of high peritransplantation mortality, late complications of chronic graft-versus-host disease (GVHD), and late infections. Promising approaches include non myeloablative conditioning ("mini") regimens for chosen patients with myeloma, either at the time of relapse or immediately after autologous stem cell transplantation.[1]

V. Drawbacks

Donating involves the use of anesthesia and risk underlying it and in some individuals it causes feeling of stiffness or sore in the part from where bone marrow is taken. Different individuals take variable time to get back to the full strength after the donation. Graft-versus host disease can develop sometimes and it involves formation of skin rash, abdominal pain, hepatitis, jaundice etc.[18]Sometimesthe immune rejection of donor cells by the host immune system is also a major drawback of transplantation.[25]

VI. Conclusion

Stem cell therapy in multiple myeloma is very effective and should be opted as one among the best for both older patients and patients younger than 65 years. Novel drugs can be indulged along with stem cell transplantation in order to improve overall outcome including health related quality of life.[19] the cellular heterogenecity both functional and phenotypical in multiple if rapidly emerging and this can be more promising in the future.[20]In future purging will become a routine practice in order to make sure that no contamination takes place. The culturing of stem cells and their expansion in the laboratories can lead to an increase in the number of transplantations and subsequently increase the rate of cure in patients. [24]

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Review on Mycobacterial Metabolic Pathways as Drug Targets

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Summary- Mycobacterium is acid fast genus of bacteria that include many pathogenic and non pathogenic species. Tuberculosis (TB) is the leading cause of death in the world from a bacterial infectious disease. The emergence of antibiotic resistance strains has raised the need towards the development of new antibiotics or drug molecules which can kill or suppress the growth of pathogenic Mycobacterium species. The increasing emergence of drug-resistant tuberculosis along with the HIV pandemic (human) threatens disease control and highlights both the need to understand how our current drugs work and the need to develop new and more effective drugs. Novel efforts in developing drugs that target the intracellular metabolism of M. tuberculosis often focus on metabolic pathways that are specific to mycobacterium. Potential drug targets were also identified from pathways related to lipid metabolism, carbohydrate metabolism, amino acid metabolism, energy metabolism, vitamin and cofactor biosynthetic pathways and nucleotide metabolism. Approximately one-fourth of the Mycobacterium tuberculosis genome contains genes that encode proteins directly involved in its metabolism. This review provides a brief historical account of tuberculosis drugs, metabolic pathways, examines the problem of current chemotherapy, discusses the targets of current tuberculosis drugs with focuses on some metabolic pathways. The identification of drug target form that unique metabolism of mycobacterium is crutial to to develop new drug for persistent and latent infection of tuberculosis.

Keywords: *anti-tuberculosis agent, drug targets,metabolic pathway, mycobacterium tuberculosis.*

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Review on Mycobacterial Metabolic Pathways as Drug Targets

Ebsa Bushura [α] & Feyera Gemeda Dima [σ]

Summary- Mycobacterium is acid fast genus of bacteria that include many pathogenic and non pathogenic species. Tuberculosis (TB) is the leading cause of death in the world from a bacterial infectious disease. The emergence of antibiotic resistance strains has raised the need towards the development of new antibiotics or drug molecules which can kill or suppress the growth of pathogenic Mycobacterium species. The increasing emergence of drug-resistant tuberculosis along with the HIV pandemic (human) threatens disease control and highlights both the need to understand how our current drugs work and the need to develop new and more effective drugs. Novel efforts in developing drugs that target the intracellular metabolism of M. tuberculosis often focus on metabolic pathways that are specific to mycobacterium. Potential drug targets were also identified from pathways related to lipid metabolism, carbohydrate metabolism, amino acid metabolism, energy metabolism, vitamin and cofactor biosynthetic pathways and nucleotide metabolism. Approximately one-fourth of the Mycobacterium tuberculosis genome contains genes that encode proteins directly involved in its metabolism. This review provides a brief historical account of tuberculosis drugs, metabolic pathways, examines the problem of current chemotherapy, discusses the targets of current tuberculosis drugs with focuses on some metabolic pathways. The identification of drug target form that unique metabolism of mycobacterium is crutial to to develop new drug for persistent and latent infection of tuberculosis.

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I. Introduction

Tuberculosis is a mycobacterium infection that affects a wide range of mammals. Human Tuberculosis is caused by *Mycobacterium tuberculosis (human),* was much more prevalent disease in the past than it is today, and it was responsible for the death of about one billion people during the last two centuries. *Mycobacterium tuberculosis* is a tenacious and remarkably successful pathogen that has latently infected one third of the world population. Each year there are eight million of new tuberculosis (TB) cases and two million deaths (WHO, 2003).

M. bovis is the causative agent of bovine tuberculosis, a chronic and occasionally fatal infectious disease primarily infecting cattle and other livestock; but is capable of infecting a wide range of mammals and other vertebrates, including humans. Bovine tuberculosis causes immense economic loss in many Countries, either from loss of livestock, disease testing, or compensation. Worldwide, agricultural losses are estimated to be around $3 billion a year (WHO, 2012).

M. bovis is very closely related to M. tuberculosis, a virulent tubercle bacillus estimated to infect a third of the world's population and cause the deaths of 1.4 million people each year. In an attempt to prevent tuberculosis infections more than 3 billion individuals have been immunised with M. bovis BCG, a live attenuated derivative of M. bovis (Brosch R et al., 2007).

The increasing emergencies of drug resistance tuberculosis and HIV infection which compromises host defense and allows latent infection to reactivate or render individual more susceptible to TB pose further challenges for effective control of the disease in human(Nachega et al., 2003).

Currently, TB chemotherapy is made up of a cocktail of first-line drugs; isoniazid (INH), rifampin (RIF), pyrazinamide (PZA) and ethambutol (EMB), given for six months (Blumberg, 2003). If the treatment fails as a result of bacterial drug resistance, or intolerance to one or more drugs, second-line drugs are used, such as para-aminosalicylate (PAS), kanamycin (KAN), fluoroquinolones (FQ), capreomycin (CAP), ethionamide (ETA) and cycloserine (CYS), that are generally either less effective or more toxic with serious side effects (Blumberg, 2003). Treatment is made quite difficult by the presence of metabolically silent, persistent or dormant bacteria within host lesions, which are not susceptible to the anti-mycobacterial drugs that usually kill growing bacteria but not persistent bacteria (Zhang, 2004).

Using metabolic pathway information as the starting point for the identification of potential targets has its advantages as each step in the pathway is validated as essential function for the survival of the bacterium (Cole, 2002). It is widely accepted that TB is dynamic disease that result from combination of phenotypically diverse population of bacilli in continually changing host environment. Understanding host-pathogen interactions would give an important clue for developing new drugs, vaccine and diagnostic tests. The release of complete genome sequence of M. tuberculosis has facilitated the development of more rational and specific methods to search for new drug targets and vaccine candidates (Cole et al., 1998).

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The recent rise in TB cases and especially the increase of drug resistant mycobacteria indicate an urgent need to develop new anti-TB drugs. The long duration of TB therapy is a consequence of persistent M. tuberculosis, not effectively killed by current anti-TB agents. Recent advances in the knowledge of the biology of the organism and the availability of the genome sequence give an opportunity to explore a wide range of novel targets for drug design. Metabolic studies on mycobacteria have been important areas of the investigation to identify that metabolic pathway as drug target (Zhang, 2005). But among many pathogenic species of mycobacterium, many researches were done on M.tuberculosis metabolic pathways as drug target.

The objective of this paper is to review on mycobacterium metabolic pathways as drug targets.

II. Metabolic Pathways used as Drug Targets

Mycobacterium metabolic pathways which do not appear in the host but present in the pathogen are identified as pathways unique to mycobacterium as compared to the host. Enzymes in these unique pathways as well as enzymes involved in other metabolic pathways under carbohydrate metabolism, amino acid metabolism, lipid metabolism, energy metabolism, vitamin and cofactor biosynthesis and nucleotide metabolism are important to identify novel drug targets (Kanehisa et al., 2002). An important question to be addressed while choosing potential drug targets is whether the biochemical pathway to be targeted is unique to bacteria. These biochemical pathways which are; Peptidoglycan biosynthesis, Mycobactin biosynthesis, d-alanine metabolism, thiamine metabolism and polyketide sugar unit biosynthesis, all absent in the host and therefore unique to the pathogen M. tuberculosis (Tatusov et al., 2003).Among these pathways, Peptidoglycan biosynthesis and d-alanine metabolism is common to all bacterial species, and cell wall biosynthetic pathways have long been targeted for anti-microbial discovery.

a) Mycobactin biosynthesis

One of the key host defense mechanism is the production of siderocalins that sequester iron-laden siderophores and M. tuberculosis replicates poorly in the absence of these siderophores (de Voss & et al., 2000). To overcome iron deficiency imposed by the host defensive system, bacteria have evolved iron acquisition systems where small molecules called siderophores, which bind extracellular iron, are secreted. These get reabsorbed along with the bound iron through specific cell surface receptors (Braun et al., 1998; Byers and Arceneaux, 1998).

The iron acquisition systems of many pathogenic and saprophytic bacteria mostly rely on the production of small molecules called siderophores. M. tuberculosis produces the mycobactin class of siderophore, which contains a salicylic acid derived moiety. Therefore, this siderophores used for mycobactin biosynthesis, d-alanine metabolism and Peptidoglycan biosynthetic pathways (De Voss et al., 2000). Mycobactin G (Mycobactin lysine-N6-hydroxylase), which catalyzes the hydroxylation of lysine moiety in mycobactin synthesis, is the potential target in this pathway. It has been shown that there is no possibility of bacterial survival on more than a few generations if it is deprived of iron. So to acquire iron from host, it relies on a siderophore mediated pathway (Hider & Kong, 2010). Another reason that why we are so interested in this pathway is that we can see that there is a possibility of lacing the siderophore with the drug. Since there is no siderophore mediate pathway that is important for the host

It is obvious that M. tuberculosis inhabits one of the most hostile environments, the alveolar macrophage. Among the various defensive mechanisms expressed by the host are a potent burst of oxygen derived radical species and a dramatic restriction of available iron to support microbial growth (Kontoghiorghes and Weinberg, 1995). Disruption of mycobactin biosynthetic pathway may affect the survival of the bacterium under these conditions of iron limitation. It has been shown that siderophore production is also important for the virulence of M. tuberculosis. MbtG (Rv2378) is therefore one of the important drug targets found and is also under consideration at the TB Structural Genomics Consortium (James et al., 2000). But some very recent research showed that even though siderophore are unique they are not the only machinery employed by the mycobacterium to acquire iron from vicinity. The mycobacterium can utilize Heme as an Iron source (Christopher & Niederweis, 2011).

An Mtb heme-uptake system has been defined (Tullius, M.V et al., 2011)that consists of the secreted protein Rv0203 and the transmembrane proteins MmpL3 and MmpL11; recent experiments showed that Rv0203 transfers heme to both MmpL3 and MmpL11 during Mtb heme uptake making these proteins potential tar- gets for TB drugs (Owens et al., 2013). Hence this pathway will have to be targeted in conjugation with the main iron acquisition pathway.

b) Peptidoglycan biosynthesis

Mycobacterium is surrounded by a lipid-rich outer capsule that protects it from the toxic radicals and hydrolytic enzymes produced as defense by macrophages (Kolattukudy et al., 1997). The peptidogly can layer of the cell wall serves as a base for the lipid-rich capsule. Peptidogly can or murein is the polymeric mesh of the bacterial cell wall, which plays a critical role in protecting the bacteria against osmotic lysis. The currently used anti-mycobacterial drugs are isoniazid

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(INH) and ethambutol (EMB). Isoniazid is known to inhibit mycolic acid synthesis (Zhang et al., 1992), where as ethambutol inhibits the polymerization step of arabinan biosynthesis of Arabinogalactan (Mikusova et al., 1995).

The primary target of inhibition is the cell wall mycolic acid synthesis pathway, where enoyl ACP (acyl carrier protein)reductase (InhA) was identified as the target of INH inhibitionThe active species for InhA inhibition has been found to be isonicotinic acyl radical, which reacts with NAD to form INH-NAD adduct and then inhibitsthe InhA enzyme. The reactive species produced during INH activation could also cause damage to DNA, carbohydrates, and lipids and inhibit NAD metabolism .Mutations in KatG involved in INH activation in the INH target InhA and Ndh II (NADH dehydrogenase II) (57) could all cause INH resistance. KatG mutation is the major mechanism of INH resistance (Ying Zhangi, 2004). Because INH is a prodrug that requires activation by M. tuberculosis catalase-peroxidase (KatG) to generate a range of reactive oxygen species and reactive organic radicals, which then attack multiple targets in the tubercle bacillus.

c) D-Alanine metabolism

D-alanine is a necessary precursor in the bacterial peptidoglycan biosynthetic pathway. The naturally occurring L-isomer is racemized to its D-form through the action of a class of enzymes called alanine racemases. These enzymes are ubiquitous among prokaryotes and are absent in eukaryotes with a few exceptions making them a logical target for the development of antibiotics. The d-alanine–d-alanine ligase (ddlA) and alanine racemase (alr) from this pathway have no similarity to any of the host proteins. Alanine racemase (alr) has been identified as a target as all the bacteria investigated contained either one or two alanine racemase genes (Strych et al., 2001). However, in mycobacteria, there is a single alanine racemase gene. One alanine racemase inhibitor, the structural d-alanine analogue d-cycloserine has been marketed clinically. Both alanine racemase (Alr) and D-Ala-D-Ala ligase are targets of D-cycloserine, a second-line anti - TB drug. These two enzymes catalyze the first and second committed steps in bacterial peptidoglycan biosynthesis. Alr is a pyridoxal 50-phosphate-containing enzyme that catalyzes the racemization of L-alanine into D-alanine, a major component in the biosynthesis of peptidoglycan (LeMagueres et al., 2005). Although, this is supposed to be an excellent inhibitor of mycobacteria and other pathogenic bacteria species, serious side effects especially CNS toxicity has limited its use (Yew et al., 1993).

d) Polyketide Sugar Unit Biosynthesis

Arabindgalactan a heteropolysacharide is connected via a linker disaccharide, a-L-Rha--a-D-GlcNAc-1 -phosphate, to the sixth position of a muramic acid residue in the peptidoglycan. The reaction is catalyzed: by the enzyme rhamnosyl transferase (Mills et al., 2004) Rhamnose residue and large portion of arabinogalactan polysaccharide are synthesized on GlcNAc-P-P-decaprenyl carrier lipid (Mikusova et al., 1996). The eventual transfer of the arabinogalaetan-Rha-GicNac-phosphate unit to the O-sixth of a muramic acid places the polysaccharide in mass on to the peptidogly can.

The rhamnose-GlcNAc disaccharide is a critical linker which connects arabinogalactan to peptidoglycan via a phosphodiester linkage. L-rhamnose transferase (WbbL) is an enzyme that utilizes dTDP-Rha as a substrate for the formation of final product L-rhamnose which plays a crucial role in the linkage of cell wall. The biosynthesis of dTDP-rhamnose is catalysed by four enzymes coded by the genes; RmlA (Rv0334), RmlB (Rv03464), RmlC (Rv3465) and RmlD (Rv3266) and ultimately synthesizes dTDP rhamnose from glucose-1-phosphate. Among these genes RmlC has no human homologue. RmlC codes for dTDPd-glucose-3, 5-epimerase which is involved in the arabinogalactan biosynthesis. The biosynthesis of arabinogalactan in M. tuberculosis begins with the transfer of N-acetylglucosamine-1-phosphate from UDP-N-acetyl glucosamine to prenylphosphate followed by an addition of rhamnose (Rha) from dTDP-Rha, forming a linker region of the arabinogalactan (=, Ma et al., 2001).

e) Targets from Other Pathways

Even amongst the pathways shared by the host and the pathogen, there are several proteins from pathways involved in lipid metabolism, carbohydrate metabolism, amino acid metabolism, energy metabolism, vitamin and cofactor biosynthetic pathways and nucleotide metabolism which do not bear similarity to host proteins. While some of them are known to be associated with virulence or important for persistence or vital for mycobacterial metabolism, others should further be invetigated for their potential to be drug targets (GIC, 2001).

A significant proportion of the M. tuberculosis genome is devoted to lipid metabolism. It possesses more than 250 enzymes involved in lipid metabolism, which includes enzymes for lipid biosynthesis as well as degradation. Degradation of host cell lipids is essential for the intracellular life of the organism. Host cell membranes provide precursors for many metabolic processes. They are also potential precursors of mycobacterial cell wall constituents through the action of beta oxidative enzymes encoded in multiple copies in the genome. Among these secreted proteins of M. tuberculosis which could act as virulence factors are a series of phospholipases C, lipases and esterases which might attack cellular or vacuolar membranes (The Genome International Consortium, 2001). Notable

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amongst these are phospholipases plcA (Rv2351c), plcB (Rv2350c), plcC (Rv2349c) and serine esterase (Rv2301) (GIC, 2001). The targets cutinases Rv3451 and Rv3452 are news (Anishetty et al., 2005)

The targets aceARv0467, aceAaRv1915, aceAbRv1916, glcBRv1837, Rv2205 identified, are related to mycobacterial persistence. They are enzymes from the glyoxylate by pass, which is important for mycobacterial persistence (e.g., Isocitrate Lyase and Malate Dehydrogenase). It has been proposed by Waynes and Lin (1982) that the enzymes of the glyoxylate cycle are activated during adaptation to the low oxygen environment of the granuloma. The glyoxylate by pass allows the bacterium to synthesize carbohydrates from fatty acids. Succinate and glyoxylate produced by this cycle are supplied to the TCA cycle and gluconeogenesis. Disrupting this pathway by targeting these enzymes has a potential in the treatment of latent tuberculosis infections (Waynes and Lin, 1982).

f) In silico comparative metabolic pathways analysis

In some recent researches five unique pathways, C5-branched dibasic acid metabolism, carbon fixation pathways in prokaryotes, methane metabolism, lipopolysaccharide biosynthesis, and peptidoglycan biosynthesis with 60 new nonhomologous targets, were identified through in silico comparative metabolic pathway analysis of Homo sapiens and M. tuberculosis H37Rv using KEGG database. Pathways which are not present in the Homo sapiens but present in the Mycobacterium are designated as unique pathways. Design and targeting inhibitors against these nonhomologous sequences could be the better approach for generation of new drugs. Thus total 5 unique metabolic pathways have been taken in M. tuberculosis (Asad Amir et al., 2014)

i. *Identification of unique pathways and potential drug*

No new anti-tuberculosis drugs have been developed for well over 20 years. In view of the increasing development of resistance to the current leading anti-tuberculosis drugs, novel strategies are desperately needed to avert the "global catastrophe" forecast by the WHO. Therefore, computational approach for drug targets identification, specifically for M. tuberculosis, can produce a list of reliable targets very rapidly. These methods have the advantage of speed and low cost and, even more importantly, provide a systems view of the whole microbe at a time. Since it is generally believed that the genomes of bacteria contain genes both with and without homologues to the host (WHO, 2005).

ii. *Identification of essential genes*

Essential genes are those indispensable for the survival of an organism, and their functions are considered as foundation of life. Total 55 enzymes out of all were found to be essential for M. tuberculosis life cycle. These targets were found to be potential targets and could be considered for rational drug design. Using metabolic pathway information as the starting point for the identification of potential targets has its advantages as each step in the pathway is validated as the essential function for the survival of the bacterium (Lamichhane et al., 2003).

iii. *Identification of drug target's functions using UniProt (Universal Protein Resource).*

The sub cellular localization analysis of all supposed essential and unique enzymes of M. tuberculosis were evaluated by UniProt server. As it was suggested that, membrane associated protein could be the better target for developing vaccines. After functional analysis unique enzymes involved in cellular components like cell wall, cytoplasm, extra cellular region, plasma membrane, and so forth, their biological processes and their functions have been retrieved (Asad Amir et al, 2014). Further, the functional analysis using Uniprot showed involvement of all the unique enzymes in the different cellular components (Asad Amir et al., 2014)

III. Possible Drug Targets

Desirable targets should be involved in vital aspects of bacterial growth, metabolism and viability, whose inactivation will lead to bacterial death or inability to persist (sunny. j et al., 2013). In recent years, a number of new genes and their products in M. tuberculosis have been identified, which can be possible drug targets for tuberculosis. The gene products that control vital aspects of mycobacterial physiology like, metabolism, persistence, virulence, two component system and cell wall synthesis would be attractive targets for new drugs. A large number of genes are being studied in the search for new drug targets using various approaches (chopra et al., 2002).

Because of the drug-resistant TB problem, it is important to develop new drugs that inhibit novel targets that are different from those of currently used drugs. To avoid significant toxicity, the targets of inhibition should be present in bacteria but not in the human host. Although modification of existing drugs for improved half-life, bioavailability, or drug delivery may be of some use, agents obtained by this approach may have a cross-resistance problem. Similarly, targeting existing TB drug targets for drug development may be limited value because of potential cross-resistance (Chopra I, 2002).

New drugs that inhibit novel targets are needed. In choosing targets for drug development, it is important that they be involved in vital aspects of bacterial growth, metabolism, and viability. Recent developments in mycobacterial molecular genetics tools such as transposon mutagenesis, signature-tagged muta genesis, gene knockout, and gene transfer will facilitate the identification and validation of new drug targets

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essential for the survival and persistence of tubercle bacilli not only in vitro but also in vivo. Below is a list of potential targets where by new drugs may be developed for improved treatment of TB (Zhang et al., 2005).

a) Targeting Mycobacterial persistence

Mycobacterial persistence refers to the ability of tubercle bacillus to survive in the face of chemotherapy and/or immunity (McDermott, 2000). The nature of the persistent bacteria is unclear but might consist of stationary phase bacteria, post-chemotherapy residual survivors and/or dormant bacteria that do not form colonies upon plating (Zhang, 2004). The presence of such persistent bacteria is considered to be the major reason for lengthy therapy. A lot of research activity is currently aimed at understanding the biology of persistence of the tubercle bacillus and developing new drugs that target the persistent bacteria (GAFTDD, 2001).

ISOCITRASE LYASE (ICL) Gene products involved in mycobacterial persistence (Mckinney et al., 2000). ICL catalyzes the conversion of isocitrate to glyoxylate and succinate and is an essential enzyme for fatty acid metabolism in the glyoxylate shunt pathway. Survival of M. tuberculosis in the adverse in vivo environment requires utilization of C2 substrates (generated by β-oxidation of fatty acids) as the carbon source (Chen B et al., 2000). ICL was induced in the Wayne "dormancy" model, inside macrophages and in the lesions of the human lung (166). ICL is not essential for the viability of tubercle bacilli in normal culture or in hypoxic conditions, but it is needed for long-term persistence in mice

Pca A (PROXIMAL CYCLOPROPANATION OF ALPHA-MYCOLATES) Using a transposon mutagenesis approach based on changes in colony morphology, a gene called pcaA encoding a novel methyl transferase involved in the modification of mycolic acids in mycobacterial cell wall was identified (Ying Zhang et al., 2005). Although the PcaA knockout mutant grew normally in vitro and replicated in mice initially like the parent strain, the mutant was defective in persisting in mice and could be a target for drug design against persistent bacilli (Glickman et al., 2000) the stringent response induced by starvation is mediated by the signaling molecule hyperphosphorylated guanine (ppGpp) synthesized by RelA (ppGpp synthase I) RelA (ppGpp synthase) (Dahl et al., 2003). In subsequent studies aimed at characterizing mycobacterium genes that are induced in the Wayne "dormancy" model, the same two-component system was
Identified by microarray analysis and named Rv3133c/Rv3132c.

DosR-Rv3133/DevR-DevS The two-component system DevR-DevSwas initially identified as being preferentially expressed in virulent M. tuberculosis strain H37Rv over that in avirulent strain H37Ra in a subtractive hybridization analysisInactivation of DosR abolished the rapid induction of hypoxia-induced gene expression,suggesting that DosR is a key regulator in the hypoxia-induced mycobacterial "dormancy" response.The DosR mutant grew as well as the wild-type strain initially in five-day incubation, but it survived significantly less well upon extended incubation up to 40 days in the Wayne model (Boon C and Dick T. 2002).A recent microarray study has found that DosR controls the expression of a 48-gene "dormancy regulon," which is induced under hypoxic conditions and by nitric oxide (NO) .DosR could be a good target for developing drugs against persisters(Ying Zhang et al., 2005). DosR (controlling a 48-gene regulon involved in mycobacterial survival under hypoxic conditions) have been identified and could be good targets for the development of drugs that target persistent bacilli (Park et al., 2003).

Rv2421c transfers phosphorous groups in nicotinate/nicotinamide salvage and de novo synthesis. Rv2043c of this pathway is the target of the highly effective drug PZA that kills persistent bacilli in the initial phase of TB therapy. Mutations in the encoding gene pncA confer resistance to PZA. Successful inhibition of Rv2421c could thus help to eradicate slowly growing persistent bacilli in TB infection (Cloete et al., 2016).

b) Targeting essential Genes

Essential genes are genes whose inactivation leads to non-viability or death of the bacteria. Transposon mutagenesis and signature-tagged mutagenesis have been used to identify genes essential for M. tuberculosis growth in vitro and survival in vivo. In a recent study, 614 genes, about one-sixth of the total number of genes in M. tuberculosis, were found to be essential for in vitro growth, whereas 194 genes were demonstrated to be essential for in vivo survival in mice (Sassetti et al., 2003).

The genes that are essential for survival in vitro and in vivo are grouped into the following categories: lipid metabolism; carbohydrate and amino acid transport and metabolism; inorganic ion transport and metabolism; nucleotide transport and metabolism; energy production and conversion; secretion; cell envelope biogenesis; cell division; DNA replication; recombination and repair; transcription and translation; post-translational modification; chaperones; coenzyme metabolism; and signal transduction (Lamichhane et al., 2003).

However, the function of a significant number of essential genes is unknown. Besides systematic analysis of essential genes by transposon mutagenesis, targeted knockout of specific genes is also a valuable approach to identifying essential genes, in other words, those whose disruption leads to non-viability of the bacilli. These essential mycobacterial genes should be good targets for TB drug development (Zhang et al., 2003).

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c) *Targeting energy production pathway*

All bacteria require energy to remain viable. Although the energy production pathways in M. tuberculosis are not well characterized, their importance as drug targets is demonstrated by the recent finding that PZA (a frontline TB drug that is more active against non-growing persistent bacilli than growing bacilli and shortens TB therapy) acts by disrupting membrane potential and depleting energy in M. tuberculosis. This study implies that energy production or maintenance is important for the viability of persistent non-growing tubercle bacilli in vivo. The recent discovery of the highly effective TB drug diarylquinoline also highlights the importance of energy production pathways for mycobacteria. It is likely that energy production pathways, such as the electron transport chain, glycolytic pathways (like the Embden–Meyerhof pathway) and fermentation pathways, could be good targets for TB drug development (Zhang et al., 2003). Isocitrate lyase (ICL) is an important enzyme in this category and also an important drug target. ICL is involved in energy production via the metabolism of acetyl-CoA and propionial CoA of the glyoxilate pathway. Inactivation of the icl gene leads to attenuation of both persistent and virulent strains of M. tuberculosis. Five candidates, Rv2984, Rv2194, Rv1311, Rv1305 and Rv2195, map to the oxidative phosphorylation pathway. The target Rv1854c (gene ndh) in this pathway is the target for INH and several mutations in this gene account for INH resistant cases. Inhibiting any of the five proposed targets could disrupt the pathway and eliminate M. tuberculosis by reducing its limited ATP availability during dormancy (Cloete et al., 2016).

d) *Targeting virulence factors*

A number of genes have been identified, using different techniques like allelic exchange, signature tagged mutagenesis, and anti-sense RNA, that show a role in the virulence of M. tuberculosis. Some of these genes include, Cell Envelope Protein erp (Rv3810)Exported repetitive protein erp (extracellular repeat protein), which has been shown to be essential for the multiplication of mycobacteria during the acute phase of infection in the mouse model. The most important point is that this gene has no homologues in other organisms, making it an attractive drug target. Recently, two gene clusters were identified and shown to be important for the growth of mycobacteria in the lungs during the early phase of infection. This gene cluster is involved in the synthesis (fadD28) and export (mmpL7) of a complex cell wall associated lipid, phthiocerol dimycocerosate (Barrett et al., 1998).

The approach of targeting virulence factors, like other approaches suffers from some serious drawbacks, like virulence factors may not be necessarily survival genes. Therefore, inhibition of virulence factors may not be lethal to the pathogen. The other very important hurdle in this approach is that drugs that target virulence factors may be of very little or of no use if the disease has already been established. However, inhibitors of these virulence gene products may be used in combination with existing drugs to improve the regime of chemotherapy (Alksne et al., 2000).

e) *Targeting two-component systems*

Mycobacterial disease is characterized by the lack of involvement of classical virulence factors; rather a dynamic balance between host and pathogen defines the outcome of an infection. Therefore those mycobacterial genes that confer an advantage to the organism in this ongoing battle would qualify as virulence factors. Infection of macrophages constitutes an early stage in the host pathogen encounter. Obvious candidates among M. tuberculosis genes that can mastermind the intracellular survival and multiplication within macrophages as also the shutdown of mycobacteria during persistence are signal transduction systems, in particular TCS. Therefore in vitro infection models have been used extensively to delineate the role of TCS during the stage of pathogen macrophage interaction. Animal models have also been used to study the effect of defined mutations in TCS on growth and virulence of the mycobacterial strains (Jaya Sivaswam et al., 2004)

Two-component systems (TCS) are vital components of signal transduction systems in a number of organisms. It consists of a sensor kinase that senses external signals and transmits the signals to the response regulator. The response regulator interacts with transcription factors which in turn will switch on/off a number of genes (Hoch, 2000).The mycobacterial genome encodes several two-component systems, which consist of histidine kinases and their associated response regulators. These control the expression of target genes in response to stimuli that are involved in chemotaxis, phototaxis, osmosis, nitrogen fixation and intracellular survival (Stock, A. M et al, 2000). The histidine kinases from various bacteria also present novel targets for the development of new kinase inhibitors. MtrA (Rv3246c) (magnesium transporter) and SenX3, histidine kinases that are essential for mycobacterial virulence and persistence in mice, could also be good targets for the development of new drugs for persistent TB bacteria (Parish, T et al, 2003).

Mycobacterium tuberculosis has shown the presence of at least 12 two-component system homologues with 8 unlinked sensor kinases or response regulators. However, the exact physiological role of most of these proteins is far from being understood. It has been shown that the inactivation of mtrA component of mtrA-mtrB complex of M. tuberculosis H37Rv was possible only in the presence of a functional copy of mtrA, suggesting that this response regulator is

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essential for the viability of M. tuberculosis (Zahrt et al., 2000).

Interestingly, another two-component system, devR-devS, was found to be over expressed in a virulent strain, H37Rv (Dasgupta et al., 2000). Disruption of the phoP component of the PhoP/PhoR in M. tuberculosis resulted in a mutant strain with impaired multiplication in the host. This mutant was also found to be attenuated in vivo in a mouse model, suggesting that PhoP is required for intracellular growth of M. tuberculosis. These observations collectively suggest that TCS in M. tuberculosis could be important drug targets (Perez et al., 2001).

Although the inhibitors that were used in this study— for example, staurosporine were relatively non-selective, some recent researches provide the first indication that protein kinases might be important in regulating the entry and phagocytosis of mycobacteria in macrophages. Subsequently, a small-molecule kinase inhibitor-1-(5-isoquinolinesulphonyl)-2-ethylpiperazine,a sulphonyl compound belonging to the H-series was found to inhibit in vitro growth of M. Bovis BCG, and also inhibited the kinase activity of the M. tuberculosis kinase PknB (Drews,S.J et al., 2001). As PknA, PknB and PknG are required for the growth of mycobacteria in vitro; any compound that specifically blocks these kinases might be a potential candidate for a new antimycobacterial agent (Young, T. et al., 2003).

f) Targeting cell wall synthesis

The mycobacterial cell wall is a complex structure that is required for cell growth, resistance to antibiotics and virulence (Bansal-Mutalik, R. & Nikaido, H, 2014) It is composed of three distinct macromolecules; peptidoglycan, arabinogalactan and mycolic acids — which are surrounded by a non-covalently linked outer capsule of proteins and polysaccharides (Sani, M. et al., 2010) The outermost, the mycolic acids (Brennan et al., 1995) are 70 to 90 carbon-containing, branched fatty acids which form an outer lipid layer in some way similar to the classical outer membrane of gram-negative bacteria (Brennan et al., 1968). Mycolic acids are strong hydrophobic molecules oriented perpendicular to the plane of the membrane and provide a special lipid barrier responsible for many physiological and disease inducing aspects of Mtb.

They are thought to be significant determinant of virulence in Mtb. Probably they prevent attack of mycobacteria by cationic proteins, lysozyme and oxygen radicals in the phagocytic granule. The mycolic acids are esterified to the middle component, arabinogalactan (AG), a polymer composed primarily of Dgalactofuranosyl and D-arabinofuranosyl residues. AG is connected via a linker disaccharide, a-L-rhamnosyl-(1-3)-a-D-A/-acetyl-glucosaminosyl-1 -phosphate, to the sixth position of a muramic acid residue of peptidoglycan (PG) (Me Neil and Brennan 1990) which is the innermost of the three cell wall core macromolecules.A complex consisting of mycolic acids, arabinogalactan and peptidoglycan constitutes “the core” of the cell wall (Crick et al., 2001) which is often referred as “mycolyl-arabinogalactan-peptidoglycan” (MGP) complex. This covalently linked structure is intercalated with numerous glycolipids such as lipoarabinomannan (LAM), the phosphatidyl inositol containing mannosides (PIMs), trehalose dimycolate (TDM; so-called cord factor), trehalose-monomycolate (TMM), which play an important role in virulence of Mtb (Glickman and Jocob 2001). The dominating heteropolysacharide LAM is noncovalently attached to cell wall and may be anchored to the cytoplasmic membrane via phophatidyl-myo-inositol (pi) unit.

The cell wall is the most common target of antituberculosis drugs, and many compounds that are in clinical use or under development target enzymes that synthesize distinct layers of the cell wall(Karen J. Kieser &Eric J. Rubin, 2014) Mycobacteria including M. tuberculosis have a unique cell wall structure. A variety of unique lipids like lipoarabinomannan (LAM), trehalose dimycolate, and phthiocerol dimycocerate which form non covalent anchorage with the cell membrane have been documented to play an important role in the virulence of M. tuberculosis (Glickman et al., 2001).

Lipids such as cord factor have been suggested to play an important role in the virulence of M. tuberculosis by inducing cytokine mediated events. LAM is also a major constituent of the mycobacterial cell wall and has been shown to induce TNF release from the macrophages which plays a significant role in bacterial killing (Puneet Chopra, 2003).

Because of the reasons cited above, genes involved in cell wall synthesis of mycobacteria have been exploited as targets for many anti-mycobacterial drugs. Several important TB drugs such as INH, ETA and EMB target mycobacterial cell wall synthesis. Enzymes involved in this pathway have always been preferred targets in drug development efforts (SchullerLevis et al., 1994).

Thiolactomycin (TLM) targets two β-ketoacyl-acyl-carrier protein synthases, KasA and KasB enzymes that belong to the fatty acid synthase type II system involved in the fatty acid and mycolic acid biosynthesis, (Puneet Chopra, 2003). TLM has also been shown to be active against MDR-TB clinical isolate. Several TLM derivatives have been found to be more potent in vitro against fatty acid and mycolic acid biosynthesis (Zhang et al., 2002).

Cerulenin, an inhibitor of fatty acid synthesis, has also been shown to inhibit mycobacterial lipid synthesis and is active against M. tuberculosis in vitro with an MIC of 1.5-12.5 mg/ml (Parrish et al., 1999).

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Octane sulphonyl acetamide (OSA) has recently been identified as an inhibitor of fatty acid and mycolic acid biosynthesis in mycobacteria. The inhibitor was found to be active against both slow growers such as M. tuberculosis and also MDR-TB strains with a MIC of about 6.25-12.5mg/ml. These reports clearly suggest that several genes of the cell wall synthesis pathway and enzymes involved in fatty acid and mycolic acid synthesis could be good candidates for further drug development (Jones et al., 2000).

g) Genes of other metabolic pathways

Genes of some other metabolic pathways can also serve as possible targets for developing drugs against tuberculosis. Some of these genes include, mgtc, which codes for a putative Mg^{+2} transporter protein. This protein has been shown to be essential for the survival of mycobacteria both in macrophages and mice. The Δ-mgtc mutant showed in vitro growth defects (Buchmeier et al., 2000).

Similarly Δ-mbtB mutant deficient in synthesis of siderophores was unable to replicate within the macrophages. Failure of mycobacteria to survive in the absence of specific iron uptake system suggests the scarcity of this important nutrient in phagosomal environment. Members of PE-PGRS family of proteins that are highly expressed within tissue granulomas have been shown to be essential for the virulence of mycobacteria. Therefore, the members of this category of genes also constitute potential drug targets (De Voss et al., 2002).

h) TB genomics and drug targets

The first bacterial genome was sequenced by Fleischmann and colleagues at The Institute for Genomic Research (TIGR) in 1995 (Fleischmann et al., 1995). So far, more than 100 bacterial genomes have been sequenced. As bacterial genome sequences become available, there is increasing interest in developing new antibacterial agents using genomics-based approaches (Dougherty et al., 2002).

The complete genome sequence of M. tuberculosis provides an opportunity for a more focused and planned approach towards the identification of new drug targets (Puneet Chopra et al., 2003). Genome sequence helps in compilation of all the potential gene products encoded by a particular organism, identification of functions (enzymes and pathways) that are missing or unique in a particular organism, and finally identifying the genes that are common to all or most prokaryotes and eukaryotes (S Gerdes , 2011).

The common targets can then be over expressed for biochemical assays in drug screens or structure determination, to be used in the drug design. So far, however, no company has been successful in developing a drug using a genomics approach. The availability of the M. tuberculosis genome sequence (Cole et al., 1998) opens up a new opportunity to understand the biology of the organism and provides a range of potential drug targets (Cole, 2002).

The recent developments in microarray technology, signature tag mutagenesis , mycobacterial transposon mutagenesis and gene knock-out technology provide important tools to identify new drug targets. Microarray has been used to identify M. tuberculosis genes that are induced by INH and ETH (Wilson et al., 1999), and by INH, TLM and triclosan (Betts et al., 2003). Microarray was also used to identify genes that are switched on in the Wayne “dormancy” model under hypoxic and nitric oxide stress conditions (Voskuil et al., 2003), a discovery that led to the identification of a 48-gene “dormancy regulon” controlled by DosR (Voskuil et al., 2003).

A proteomic approach was used to identify potential proteins that are induced in starvation as an in vitro model of persistence (Betts et al., 2002). Two unique M. tuberculosis proteins with homology to each other were identified: Rv2557 and Rv2558. Rv2557 were also induced in side granulomatous lesions in the lung. Genes identified by microarray analysis or proteins identified by a proteomic approach should be further validated as potential drug targets by gene knockout and in vivo testing in mice before they are selected as targets for drug development (Fenhalls et al., 2002).

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Table 1: Current tuberculosis drugs and their targets

Drugs	MIC (g/ml)	Mechanism of action	Targets	Gene involved in resistance
Isoniazid	0.01-0.20	Inhibition of cell wall Mycoli acid synthesis	Enoyl acyl carrier protein reductase (InhA)	KatG, inhA
Rifampin	0.05 - 0.50	Inhibition of RNA synthesis	RNA polymerase, subunit	rpoB
Pyrazinamide	20 -100	Depletion of membrane energy	Membrane energy metabolism	pncA
Ethambutol	1-5	Inhibion of cell wall arabinogalactan synthesis	Arabinsyltransferase	embCA
Streptomycin	2-8	Inhibition of protein synthesis	Ribosomal S12 protein & 16s rRNA	rpcl, rrs
Kanamycin	1-8	Nhibition of protein synthesis	16s rRNA	Rrs
Capreomycin	4	Inhibition of protein synthesis	16s rRNA, 50s ribosome, rRNA Methyl transferase (TlyA)	
Fluoroquinolone	0.2-4.0	Inhibition of DNA synthesis	DNA gyrase	gyrA,gyrB
Ethionamide	0.6-2.5	Inhibition of mycolic acid	Acyl carrier protein reductase (inhA)	inhA,etaA/ethA
PAS	1-8	Inhibition of folate pathway and mycobactin synthesis	Thymidylatesynthase(TlyA)	

Source; (Maus et al., 2005)

IV. Anti-Tuberculosis Drugs in Current Clinical Practice

As described recently the chemotherapy of tuberculosis has much evolved along the years since it started with the introduction of streptomycin 1946. By 1955, the combination of streptomycin, para-aminosalicylic acid and isoniazid was adopted as a standard treatment by the western world (Datta et al., 1993).More recent clinical data suggest using at least five regimens of adequate anti-tuberculosis drugs (Mirsaeidi et al., 2005). The choice of these drugs will be driven by the actual or presumed (in view of past failed treatment) resistance characteristic of the strains of M. tuberculosis considered. In order of preference they can be chosen from the following.

- In any case, the first line agents still active on the patient: isoniazid, rifampin, pyrazinamide and ethambutol.
- This is followed by the group of injectable drugs: streptomycin, kanamycin, amikacin, capreomycin or viomycin/tuberactinomycin B and the related tuberactinomycins A, N and O.
- One of the many related antibacterial fluoroquinolones such as ciprofloxacin, ofloxacin, levofloxacin or the more recent sparfloxacin, gatifloxacin, moxifloxacin and sitafloxacin should be included in the regimen. This class of antibiotics has now been proven as indispensable treatment for MDR tuberculosis (Chan et al., 2004).
- Second line bacteriostatics, with established clinical efficacy, usually have more important side effects (Newton et al., 1975). They are para-aminosalicylic acid, ethionamide (the propyl analogue prothionamide is also used) and cycloserine.
- Other drugs are also considered. Their use is the subject of debate and only time and proper observations will provide the necessary data. Clofazimine is among these compounds and is also used against M. leprae. The combination of amoxicillin and the penicillinase inhibitor clavulanic acid has an anti-mycobacterial effect in vitro. The same is true for clarithromycin although its clinical efficacy remains to be established (Forget et al., 2006).

a) Status of current tuberculosis drug therapy

The current live vaccine Bacillus Calmette Gurein (BCG) attenuated strain of M bovis was introduced in 1922. It does not protect all age groups as its efficacy is globally variable, and it does not provide protection in most parts of the world where TB is effectively prevalent. It is not suitable to use for immune compromised patients. In addition to this, BCG only reduces dissemination of Mtb to the spleen and other organs, but it does not prevent mycobacterial growth in the lungs (Andrea M. and Shabaana A, 2009).

Current TB therapy, also known as DOTS (directly observed treatment, short-course) consists of an initial phase of treatment with 4 drugs, INH, RIF, PZA and EMB, for 2 months daily, followed by treatment with INH and RIF for another 4 months, three times a week(WHO,2000). The targets of these drugs are varied. INH inhibits synthesis of mycolic acid, a cell well component (PZA targets cell membrane whereas rifampin and streptomycin interferes with the initiation and streptomycin interferes with the initiation of RNA and protein synthesis respectively EMB blocks biosynthesis of arabinogalactan, a major polysaccharide present in the mycobacterial cell wall and kanamycin and capreomycin, like streptomycin, inhibit protein synthesis through modification of ribosomal structures at the 16S rRNA (Zhang et al., 2000). Cycloserine prevents the synthesis of peptidoglycan, a constituent of cell wall (Puneet Chopra, 2003).

b) Limitation of current tuberculosis therapy

In the present scenario, due to the emergence of multi drug resistant tuberculosis (MDR-TB) and association between immune compromising disease and TB, DOTS is becoming rapidly ineffective in

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controlling tuberculosis in human. Recent reports indicate that, areas where there is a high incidence of MDR-TB, DOTS is failing to control the disease. In such circumstances, the second line drugs are prescribed in combination with DOTS. However, this combination of drugs is very expensive, has to be administered for a longer duration and has significant side effects. One major drawback of current TB therapy is that the drugs are administered for at least 6 months (Kimerling et al., 1999).

The length of therapy makes patient compliance difficult, and such patients become potent source of drug-resistant strains. The second major and serious problem of current therapy is that most of the TB drugs available today are ineffective against persistent bacilli, except for RIF and PZA. RIF is active against both actively growing and slow metabolizing non-growing bacilli, whereas PZA is active against semi-dormant non-growing bacilli. However, there are still persistent bacterial populations that are not killed by any of the available TB drugs. Therefore, there is a need to design new drugs that are more active against slowly growing or non-growing persistent bacilli to treat the population at risk (Zhang et al., 2002)

V. Conclusion and Recommendations

Tuberculosis is still a leading infectious disease worldwide. Along with the socio-economic and host factors that underlie this problem, a fundamental problem that hinders more effective TB control is the tenacious ability of Mycobacterium bacteria to persist in the host and to develop drug resistance, often as a consequence of poor compliance to lengthy therapy. Major obstacle in the cure and prevention of tuberculosis is posed by the latent or persistent mycobacterium infection. This is due to the fact that most of the currently available drugs are ineffective against latent infection. A better understanding on the physiology of mycobacteria during the latent period will help in the identification of new drug targets that can act on the persistent mycobacteria. The list of potential drug targets encoded in the genome of M. tuberculosis include genes involved in persistence or latency, cell wall synthesis, virulence, signal transduction, genes encoding transcription factors and enzymes of other metabolic pathways. Identification of these targets will to produce new drugs against tuberculosis that will overcome the limitations of existing drugs such as, prolonged chemotherapy, failure against persistent infection and multidrug resistance.

Based on above conclusion the following recommendations are forwarded

- The lists of potential drug targets encoded in the genome of M. tuberculosis should be explored to identify new drug against tuberculosis that will overcome the limitation of existing drugs.
- Research should involve testing new or reformulated drug, combination of different drugs to shorten therapy, supplementation and enhancements of existing drugs.
- The existing (currently in use) drugs should be modified because of continuous development of drug resistance.
- TB drugs should be tested and combined with different drugs to shorten therapy, to reduce toxicity and to enhance its activity.
- More research should be conducted on molecular targets of Mycobacterium
- Researcher should actively participate in finding better and more effective drugs that reduce time of treatment and less toxic

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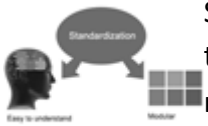

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A major lynchpin of research work for the writing of research papers is the keyword search, which one will employ to find both library and internet resources. Up to eleven keywords or very brief phrases have to be given to help data retrieval, mining, and indexing.

One must be persistent and creative in using keywords. An effective keyword search requires a strategy: planning of a list of possible keywords and phrases to try.

Choice of the main keywords is the first tool of writing a research paper. Research paper writing is an art. Keyword search should be as strategic as possible.

One should start brainstorming lists of potential keywords before even beginning searching. Think about the most important concepts related to research work. Ask, “What words would a source have to include to be truly valuable in a research paper?” Then consider synonyms for the important words.

It may take the discovery of only one important paper to steer in the right keyword direction because, in most databases, the keywords under which a research paper is abstracted are listed with the paper.

Numerical Methods

Numerical methods used should be transparent and, where appropriate, supported by references.

Abbreviations

Authors must list all the abbreviations used in the paper at the end of the paper or in a separate table before using them.

Formulas and equations

Authors are advised to submit any mathematical equation using either MathJax, KaTeX, or LaTeX, or in a very high-quality image.

Tables, Figures, and Figure Legends

Tables: Tables should be cautiously designed, uncrowned, and include only essential data. Each must have an Arabic number, e.g., Table 4, a self-explanatory caption, and be on a separate sheet. Authors must submit tables in an editable format and not as images. References to these tables (if any) must be mentioned accurately.

Figures

Figures are supposed to be submitted as separate files. Always include a citation in the text for each figure using Arabic numbers, e.g., Fig. 4. Artwork must be submitted online in vector electronic form or by emailing it.

Preparation of Eletronic Figures for Publication

Although low-quality images are sufficient for review purposes, print publication requires high-quality images to prevent the final product being blurred or fuzzy. Submit (possibly by e-mail) EPS (line art) or TIFF (halftone/ photographs) files only. MS PowerPoint and Word Graphics are unsuitable for printed pictures. Avoid using pixel-oriented software. Scans (TIFF only) should have a resolution of at least 350 dpi (halftone) or 700 to 1100 dpi (line drawings). Please give the data for figures in black and white or submit a Color Work Agreement form. EPS files must be saved with fonts embedded (and with a TIFF preview, if possible).

For scanned images, the scanning resolution at final image size ought to be as follows to ensure good reproduction: line art: >650 dpi; halftones (including gel photographs): >350 dpi; figures containing both halftone and line images: >650 dpi.

Color charges: Authors are advised to pay the full cost for the reproduction of their color artwork. Hence, please note that if there is color artwork in your manuscript when it is accepted for publication, we would require you to complete and return a Color Work Agreement form before your paper can be published. Also, you can email your editor to remove the color fee after acceptance of the paper.

Tips for writing a good quality Medical Research Paper

1. Choosing the topic: In most cases, the topic is selected by the interests of the author, but it can also be suggested by the guides. You can have several topics, and then judge which you are most comfortable with. This may be done by asking several questions of yourself, like "Will I be able to carry out a search in this area? Will I find all necessary resources to accomplish the search? Will I be able to find all information in this field area?" If the answer to this type of question is "yes," then you ought to choose that topic. In most cases, you may have to conduct surveys and visit several places. Also, you might have to do a lot of work to find all the rises and falls of the various data on that subject. Sometimes, detailed information plays a vital role, instead of short information. Evaluators are human: The first thing to remember is that evaluators are also human beings. They are not only meant for rejecting a paper. They are here to evaluate your paper. So present your best aspect.

2. Think like evaluators: If you are in confusion or getting demotivated because your paper may not be accepted by the evaluators, then think, and try to evaluate your paper like an evaluator. Try to understand what an evaluator wants in your research paper, and you will automatically have your answer. Make blueprints of paper: The outline is the plan or framework that will help you to arrange your thoughts. It will make your paper logical. But remember that all points of your outline must be related to the topic you have chosen.

3. Ask your guides: If you are having any difficulty with your research, then do not hesitate to share your difficulty with your guide (if you have one). They will surely help you out and resolve your doubts. If you can't clarify what exactly you require for your work, then ask your supervisor to help you with an alternative. He or she might also provide you with a list of essential readings.

4. Use of computer is recommended: As you are doing research in the field of medical research then this point is quite obvious. Use right software: Always use good quality software packages. If you are not capable of judging good software, then you can lose the quality of your paper unknowingly. There are various programs available to help you which you can get through the internet.

5. Use the internet for help: An excellent start for your paper is using Google. It is a wondrous search engine, where you can have your doubts resolved. You may also read some answers for the frequent question of how to write your research paper or find a model research paper. You can download books from the internet. If you have all the required books, place importance on reading, selecting, and analyzing the specified information. Then sketch out your research paper. Use big pictures: You may use encyclopedias like Wikipedia to get pictures with the best resolution. At Global Journals, you should strictly follow here.

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6. Bookmarks are useful: When you read any book or magazine, you generally use bookmarks, right? It is a good habit which helps to not lose your continuity. You should always use bookmarks while searching on the internet also, which will make your search easier.

7. Revise what you wrote: When you write anything, always read it, summarize it, and then finalize it.

8. Make every effort: Make every effort to mention what you are going to write in your paper. That means always have a good start. Try to mention everything in the introduction—what is the need for a particular research paper. Polish your work with good writing skills and always give an evaluator what he wants. Make backups: When you are going to do any important thing like making a research paper, you should always have backup copies of it either on your computer or on paper. This protects you from losing any portion of your important data.

9. Produce good diagrams of your own: Always try to include good charts or diagrams in your paper to improve quality. Using several unnecessary diagrams will degrade the quality of your paper by creating a hodgepodge. So always try to include diagrams which were made by you to improve the readability of your paper. Use of direct quotes: When you do research relevant to literature, history, or current affairs, then use of quotes becomes essential, but if the study is relevant to science, use of quotes is not preferable.

10. Use proper verb tense: Use proper verb tenses in your paper. Use past tense to present those events that have happened. Use present tense to indicate events that are going on. Use future tense to indicate events that will happen in the future. Use of wrong tenses will confuse the evaluator. Avoid sentences that are incomplete.

11. Pick a good study spot: Always try to pick a spot for your research which is quiet. Not every spot is good for studying.

12. Know what you know: Always try to know what you know by making objectives, otherwise you will be confused and unable to achieve your target.

13. Use good grammar: Always use good grammar and words that will have a positive impact on the evaluator; use of good vocabulary does not mean using tough words which the evaluator has to find in a dictionary. Do not fragment sentences. Eliminate one-word sentences. Do not ever use a big word when a smaller one would suffice.

Verbs have to be in agreement with their subjects. In a research paper, do not start sentences with conjunctions or finish them with prepositions. When writing formally, it is advisable to never split an infinitive because someone will (wrongly) complain. Avoid clichés like a disease. Always shun irritating alliteration. Use language which is simple and straightforward. Put together a neat summary.

14. Arrangement of information: Each section of the main body should start with an opening sentence, and there should be a changeover at the end of the section. Give only valid and powerful arguments for your topic. You may also maintain your arguments with records.

15. Never start at the last minute: Always allow enough time for research work. Leaving everything to the last minute will degrade your paper and spoil your work.

16. Multitasking in research is not good: Doing several things at the same time is a bad habit in the case of research activity. Research is an area where everything has a particular time slot. Divide your research work into parts, and do a particular part in a particular time slot.

17. Never copy others' work: Never copy others' work and give it your name because if the evaluator has seen it anywhere, you will be in trouble. Take proper rest and food: No matter how many hours you spend on your research activity, if you are not taking care of your health, then all your efforts will have been in vain. For quality research, take proper rest and food.

18. Go to seminars: Attend seminars if the topic is relevant to your research area. Utilize all your resources.

19. Refresh your mind after intervals: Try to give your mind a rest by listening to soft music or sleeping in intervals. This will also improve your memory. Acquire colleagues: Always try to acquire colleagues. No matter how sharp you are, if you acquire colleagues, they can give you ideas which will be helpful to your research.

20. Think technically: Always think technically. If anything happens, search for its reasons, benefits, and demerits. Think and then print: When you go to print your paper, check that tables are not split, headings are not detached from their descriptions, and page sequence is maintained.

21. Adding unnecessary information: Do not add unnecessary information like "I have used MS Excel to draw graphs." Irrelevant and inappropriate material is superfluous. Foreign terminology and phrases are not apropos. One should never take a broad view. Analogy is like feathers on a snake. Use words properly, regardless of how others use them. Remove quotations. Puns are for kids, not grunt readers. Never oversimplify: When adding material to your research paper, never go for oversimplification; this will definitely irritate the evaluator. Be specific. Never use rhythmic redundancies. Contractions shouldn't be used in a research paper. Comparisons are as terrible as clichés. Give up ampersands, abbreviations, and so on. Remove commas that are not necessary. Parenthetical words should be between brackets or commas. Understatement is always the best way to put forward earth-shaking thoughts. Give a detailed literary review.

22. Report concluded results: Use concluded results. From raw data, filter the results, and then conclude your studies based on measurements and observations taken. An appropriate number of decimal places should be used. Parenthetical remarks are prohibited here. Proofread carefully at the final stage. At the end, give an outline to your arguments. Spot perspectives of further study of the subject. Justify your conclusion at the bottom sufficiently, which will probably include examples.

23. Upon conclusion: Once you have concluded your research, the next most important step is to present your findings. Presentation is extremely important as it is the definite medium though which your research is going to be in print for the rest of the crowd. Care should be taken to categorize your thoughts well and present them in a logical and neat manner. A good quality research paper format is essential because it serves to highlight your research paper and bring to light all necessary aspects of your research.

Informal Guidelines of Research Paper Writing

Key points to remember:

- Submit all work in its final form.
- Write your paper in the form which is presented in the guidelines using the template.
- Please note the criteria peer reviewers will use for grading the final paper.

Final points:

One purpose of organizing a research paper is to let people interpret your efforts selectively. The journal requires the following sections, submitted in the order listed, with each section starting on a new page:

The introduction: This will be compiled from reference matter and reflect the design processes or outline of basis that directed you to make a study. As you carry out the process of study, the method and process section will be constructed like that. The results segment will show related statistics in nearly sequential order and direct reviewers to similar intellectual paths throughout the data that you gathered to carry out your study.

The discussion section:

This will provide understanding of the data and projections as to the implications of the results. The use of good quality references throughout the paper will give the effort trustworthiness by representing an alertness to prior workings.

Writing a research paper is not an easy job, no matter how trouble-free the actual research or concept. Practice, excellent preparation, and controlled record-keeping are the only means to make straightforward progression.

General style:

Specific editorial column necessities for compliance of a manuscript will always take over from directions in these general guidelines.

To make a paper clear: Adhere to recommended page limits.

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Mistakes to avoid:

- Insertion of a title at the foot of a page with subsequent text on the next page.
- Separating a table, chart, or figure—confine each to a single page.
- Submitting a manuscript with pages out of sequence.
- In every section of your document, use standard writing style, including articles ("a" and "the").
- Keep paying attention to the topic of the paper.
- Use paragraphs to split each significant point (excluding the abstract).
- Align the primary line of each section.
- Present your points in sound order.
- Use present tense to report well-accepted matters.
- Use past tense to describe specific results.
- Do not use familiar wording; don't address the reviewer directly. Don't use slang or superlatives.
- Avoid use of extra pictures—include only those figures essential to presenting results.

Title page:

Choose a revealing title. It should be short and include the name(s) and address(es) of all authors. It should not have acronyms or abbreviations or exceed two printed lines.

Abstract: This summary should be two hundred words or less. It should clearly and briefly explain the key findings reported in the manuscript and must have precise statistics. It should not have acronyms or abbreviations. It should be logical in itself. Do not cite references at this point.

An abstract is a brief, distinct paragraph summary of finished work or work in development. In a minute or less, a reviewer can be taught the foundation behind the study, common approaches to the problem, relevant results, and significant conclusions or new questions.

Write your summary when your paper is completed because how can you write the summary of anything which is not yet written? Wealth of terminology is very essential in abstract. Use comprehensive sentences, and do not sacrifice readability for brevity; you can maintain it succinctly by phrasing sentences so that they provide more than a lone rationale. The author can at this moment go straight to shortening the outcome. Sum up the study with the subsequent elements in any summary. Try to limit the initial two items to no more than one line each.

Reason for writing the article—theory, overall issue, purpose.

- Fundamental goal.
- To-the-point depiction of the research.
- Consequences, including definite statistics—if the consequences are quantitative in nature, account for this; results of any numerical analysis should be reported. Significant conclusions or questions that emerge from the research.

Approach:

- Single section and succinct.
- An outline of the job done is always written in past tense.
- Concentrate on shortening results—limit background information to a verdict or two.
- Exact spelling, clarity of sentences and phrases, and appropriate reporting of quantities (proper units, important statistics) are just as significant in an abstract as they are anywhere else.

Introduction:

The introduction should "introduce" the manuscript. The reviewer should be presented with sufficient background information to be capable of comprehending and calculating the purpose of your study without having to refer to other works. The basis for the study should be offered. Give the most important references, but avoid making a comprehensive appraisal of the topic. Describe the problem visibly. If the problem is not acknowledged in a logical, reasonable way, the reviewer will give no attention to your results. Speak in common terms about techniques used to explain the problem, if needed, but do not present any particulars about the protocols here.

The following approach can create a valuable beginning:

- Explain the value (significance) of the study.
- Defend the model—why did you employ this particular system or method? What is its compensation? Remark upon its appropriateness from an abstract point of view as well as pointing out sensible reasons for using it.
- Present a justification. State your particular theory(-ies) or aim(s), and describe the logic that led you to choose them.
- Briefly explain the study's tentative purpose and how it meets the declared objectives.

Approach:

Use past tense except for when referring to recognized facts. After all, the manuscript will be submitted after the entire job is done. Sort out your thoughts; manufacture one key point for every section. If you make the four points listed above, you will need at least four paragraphs. Present surrounding information only when it is necessary to support a situation. The reviewer does not desire to read everything you know about a topic. Shape the theory specifically—do not take a broad view.

As always, give awareness to spelling, simplicity, and correctness of sentences and phrases.

Procedures (methods and materials):

This part is supposed to be the easiest to carve if you have good skills. A soundly written procedures segment allows a capable scientist to replicate your results. Present precise information about your supplies. The suppliers and clarity of reagents can be helpful bits of information. Present methods in sequential order, but linked methodologies can be grouped as a segment. Be concise when relating the protocols. Attempt to give the least amount of information that would permit another capable scientist to replicate your outcome, but be cautious that vital information is integrated. The use of subheadings is suggested and ought to be synchronized with the results section.

When a technique is used that has been well-described in another section, mention the specific item describing the way, but draw the basic principle while stating the situation. The purpose is to show all particular resources and broad procedures so that another person may use some or all of the methods in one more study or referee the scientific value of your work. It is not to be a step-by-step report of the whole thing you did, nor is a methods section a set of orders.

Materials:

Materials may be reported in part of a section or else they may be recognized along with your measures.

Methods:

- Report the method and not the particulars of each process that engaged the same methodology.
- Describe the method entirely.
- To be succinct, present methods under headings dedicated to specific dealings or groups of measures.
- Simplify—detail how procedures were completed, not how they were performed on a particular day.
- If well-known procedures were used, account for the procedure by name, possibly with a reference, and that's all.

Approach:

It is embarrassing to use vigorous voice when documenting methods without using first person, which would focus the reviewer's interest on the researcher rather than the job. As a result, when writing up the methods, most authors use third person passive voice.

Use standard style in this and every other part of the paper—avoid familiar lists, and use full sentences.

What to keep away from:

- Resources and methods are not a set of information.
- Skip all descriptive information and surroundings—save it for the argument.
- Leave out information that is immaterial to a third party.

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Results:

The principle of a results segment is to present and demonstrate your conclusion. Create this part as entirely objective details of the outcome, and save all understanding for the discussion.

The page length of this segment is set by the sum and types of data to be reported. Use statistics and tables, if suitable, to present consequences most efficiently.

You must clearly differentiate material which would usually be incorporated in a study editorial from any unprocessed data or additional appendix matter that would not be available. In fact, such matters should not be submitted at all except if requested by the instructor.

Content:

- Sum up your conclusions in text and demonstrate them, if suitable, with figures and tables.
- In the manuscript, explain each of your consequences, and point the reader to remarks that are most appropriate.
- Present a background, such as by describing the question that was addressed by creation of an exacting study.
- Explain results of control experiments and give remarks that are not accessible in a prescribed figure or table, if appropriate.
- Examine your data, then prepare the analyzed (transformed) data in the form of a figure (graph), table, or manuscript.

What to stay away from:

- Do not discuss or infer your outcome, report surrounding information, or try to explain anything.
- Do not include raw data or intermediate calculations in a research manuscript.
- Do not present similar data more than once.
- A manuscript should complement any figures or tables, not duplicate information.
- Never confuse figures with tables—there is a difference.

Approach:

As always, use past tense when you submit your results, and put the whole thing in a reasonable order.

Put figures and tables, appropriately numbered, in order at the end of the report.

If you desire, you may place your figures and tables properly within the text of your results section.

Figures and tables:

If you put figures and tables at the end of some details, make certain that they are visibly distinguished from any attached appendix materials, such as raw facts. Whatever the position, each table must be titled, numbered one after the other, and include a heading. All figures and tables must be divided from the text.

Discussion:

The discussion is expected to be the trickiest segment to write. A lot of papers submitted to the journal are discarded based on problems with the discussion. There is no rule for how long an argument should be.

Position your understanding of the outcome visibly to lead the reviewer through your conclusions, and then finish the paper with a summing up of the implications of the study. The purpose here is to offer an understanding of your results and support all of your conclusions, using facts from your research and generally accepted information, if suitable. The implication of results should be fully described.

Infer your data in the conversation in suitable depth. This means that when you clarify an observable fact, you must explain mechanisms that may account for the observation. If your results vary from your prospect, make clear why that may have happened. If your results agree, then explain the theory that the proof supported. It is never suitable to just state that the data approved the prospect, and let it drop at that. Make a decision as to whether each premise is supported or discarded or if you cannot make a conclusion with assurance. Do not just dismiss a study or part of a study as "uncertain."

Research papers are not acknowledged if the work is imperfect. Draw what conclusions you can based upon the results that you have, and take care of the study as a finished work.

- You may propose future guidelines, such as how an experiment might be personalized to accomplish a new idea.
- Give details of all of your remarks as much as possible, focusing on mechanisms.
- Make a decision as to whether the tentative design sufficiently addressed the theory and whether or not it was correctly restricted. Try to present substitute explanations if they are sensible alternatives.
- One piece of research will not counter an overall question, so maintain the large picture in mind. Where do you go next? The best studies unlock new avenues of study. What questions remain?
- Recommendations for detailed papers will offer supplementary suggestions.

Approach:

When you refer to information, differentiate data generated by your own studies from other available information. Present work done by specific persons (including you) in past tense.

Describe generally acknowledged facts and main beliefs in present tense.

The Administration Rules

Administration Rules to Be Strictly Followed before Submitting Your Research Paper to Global Journals Inc.

Please read the following rules and regulations carefully before submitting your research paper to Global Journals Inc. to avoid rejection.

Segment draft and final research paper: You have to strictly follow the template of a research paper, failing which your paper may get rejected. You are expected to write each part of the paper wholly on your own. The peer reviewers need to identify your own perspective of the concepts in your own terms. Please do not extract straight from any other source, and do not rephrase someone else's analysis. Do not allow anyone else to proofread your manuscript.

Written material: You may discuss this with your guides and key sources. Do not copy anyone else's paper, even if this is only imitation, otherwise it will be rejected on the grounds of plagiarism, which is illegal. Various methods to avoid plagiarism are strictly applied by us to every paper, and, if found guilty, you may be blacklisted, which could affect your career adversely. To guard yourself and others from possible illegal use, please do not permit anyone to use or even read your paper and file.

Criterion for Grading a Research Paper (Compilation) by Global Journals

Please note that following table is only a Grading of "Paper Compilation" and not on "Performed/Stated Research" whose grading solely depends on Individual Assigned Peer Reviewer and Editorial Board Member. These can be available only on request and after decision of Paper. This report will be the property of Global Journals.

Topics	Grades		
	A-B	**C-D**	**E-F**
Abstract	Clear and concise with appropriate content, Correct format. 200 words or below	Unclear summary and no specific data, Incorrect form Above 200 words	No specific data with ambiguous information Above 250 words
Introduction	Containing all background details with clear goal and appropriate details, flow specification, no grammar and spelling mistake, well organized sentence and paragraph, reference cited	Unclear and confusing data, appropriate format, grammar and spelling errors with unorganized matter	Out of place depth and content, hazy format
Methods and Procedures	Clear and to the point with well arranged paragraph, precision and accuracy of facts and figures, well organized subheads	Difficult to comprehend with embarrassed text, too much explanation but completed	Incorrect and unorganized structure with hazy meaning
Result	Well organized, Clear and specific, Correct units with precision, correct data, well structuring of paragraph, no grammar and spelling mistake	Complete and embarrassed text, difficult to comprehend	Irregular format with wrong facts and figures
Discussion	Well organized, meaningful specification, sound conclusion, logical and concise explanation, highly structured paragraph reference cited	Wordy, unclear conclusion, spurious	Conclusion is not cited, unorganized, difficult to comprehend
References	Complete and correct format, well organized	Beside the point, Incomplete	Wrong format and structuring

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