# EVALUATING GENOMIC DEOXYRIBONUCLEIC ACID (gDNA) OF PARENTS AND OFFSPRING TOWARDS DETERMINING RICKETS HEREDITARY LINK IN GONIN GORA, KASO AND JANKASA COMMUNITIES IN KADUNA STATE

**BY**

# ADAMU SALLAH ALHAJI

**DEPARTMENT OF PHARMACEUTICAL AND MEDICINAL CHEMISTRY FACULTY OF PHARMACEUTICAL SCIENCES AHMADU BELLO UNIVERSITY**

# ZARIA, NIGERIA

**MACH, 2011**

# EVALUATING GENOMIC DEOXYRIBONUCLEIC ACID (gDNA) OF PARENTS AND OFFSPRING TOWARDS DETERMINING RICKETS HEREDITARY LINK IN GONIN GORA, KASO AND JANKASA COMMUNITIES IN KADUNA STATE

**BY**

# ADAMU SALLAH ALHAJI

**B. PHARM. ABU ZARIA (1995)**

# A THESIS SUBMITTED TO THE SCHOOL OF POST GRADUATE STUDIES AHMADU BELLO UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF M.Sc. IN PHARMACEUTICAL AND MEDICINAL CHEMISTRY

**DEPARTMENT OF PHARMACEUTICAL AND MEDICINAL CHEMISTRY, FACULTY OF PHARMACEUTICAL SCIENCES AHMADU BELLO UNIVERSITY**

# ZARIA, NIGERIA

**MARCH, 2011**

# DECLARATION

I Adamu Sallah Alhaji hereby solemnly declares that the thesis work was solely and wholly undertaken by me under the supervision of Prof. (Mrs) M. T. Odunola and Dr.

P.A. Wuyep of the Department of Pharmaceutical and Medicinal Chemistry and Department of Biological Sciences A. B. U. Zaria respectively. The work of other investigators was acknowledged and referred to accordingly.

# …………………………….. …………………………… ADAMU SALLAH ALHAJI DATE

**CERTIFICATION**

This thesis titled Evaluating Genomic DNA of parents and offspring towards determining Rickets hereditary link in a chosen population, meets the regulations governing the award of the degree of Masters in Pharmaceutical and medicinal Chemistry of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

……………………………… ………..

Prof. Mrs M.T. Odunola Date

(Chairman Supervisory Committee)

………………………………. ………...

Dr. P. A. Wuyep Date

(Member, Supervisory Committee)

……………………………… ………...

Prof. M. Garba Date

Dean Faculty of Pharmaceutical Sciences Ahmadu Bello University, Zaria

……………………………… ………...

Prof. A A. Joshua` Date

Dean School of Postgraduate Studies Ahmadu Bello University, Zaria

# ACKNOWLEDGEMENT

I wish to give thanks to the Almighty Allah who made all these possible. If not for His grace I will not have accomplish this, therefore all praise be to Him only.

My gratitude also goes to my wife Fatima Bala Maina for her patience and understanding during the period of my studies.

My sincere appreciation goes to my supervisors Prof. (Mrs) Odunola M.T, and Dr.Wuyep P.A. whom despite their tight schedule find time to attend and participate in the project.

My special thanks goes to the staff of the centre for biotechnology A.B.U. Zaria especially Dr. Ellae. Mrs B. Afolabi, Mallam Sameer, Mallam YY for their contribution and understanding.

My gratitude goes to my children Ummusalma and Muhammad for their understanding during the period of my absence.

I will like to acknowledge the following people Dr. Musa Usman, Njinga N.S., Atiku Ibrahim, Abbas, Ajima, Khadija, Marafa, FMC Gombe, Alhajijo, Abba, Mamman, who all stood by me during this critical moment.

# ABSTRACT

Rickets is still a debilitating disease in various communities of Jankasa, Kaso and Gonin Gora in which about 3-7 percent of the population is affected. Genomic Deoxyribonucleic acid (gDNA) of parents and offspring were evaluated towards determining rickets hereditary link in the population. Four families from Jankasa, Kaso and two families from Gonin Gora composed of father, mother, child without rickets and child with rickets each were used for the study.

The DNA were extracted using zymo DNA extraction kits, they were quantified and purified using Biophotometer and gel electrophoresis respectively. Gel was viewed under ultra violet light and documented. The DNA was then restricted and amplified using polymerase chain reaction (PCR) technique.

The blood samples of parents and offspring from Kaso, Jankasa and Gonin Gora showed bands which indicated the presence of DNA. Lower concentrations of gDNA were observed for the rickets children who could be due to the fact that blood formation occurs in the long bone and Rickets is the long bone disease. Comparison of mean concentration (µg/ml) between rickets and non Rickets afflicted children using T-test gives 1.6 calculated as opposed 1.73 tabulated suggesting no significant difference at 95% confidence interval.

The isolated DNA from all blood samples were restricted, the restricted fragment length polymorph of DNA used in PCR showed that the vitamin D receptor (VDR) gene for hereditary vitamin D resistant rickets (HVDRR) was successfully amplified in rickets children and gave smear amplification in parents and children without rickets at the same PCR cycle.

The study concluded that there is an hereditary link in the rickets disorder affecting Kaso, Jankasa and Gonin Gora communities. .

# TABLE OF CONTENTS

|  |  |  |
| --- | --- | --- |
| Title page | ……………………………………………… | i |
| Declaration | ……………………………………………. | ii |
| Certification | ………………………………………….. | iii |
| Acknowledgement | ……………………………………. | iv |
| Abstract | ……………………………………………….. | v |
| Table of content | ………………………………………. | vi |
| List of figures | …………………………………………. | ix |
| List of Tables | ………………………………………….. | x |
| Abbreviation | ………………………………………….. | xi |
| Dedication | …………………………………………….. | xiii |

**CHAPTER 1 : INTRODUCTION**

1.0 Introduction …………………………………….. 1

[1.1 Rickets ………………………………………….. 1](#_TOC_250044)

[1.2 Communities …………………………… 4](#_TOC_250043)

[1.3 Justification ……………………………………….. 4](#_TOC_250042)

[1.4 Aim and Objectives of the study ………………… 5](#_TOC_250041)

[CHAPTER 2: LITERATURE REVIEW](#_TOC_250040)

[2.1 Review of Rickets DNA Research ………………….. 6](#_TOC_250039)

[2.1.1 Epidemiology …………………………………… 11](#_TOC_250038)

[2.1.2 Etiology ………………………………………… 12](#_TOC_250037)

[2.1.3 Symptoms ……………………………………… 13](#_TOC_250036)

[2.1.4 Diagnosis ………………………………………. 14](#_TOC_250035)

[2.1.5 Treatment and prevention ……………………… 15](#_TOC_250034)

[2.1.5.1 Diet and Sunlight …………………………....... 15](#_TOC_250033)

[2.1.5.2 Supplementation ……………………………… 15](#_TOC_250032)

[2.2. DNA …………………………………………….. 16](#_TOC_250031)

[2.2.1 History and Anthropology …………………….. 17](#_TOC_250030)

[2.2.2. Evolution ……………………………………… 18](#_TOC_250029)

* + 1. [Uses in Technology …………………………… 19](#_TOC_250028)
       1. [Genetic Engineering …………………….. 19](#_TOC_250027)

[2.2.3.2 Forensics ………………………………… 19](#_TOC_250026)

[2.2.3.3 Bioinformatics ………………………….. 20](#_TOC_250025)

[2.2.3.4 DNA nanotechnology …………………… 21](#_TOC_250024)

[2.3. DNA Extraction …………………………………. 21](#_TOC_250023)

[2.3.1 Detecting DNA ………………………………… 22](#_TOC_250022)

* 1. Restriction Enzymes …………………………….. 23
  2. [DNA Quantification ……………………………. 25](#_TOC_250021)
     1. [Estimation of DNA Concentration …………… 25](#_TOC_250020)
     2. [Procedure of DNA Quantification ……………… 26](#_TOC_250019)
  3. Characterisation of DNA by Agarose Gel Electrophoresis. …………… 26
  4. [Polymerase Chain reaction ……………………… 27](#_TOC_250018)
     1. [Principles of PCR ……………………………. 28](#_TOC_250017)
     2. [Application of PCR …………………………… 29](#_TOC_250016)
        1. [Selective DNA isolation …………………….. 29](#_TOC_250015)
        2. [Amplification and quantification of DNA ………………..….. 30](#_TOC_250014)

2.7.2.3 Uses of PCR ………………………………. 31

CHAPTER 3: MATERIAL AND METHOD

[3.1 Materials …………………………………………. 32](#_TOC_250013)

[3.1.1 Reagents ………………………………………. 32](#_TOC_250012)

[3.1.2 Equipments ……………………………………. 33](#_TOC_250011)

[3.2 Method …………………………………………… 33](#_TOC_250010)

[3.2.1 Preparation of reagents ………………………… 33](#_TOC_250009)

[3.2.2 Extraction of DNA …………………………….. 34](#_TOC_250008)

[3.2.3 Procedure of DNA Quantification ……………… 35](#_TOC_250007)

[3.2.4. Protocol ( gel preparation) ………………….. 35](#_TOC_250006)

3.2.6 Polymerase Chain Reaction (PCR) …………. 36

[CHAPTER 4: RESULTS](#_TOC_250005)

* 1. The genomic DNA isolated from blood of parents and children……… 38
  2. [DNA Quantification of parents, rickets and non rickets children…… 41](#_TOC_250004)
  3. Restriction fragment length polymorphs of DNA from

DNA of parents and offspring’s ………………………………. 44

* 1. [Polymerase Chain Reaction ………………………………. 45](#_TOC_250003)

[CHAPTER 5: DISCUSSION](#_TOC_250002)

* 1. The genomic DNA isolated from blood of parents and children……… 48
  2. [DNA Quantification of parents, rickets and non rickets children……… 48](#_TOC_250001)
  3. Restriction fragment length polymorphs of DNA from DNA of parents

and offspring’s ………………………………. 49

* 1. [Polymerase Chain Reaction ………………………………………. 49](#_TOC_250000)

# CHAPTER 6: SUMMARY, CONCLUSION AND RECOMMENDATIONS

|  |  |  |
| --- | --- | --- |
| 6.1 Summary | ……………………………………………… | 50 |
| 6.2 Conclusion | ……………………………………………………… | 50 |
| 6.3 Recommendations | …………………………………………….. .. | 51 |
| References | ………………………………………………………. | 52 |

**LIST OF FIGURES**

Figure 1 The Structure of the DNA ... ... ... ... ...... 17

Figure 4.1a. Electrophoregraph of the genomic DNA isolated from the blood of parents and children. … … … … … … 38

Figure 4.1b. Electrophoregraph of the genomic DNA isolated from the blood of parents and children. … … … … … … … 39

Figure 4.1c. Electrophoregraph of the genomic DNA isolated from the blood of parents and children. … … … … … … … 40

Figure 4.2. Restriction fragment length polymorphs of DNA from blood of parents and offspring’s**.** … … … … … … … 44

Figure 4.3a. Amplified fragment length polymorphs from restriction fragment

length polymorphs of parent and offspring’s. (Before optimisation) … 46

Figure 4.3b. Amplified fragment length polymorphs from restriction fragment

length polymorphs of parent and offspring’s (After optimisation) … 47

# LIST OF TABLES

Table 1 DNA Quantification of parents, rickets and non rickets afflicted childre… 42 Table 2 Comparison of mean concentration (µg/ml) and absorbance between

rickets and non rickets children … … … … … … 43

# ABBREVIATIONS

DNA- Deoxyribonucleic acid PCR- Polymerase chain reaction TAE- tris acetate EDTA

IEC – Information, Education and Communication VDR – Vitamin D receptor

HVDRR – Hereditary vitamin D resistant rickets µg – microgramme

ml- millilitre

RNA – Ribonucleic acid F1 – Father family 1 M1 – Mother family 1

C1 – Child without rickets family 1 R1 – Child with rickets family 1 F2 – Father family 2

M2 – Mother family 2

C2 – Child without rickets family 2 R2 – Child with rickets family 2 F3 – Father family 3

M3 – Mother family 3

C3 – Child without rickets family 3 R3 – Child with rickets family 3 F4 – Father family 4

M4 – Mother family 4

C4 – Child without rickets family 4

R4 – Child with rickets family 4 F5 – Father family 5

M5 – Mother family 5

C5 – Child without rickets family 5 R5 – Child with rickets family 5 F6 – Father family 6

M6 – Mother family 6

C6 – Child without rickets family 6 R6 – Child with rickets family 6 F7 – Father family 7

M7 – Mother family 7

C7 – Child without rickets family 7 R7 – Child with rickets family 7 F8 – Father family 8

M8 – Mother family 8

C8 – Child without rickets family 8 R8 – Child with rickets family 8

# DEDICATION

To my late father, my mother and the entire communities of Kaso, Jankasa and Gonin Gora.

# CHAPTER 1 INTRODUCTION

# RICKETS

Rickets is an abnormal bone formation in children resulting from inadequate calcium in their bones. This lack of calcium can result from inadequate dietary calcium, inadequate exposure to sunshine (needed to make vitamin D), a nutrient needed for calcium absorption. (Atiq *et al.,* 1998) Vitamin D is found in animal foods, such as egg yolks and dairy products. (Bhattachaya *et al*., 1992)

Vitamin D is made by the body when it is exposed to ultraviolet light (sunlight). Vitamin D is also added to milk, milk products, and multi-vitamin pills. Some people who do not get enough sun exposure, milk products, or green vegetables may also develop the disease, but that rarely happens anymore. Hereditary rickets, is caused by an inherited disease that interferes with the resorption of renal tubular phosphate in the kidney. (Greer *et al*., 2003)

Rickets is a failure to mineralize bone. This softens bone (producing osteomalacia) and permits marked bending and distortion of bones. Up through the first third of the 20th century, rickets was largely due to lack of direct exposure to sunlight or lack of vitamin

D. Once the role of vitamin D in rickets was discovered, cod liver oil (which is rich in vitamin D) became a favoured remedy. Thanks to such supplements of vitamin D, nutritional rickets has become relatively rare in industrialized nations. (Fisher *et al*., 1999) It still occurs, for example, in breast-fed babies whose mothers are underexposed to sunlight and in dark-skinned babies who are not given vitamin D supplements. And

in unindustrialized countries, vitamin D deficiency rickets continues to be a problem. (Thatcher *et al.,* 1997)

Rickets most commonly affects children, who may have low vitamin D levels due to poor diet or a condition (such as celiac disease) that makes it difficult for the body to absorb vitamin D and calcium. (Thatcher *et al*., 2000) Rickets is most likely to occur during periods of rapid growth, when the body demands high levels of calcium and phosphate.

Causes

The main cause of rickets is the deficiency of vitamin D caused in the body during childhood. Vitamin D is a fat-soluble vitamin that may be absorbed from the intestines or may be produced by the skin when the skin is exposed to sunlight (ultraviolet light of sunlight helps the body to form vitamin D (Thatcher *et al.,* 2000). The absorbed vitamin D is converted into its active form to act as a hormone to regulate calcium absorption from the intestine and to regulate levels of calcium and phosphate in the bones (De lucia *et al*., 2003).

Lack of vitamin D production by the skin may occur if a person is confined indoors, or works indoors during the daylight hours, or lives in climates with little exposure to sunlight. Nutritional causes of rickets occur because of a lack of vitamin D in the diet or in association with malabsorption disorders characterized by poor fat absorption. (Lawson *et al*., 1998).

A dietary lack of vitamin D may occasionally occur in people on a vegetarian diet who do not drink milk products or in people who are lactose intolerant (have trouble digesting milk products. Hereditary rickets is an inherited form of the disease caused

when the kidneys are unable to retain phosphate. Rickets may also be caused by kidney disorders involving renal tubular acidosis. Occasionally, it can also affect children who have disorders of the liver, do not adequately absorb fats and vitamin D, or cannot convert vitamin D to its active form. (Lawson *et al*., 1998)

Symptoms

The symptoms of rickets include bowed legs and bowed arms. The bowed appearance is due to the softening of bones, and their bending if the bones are weight-bearing Other symptoms of rickets include particular bony bumps on the ribs called rachitic rosary (beadlike prominences at the junction of the ribs with their cartilages) and knock-knees. Seizures may also occasionally occur in a child with rickets, because of reduced levels of dissolved calcium in the bloodstream. Hypophosphatemic rickets usually begins in the first year of life. It ranges from so mild that it produces no noticeable symptoms to so severe that it produces bowing of the legs and other bone deformities, bone pain, and a short stature. (Chapuy *et al.,* 1997)

Diagnosis

Rickets is diagnosed by x-ray examination of leg bones. A distinct pattern of irregularities, abnormalities, and a coarse appearance can be clearly seen with rickets. A blood test may be done to measure the amounts of the minerals calcium and phosphorus. X-rays of the affected bones are obtained and a musculoskeletal examination reveals tenderness or pain of the bone itself, rather than in the joints or muscles. (Thatcher *et al.,* 2000)

Treatment

The treatment goals for rickets are to relieve symptoms and correct the cause of the condition. The underlying cause must be treated to prevent recurrence. The replacement of deficient calcium, phosphorus, or vitamin D will eliminate most symptoms of rickets. Dietary sources of vitamin D include fish, liver, and processed milk. Exposure to moderate amounts of sunlight is encouraged.. Children should also be encouraged to play outside. (Thatcher *et al*., 2000)

# COMMUNITIES

The three communities that were involved in the research are Jankasa, Kaso and Gonin Gora which are located in Kaduna state Nigeria. Four families each from Kaso and Jankasa were selected and two families from Gonin gora, involving father, mother, child without rickets and child with rickets disorder.

# JUSTIFICATION

* + - There is need to establish whether the anomaly in genomic composition exist between parents and their siblings.
    - Whether the rickets has hereditary factor apart from the nutritional pattern already established.

# AIM AND OBJECTIVES OF THE STUDY

This work is aimed to characterize genomic DNA toward the determination of hereditary link in rickets from parents and offspring**.** The aim will be achieved by:

1. Extraction of genomic DNA of parents and children
2. Quantification of genomic DNA of parents and children
3. Isolation of unique fragment by restriction endonucleases
4. Amplification of fragment

# CHAPTER 2 LITERATURE REVIEW

# REVIEW OF RICKETS DNA RESEARCH

Rickets is a softening of bones in children potentially leading to fractures and deformity. Rickets is among the most frequent childhood diseases in many developing countries (Olivieri *et al.,* 1993). The predominant cause is a vitamin D deficiency, but lack of adequate calcium in the diet may also lead to rickets (cases of severe diarrhea and vomiting may be the cause of the deficiency). Although it can occur in adults, the majority of cases occur in children suffering from severe malnutrition, usually resulting from famine or starvation during the early stages of childhood. Osteomalacia is the term used to describe a similar condition occurring in adults, generally due to a deficiency of vitamin D. The origin of the word "rickets" is probably from the Old English dialect word 'wrickken', to twist. The Greek derived word "rachitis" (ραχίτις, meaning "inflammation of the spine") was later adopted as the scientific term for rickets, due chiefly to the words' similarity in sound. (Thatcher *et al*., 2000)

Hereditary hypophosphatemic rickets is a disorder related to low levels of phosphate in the blood (hypophosphatemia). Phosphate is a mineral that is essential for the normal formation of bones and teeth. (Sattur *et al.,* 2009)

Researchers have described several forms of hereditary hypophosphatemic rickets, which are distinguished by their pattern of inheritance and genetic cause (Outila et al., 2001) The most common form of the disorder is known as X-linked hypophosphatemic rickets (XLH). It has an X-linked dominant pattern of inheritance. X-linked recessive,

autosomal dominant, and autosomal recessive forms of the disorder are much rarer. The different inheritance patterns are described below (Zeghoud *et al.,* 2002).

Another rare type of the disorder is known as hereditary hypophosphatemic rickets with hypercalciuria (HHRH). In addition to hypophosphatemia, this condition is characterized by the excretion of high levels of calcium in the urine (hypercalciuria). (Sattur *et al*., 2009)

X-linked hypophosphatemic rickets is the most common form of rickets that runs in families ( Mohapatra *et al.,* 2003). It affects about 1 in 20,000 newborns. Each of the other forms of hereditary hypophosphatemic rickets has been identified in only a few families. Hereditary hypophosphatemic rickets can result from mutations in several genes. Mutations in the *PHEX* gene, which are responsible for X-linked hypophosphatemic rickets, occur most frequently (Oginni *et al.,* 2003). Mutations in other genes cause the less common forms of the condition.

Hereditary hypophosphatemic rickets is characterized by a phosphate imbalance in the body. Among its many functions, phosphate plays a critical role in the formation and growth of bones in childhood and helps maintain bone strength in adults. Phosphate levels are controlled in large part by the kidneys. The kidneys normally excrete excess phosphate in urine, and they reabsorb this mineral into the bloodstream when more is needed. However, in people with hereditary hypophosphatemic rickets, the kidneys cannot reabsorb phosphate effectively and too much of this mineral is excreted from the body in urine. As a result, not enough phosphate is available in the bloodstream to participate in normal bone development and maintenance.

The genes associated with hereditary hypophosphatemic rickets are involved in maintaining the proper balance of phosphate. Many of these genes, including

the *PHEX* gene, may directly or indirectly regulate a protein called fibroblast growth factor 23 (produced from the *FGF23* gene) ( Sattur *et al.,* 2009). This protein normally inhibits the kidneys' ability to reabsorb phosphate into the bloodstream. Gene mutations increase the production or reduce the breakdown of fibroblast growth factor 23. The resulting overactivity of this protein reduces phosphate reabsorption by the kidneys, leading to hypophosphatemia and the related features of hereditary hypophosphatemic rickets. (Sattur *et al.,* 2009)

Hereditary hypophosphatemic rickets can have several patterns of inheritance. When the condition results from mutations in the *PHEX* gene, it is inherited in an X-linked dominant pattern. The *PHEX* gene is located on the X chromosome, which is one of the two sex chromosomes. In females (who have two X chromosomes), a mutation in one of the two copies of the gene in each cell is sufficient to cause the disorder. In males (who have only one X chromosome), a mutation in the only copy of the gene in each cell causes the disorder. A characteristic of X-linked inheritance is that fathers cannot pass X-linked traits to their sons**.** (Sattur *et al.,* 2009)

Less commonly, hereditary hypophosphatemic rickets can have an X-linked recessive pattern of inheritance. This form of the condition is often called Dent disease. Like the *PHEX* gene, the gene associated with Dent disease is located on the X chromosome. In males, one altered copy of the gene in each cell is sufficient to cause the condition. In females, a mutation would have to occur in both copies of the gene to cause the disorder. Because it is unlikely that females will have two altered copies of this gene, males are affected by X-linked recessive disorders much more frequently than females. (Sattur *et al.,* 2009)

In a few families, hereditary hypophosphatemic rickets has had an autosomal dominant inheritance pattern, which means one copy of an altered gene in each cell is sufficient to cause the disorder. The rare condition HHRH has an autosomal recessive pattern of inheritance, which means both copies of a gene in each cell have mutations. The parents of an individual with an autosomal recessive condition each carry one copy of the mutated gene, but they typically do not show signs and symptoms of the condition. However, some parents of children with HHRH have experienced hypercalcuria and kidney stones.

To study the vitamin D receptor (VDR) gene in a young girl with severe rickets and clinical features of hereditary vitamin D resistant rickets, including hypocalcemia, hypophosphatemia, partial alopecia, and elevated serum levels of 1,25- dihydroxyvitamin D. Ma and Malloy (2009). Amplified and sequenced DNA samples from blood from the patient, her mother, and the patient's two siblings. Also amplified and sequenced the VDR cDNA from RNA isolated from the patient's blood.

DNA sequence analyses of the VDR gene showed that the patient was homozygous for a novel guanine to thymine substitution in the 5'-splice site in the exon 8-intron J junction. Analysis of the VDR cDNA using reverse transcriptase-polymerase chain reaction showed that exons 7 and 9 were fused, and that exon 8 was skipped. The mother was heterozygous for the mutation and the two siblings were unaffected. (Ma and Malloy 2009)

A novel splice site mutation was identified in the VDR gene that caused exon 8 to be skipped. The mutation deleted amino acids 303-341 in the VDR ligand-binding domain, which is expected to render the VDR non-functional. Nevertheless, successful

outpatient treatment was achieved with frequent high doses of oral calcium. (Ma and Malloy 2009).

Nutritional rickets has been increasingly reported in recent years, particularly among vulnerable populations of children (Rudolf *et al.,* 1980, Eugster *et al.,* 1996 and Tomashek *et al.,* 2001). Vitamin D deficiency is widely assumed to be the primary cause of this disorder. Inadequate vitamin D acquisition through either poor dietary intake or limited sunlight exposure leads to depletion of vitamin D stores, with resultant decreased calcium absorption in the small intestine, thereby decreasing the available calcium for epiphyseal cartilage and skeletal mineralization. Limited calcium availability with resultant secondary hyperparathyroidism and attendant renal phosphate losses contribute to the bone and growth plate pathophysiology that lead to the clinical manifestations of rickets (Guillemant *et al.,* 2001).

Historical features associated with nutritional rickets include lengthy duration of breastfeeding, absence of vitamin supplementation, limited sunlight exposure, dark skin pigmentation, and limited intake of dairy products, particularly those supplemented with vitamin D. The recent increase in reported cases may reflect an increasing influence of these factors. Considerable public health efforts have resulted in an increased number of infants being breastfed (Ryan., 1997); this expanded practice of breastfeeding may have unintentionally increased the number of infants at risk for the development of rickets, as there is minimal vitamin D present in breast milk (Hollis *et al.,* 1986). However, another potential contributor to the development of nutritional rickets is the diet to which infants are weaned after breastfeeding. Unfortified juices, which are high in carbohydrates and lacking in calcium and other key bone nutrients, have replaced milk in the diets of many children (American Academy of Paediatics

Committee on Nutrition 2001). As a result, appropriate dietary intake of calcium, vitamin D, protein, and other nutrients important for the maintenance of skeletal health may be lacking.

Four affected children from three families of Arab isreal were used to determine the incidence of mutation in the vitamin D receptor genes which causes hereditary 1, 25- dihydroxyvitamin D3-resistant rickets because of high incidence of rickets in that area (Keller *et al*., 1999). The disease was found to be as a result of mutation in the VDR protein. In all the four children the DNA exhibited a single C -> A base substitution within exon 7 at nucleotide 970 that resulted in the conversion of the normal codon for tyrosine (TAC) into a premature termination codon (TAA) at amino acid 292.( Keller *et al.,* 1999)

This mutation causes truncation of the VDR protein thereby deleting a large portion of the steroid hormone binding dormain. The functional consequence of this mutation was the receptor resistant to the action of vitamin D. (Keller *et al.,* 1999)

# Epidemiology

Those at higher risk for developing rickets include:

* + - * Breast-fed infants whose mothers are not exposed to sunlight
      * Breast-fed infants who are not exposed to sunlight
      * Babies with dark complexions (e.g. black, South African), particularly when breastfed and exposed to little sunlight
      * Individuals not consuming milk, such as those who are lactose intolerant

Individuals with red hair have been speculated to have a decreased risk for rickets due to their greater production of vitamin D in sunlight.

Children ages 6 months to 24 months are at highest risk, because their bones are rapidly growing. Long-term consequences include permanent bends or disfiguration of the long bones, and a curved back.

# Etiology

**ergocalciferol (D2)**

# cholecalciferol (D3)

Vitamin D is required for proper calcium absorption from the gut. Sunlight, especially ultraviolet light, lets human skin cells convert Vitamin D from an inactive to active state. In the absence of vitamin D, dietary calcium is not properly absorbed, resulting in hypocalcemia, leading to skeletal and dental deformities and neuromuscular symptoms,

e.g. hyperexcitability. Foods that contain vitamin D include butter, eggs, fish liver oils, margarine, fortified milk and juice, and oily fishes such as tuna, herring, and salmon. A rare X-linked dominant form exists called Vitamin D resistant rickets. (Chapuy *et al*., 1997)

# Symptoms

Signs and symptoms of rickets include:

* + - * Bone pain or tenderness
      * dental problems
      * muscle weakness (rickety myopathy or "floppy baby syndrome" or "slinky baby" (where the baby is floppy or slinky-like))
      * increased tendency for fractures (easily broken bones), especially greenstick fractures
      * Skeletal deformity
      * Toddlers: Bowed legs (genu varum)
      * Older children: Knock-knees (genu valgum) or "windswept knees"
      * Cranial, spinal, and pelvic deformities
      * Growth disturbance
      * Hypocalcemia (low level of calcium in the blood), and
      * Tetany (uncontrolled muscle spasms all over the body).
      * Craniotabes (soft skull)
      * Costochondral swelling (aka "rickety rosary" or "rachitic rosary")
      * Harrison's groove
      * Double malleoli sign due to metaphyseal hyperplasia
      * Widening of wrist raises early suspicion, it is due to metaphysial cartilage hyperplasia.

An X-ray or radiograph of an advanced sufferer from rickets tends to present in a classic way: bow legs (outward curve of long bone of the legs) and a deformed chest. Changes in the skull also occur causing a distinctive "square headed" appearance. These deformities persist into adult life if not treated. (Lawson *et al.,* 1998)

Long-term consequences include permanent bends or disfiguration of the long bones, and a curved back.

# Diagnosis

Blood tests:

* + - * Serum calcium may show low levels of calcium, serum phosphorus may be low, and serum alkaline phosphatase may be high.
      * Arterial blood gases may reveal metabolic acidosis
      * X-rays of affected bones may show loss of calcium from bones or changes in the shape or structure of the bones.
      * Bone biopsy is rarely performed but will confirm rickets.

# Treatment and prevention

# Diet and sunlight

Treatment involves increasing dietary intake of calcium, phosphates and vitamin D. Exposure to ultraviolet B light (sunshine when the sun is highest in the sky), cod liver oil, halibut-liver oil, and viosterol are all sources of vitamin D. (Chapuy *et al.,* 1997)

A sufficient amount of ultraviolet B light in sunlight each day and adequate supplies of calcium and phosphorus in the diet can prevent rickets

# Supplementation

Sufficient vitamin D levels can also be achieved through dietary supplementation and/or exposure to sunlight. Vitamin D3 (cholecalciferol) is the preferred form since it is more readily absorbed than vitamin D2. Most dermatologists recommend vitamin D supplementation as an alternative to unprotected ultraviolet exposure due to the increased risk of skin cancer associated with sun exposure. Note that in July in New York City at noon with the sun out, a white male in tee shirt and shorts will produce 20000 IU of Vitamin D from 20 minutes of non-sunscreen sun exposure .(Chapuy *et al.,* 1997)

According to the American Academy of Pediatrics (AAP), infants who are breast-fed may not get enough vitamin D from breast milk alone. For this reason, the AAP recommends that infants who are exclusively breast-fed receive daily supplements of vitamin D from age 2 months until they start drinking at least 17 ounces of vitamin D-

fortified milk or formula a day. This requirement for supplemental vitamin D is not a defect in the evolution of human breastmilk, but is instead a result of the modern-day infant's decreased exposure to sunlight (i.e. breast-fed infants who receive adequate sun exposure are less likely to develop rickets, though supplementation may still be indicated in the winter, depending on geographical latitude). (Lawson *et al.,* 1998)

# DNA

Deoxyribonucleic acid (DNA) is a nucleic acid that contains the genetic instructions used in the development and functioning of all known living organisms and some viruses. The main role of DNA molecules is the long-term storage of information. DNA is often compared to a set of blueprints or a recipe, or a code, since it contains the instructions needed to construct other components of cells, such as proteins and RNA molecules. The DNA segments that carry this genetic information are called genes, but other DNA sequences have structural purposes, or are involved in regulating the use of this genetic information. (Mandekarn *et al.,* 1981)

**The Structure of DNA**

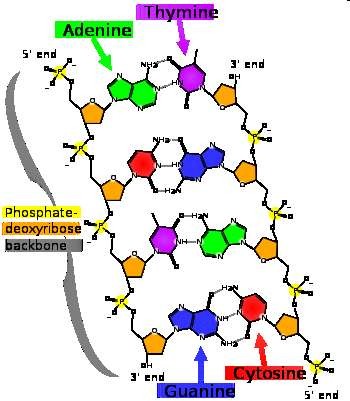


Figure 1 The structure of DNA (Mandekarn *et al.,* 1981)

Chemically, DNA (shown in fig. 1) consists of two long polymers of simple units called nucleotides, with backbones made of sugars and phosphate groups joined by ester bonds. These two strands run in opposite directions to each other and are therefore anti-parallel. Attached to each sugar is one of four types of molecules called bases. It is the sequence of these four bases along the backbone that encodes information. This information is read using the genetic code, which specifies the sequence of the amino acids within proteins. The code is read by copying stretches of DNA into the related nucleic acid RNA, in a process called transcription.

Within cells, DNA is organized into long structures called chromosomes. These chromosomes are duplicated before cells divide, in a process called DNA replication. Eukaryotic organisms (animals, plants, fungi, and protists) store most of their DNA

inside the cell nucleus and some of their DNA in organelles, such as mitochondria or chloroplasts. In contrast, prokaryotes (bacteria and archaea) store their DNA only in the cytoplasm. Within the chromosomes, chromatin proteins such as histones compact and organize DNA. These compact structures guide the interactions between DNA and other proteins, helping control which parts of the DNA are transcribed. (Mandekarn *et al.,* 1981)

# History and anthropology

Because DNA collects mutations over times, which are then inherited, it contains historical information and by comparing DNA sequences, geneticists can infer the evolutionary history of organisms, their phylogeny. This field of phylogenetics is a powerful tool in evolutionary biology. If DNA sequences within a species are compared, population geneticists can learn the history of particular populations. This can be used in studies ranging from ecological genetics to anthropology; for example, DNA evidence is being used to try to identify the Ten Lost Tribes of Israel (Mandekarn *et al.,* 1981)

.

DNA has also been used to look at modern family relationships, such as establishing family relationships. This usage is closely related to the use of DNA in criminal investigations. Indeed, some criminal investigations have been solved when DNA from crime scenes has matched relatives of the guilty individual. .(Mandekarn *et al.,* 1981)

# Evolution

DNA contains the genetic information that allows all modern living things to function, grow and reproduce. However, it is unclear how long in the 4 -billion-year history of life DNA has performed this function, as it has been proposed that the earliest forms of life may have used RNA as their genetic material. RNA may have acted as the central part of early cell metabolism as it can both transmit genetic information and carry out catalysis as part of ribozymes. This ancient RNA world where nucleic acid would have been used for both catalysis and genetics may have influenced the evolution of the current genetic code based on four nucleotide bases. This would occur since the number of different bases in such an organism is a trade-off between a small number of bases increasing replication accuracy and a large number of bases increasing the catalytic efficiency of ribozymes (Seanger *et al.,* 1984).

Unfortunately, there is no direct evidence of ancient genetic systems, as recovery of DNA from most fossils is impossible. This is because DNA will survive in the environment for less than one million years and slowly degrades into short fragments in solution. Claims for older DNA have been made, most notably a report of the isolation of a viable bacterium from a salt crystal 250 million years old, but these claims are controversial (Seanger *et al.,* 1984).

# Uses in technology

# Genetic engineering

Methods have been developed to purify DNA from organisms, such as phenol- chloroform extraction and manipulate it in the laboratory, such as restriction digests and the polymerase chain reaction. Modern biology and biochemistry make intensive use of these techniques in recombinant DNA technology. Recombinant DNA is a man- made DNA sequence that has been assembled from other DNA sequences. They can be transformed into organisms in the form of plasmids or in the appropriate format, by using a viral vector. The genetically modified organisms produced can be used to produce products such as recombinant proteins, used in medical research, or be grown in agriculture.

This highly simplified description of rDNA technology does not fully convey the enormous complexity and awesome economy and efficiency of genetic processes. But we can begin to understand how, by using rDNA, it is possible to produce substances of medical and economic value (Saenger *et al.,* 1984).

# Forensics

Forensic scientists can use DNA in blood, semen, skin, saliva or hair found at a crime scene to identify a matching DNA of an individual, such as a perpetrator. This process is called genetic fingerprinting, or more accurately, DNA profiling. In DNA profiling, the lengths of variable sections of repetitive DNA, such as short tandem repeats and minisatellites, are compared between people. This method is usually an extremely reliable technique for identifying a matching DNA. However, identification can be complicated if the scene is contaminated with DNA from several people. DNA profiling was developed in 1984 by British geneticist Sir Alec Jeffreys, and first used in

forensic science to convict Colin Pitchfork in the 1988 Enderby murders case (Saenger

*et al.,* 1984).

People convicted of certain types of crimes may be required to provide a sample of DNA for a database. This has helped investigators solve old cases where only a DNA sample was obtained from the scene. DNA profiling can also be used to identify victims of mass casualty incidents. On the other hand, many convicted people have been released from prison on the basis of DNA techniques, which were not available when a crime had originally been committed (Butler *et al.,* 2001).

# Bioinformatics

Bioinformatics involves the manipulation, searching, and data mining of DNA sequence data. The development of techniques to store and search DNA sequences have led to widely applied advances in computer science, especially string searching algorithms, machine learning and database theory. String searching or matching algorithms, which find an occurrence of a sequence of letters inside a larger sequence of letters, were developed to search for specific sequences of nucleotides. In other applications such as text editors, even simple algorithms for this problem usually suffice, but DNA sequences cause these algorithms to exhibit near-worst-case behaviour due to their small number of distinct characters. The related problem of sequence alignment aims to identify homologous sequences and locate the specific mutations that make them distinct. These techniques, especially multiple sequence alignment, are used in studying phylogenetic relationships and protein function. Data sets representing entire genomes' worth of DNA sequences, such as those produced by the Human Genome Project, are difficult to use without annotations, which label the locations of genes and regulatory elements on each chromosome. Regions of DNA

sequence that have the characteristic patterns associated with protein- or RNA-coding genes can be identified by gene finding algorithms, which allow researchers to predict the presence of particular gene products in an organism even before they have been isolated experimentally (Gregory *et al.,* 2006).

# DNA nanotechnology

DNA nanotechnology uses the unique molecular recognition properties of DNA and other nucleic acids to create self-assembling branched DNA complexes with useful properties. DNA is thus used as a structural material rather than as a carrier of biological information. This has led to the creation of two-dimensional periodic lattices (both tile-based as well as using the "DNA origami" method) as well as three- dimensional structures in the shapes of polyhedra. Nanomechanical devices and algorithmic self-assembly have also been demonstrated, and these DNA structures have been used to template the arrangement of other molecules such as gold nanoparticles and streptavidin proteins.

# DNA EXTRACTION

DNA isolation is a routine procedure to collect DNA for subsequent molecular or forensic analysis. There are three basic and one optional steps in a DNA extraction: (Tian *et al.,* 2000)

* + - Breaking the cells open, commonly referred to as cell disruption or cell lysis, to expose the DNA within. This is commonly achieved by grinding or sonicating the sample.
    - Removing membrane lipids by adding a detergent.
    - Removing proteins by adding a protease (optional but almost always done).

Precipitating the DNA with an alcohol — usually ice-cold ethanol or isopropanol. Since DNA is insoluble in these alcohols, it will aggregate together, giving a pellet upon centrifugation. This step also removes alcohol-soluble salt.

Refinements of the technique include adding a chelating agent to sequester divalent cations such as Mg2+ and Ca2+. This stops dnase enzymes from degrading the DNA (Wolfe *et al.,* 2002).

Cellular and histone proteins bound to the DNA can be removed either by adding a protease or by having precipitated the proteins with sodium or ammonium acetate, or extracted them with a phenol-chloroform mixture prior to the DNA-precipitation.

If desired, the DNA can be resolubilized in a slightly alkaline buffer or in ultra-pure water. (Tian *et al.,* 2000)

# 2.3.1 Detecting DNA

A diphenylamine (DPA) indicator will confirm the presence of DNA. This procedure involves chemical hydrolysis of DNA: when heated (e.g. ≥95 °C) in acid, the reacti on requires a deoxyribose sugar and therefore is specific for DNA. Under these conditions, the 2-deoxyribose is converted to w-hydroxylevulinyl aldehyde, which reacts with the compound, diphenylamine, to produce a blue-colored compound. DNA concentration can be determined measuring the intensity of absorbance of the solution at the 600 nm with a spectrophotometer and comparing to a standard curve of known DNA concentrations. (Tian *et al.,* 2000)

Measuring the intensity of absorbance of the DNA solution at wavelengths 260 nm and 280 nm is used as a measure of DNA purity. DNA absorbs UV light at 260 and 280

nanometres, and aromatic proteins absorb UV light at 280 nm; a pure sample of DNA has the 260/280 ratio at 1.8 and is relatively free from protein contamination. A DNA preparation that is contaminated with protein will have a 260/280 ratio lower than 1.8.

DNA can be quantified by cutting the DNA with a restriction enzyme, running it on an agarose gel, staining with ethidium bromide or a different stain and comparing the intensity of the DNA with a DNA marker of known concentration.

Using the Southern blot technique this quantified DNA can be isolated and examined further using PCR and RFLP analysis. These procedures allow differentiation of the repeated sequences within the genome. It is these techniques which forensic scientists use for comparison, identification, and analysis. (Wolfe *et al.,* 2002).

# RESTRICTION ENZYME

A restriction enzyme (or restriction endonuclease) is an enzyme that cuts double- stranded or single stranded DNA at specific recognition nucleotide sequences known as restriction sites. Such enzymes, found in bacteria and archaea, are thought to have evolved to provide a defense mechanism against invading viruses. Inside a bacterial host, the restriction enzymes selectively cut up foreign DNA in a process called restriction; host DNA is methylated by a modification enzyme (a methylase) to protect it from the restriction enzyme’s activity. Collectively, these two processes form the restriction modification system. To cut the DNA, a restriction enzyme makes two incisions, once through each sugar-phosphate backbone (i.e. each strand) of the DNA double helix. ( Kessel and Manta 1990)

Restriction enzymes recognize a specific sequence of nucleotides and produce a double-stranded cut in the DNA. While recognition sequences vary between 4 and 8 nucleotides, many of them are palindromic, which correspond to nitrogenous base sequences that read the same backwards and forwards. In theory, there are two types of palindromic sequences that can be possible in DNA. The mirror-like palindrome is similar to those found in ordinary text, in which a sequence reads the same forward and backwards on the same DNA strand (i.e., single stranded) as in GTAATG. The inverted repeat palindrome is also a sequence that reads the same forward and backwards, but the forward and backward sequences are found in complementary DNA strands (i.e., double stranded) as in GTATAC (Notice that GTATAC is complementary to CATATG)]. The inverted repeat is more common and has greater biological importance than the mirror-like. (Robert *et al.,* 1976)

Recognition sequences in DNA differ for each restriction enzyme, producing differences in the length, sequence and strand orientation (5' end or the 3' end) of a sticky-end "overhang" of an enzyme restriction.

Different restriction enzymes that recognize the same sequence are known as neoschizomers. These often cleave in a different locales of the sequence; however, different enzymes which recognize and cleave in the same location are known as an isoschizomer. (Robert *et al.,* 1976).

Restriction endonucleases are categorized into three general groups (Types I, II and III) based on their composition and enzyme cofactor requirements, the nature of their target

sequence, and the position of their DNA cleavage site relative to the target sequence. The restriction enzymes used in this research is hindi III.

# DNA QUANTIFICATION

# Estimation of DNA concentration

Nucleic acid absorbs UV light at 260nm so it possible to calculate the concentration of DNA in a sample, certain protein also absorbs light at this wavelength and so high level of contamination can give a false result. However, protein also absorb light at 280nm therefore by measuring both the absorbance at 260nm and 280nm it is possible to calculate the ratio of nucleic acid to protein in the solution and thus estimate the accuracy of DNA concentration.

A ratio of A260/A280 = 1.8 is considered acceptable. A higher value is the reflection of purer sample. A lower value indicates high level of protein contamination and the estimation of the DNA will not be accurate. For higher molecular weight DNA 1.0 OD is equivalent to 35µg DNA.

It is known that double strand DNA at a concentration of approximately 50µg/ml reads A260 = 1.0 (Manual centre of biotechnology ABU Zaria 2008).

# Procedure Of DNA Quantification

Elution buffer was used as blank to zero the machine and to get the baseline, 20µl of DNA sample was transferred to 1ml quartz cuvettes at time, and the reading was

recorded. This procedure was done for the entire sample. The cuvettes were washed thoroughly with distilled water before applying another sample to minimise contamination. For each sample the machine calculates absorbance at 260nm and 280nm and automatically gives the concentration of the DNA in that particular sample this was repeated for the entire sample and the results were documented.

# CHARACTERIZATION OF DNA BY AGAROSE GEL ELECTROPHORESIS

Agarose gel electrophoresis is a standard to separate, identify and purify DNA and occasionally RNA fragment. The location of DNA within the gel can be conveniently determined by staining with low concentration of ethidium bromide, an intercalating fluorescent dye. The DNA bands in an agarose gel can be cut out to recover the DNA with high yield.

Agarose gel electrophoresis of DNA is the most successful in buffer of 8.0, there are several buffer used the most commonly employed being tri acetate, tri phosphate and tri borate

Tri acetate has lowest buffer capacity, and is recommended for routine analysis. Tri phosphate has advantage over tri borate that it gel dissolved in sodium perchlorate or potassium iodide (Manual centre of biotechnology ABU Zaria 2008)

# POLYMERASE CHAIN REACTION

The polymerase chain reaction (PCR) is a technique to amplify a single or few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. The method relies on thermal cycling,

consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Primers (short DNA fragments) containing sequences complementary to the target region along with a DNA polymerase (after which the method is named) are key components to enable selective and repeated amplification. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified. PCR can be extensively modified to perform a wide array of genetic manipulations.

Almost all PCR applications employ a heat-stable DNA polymerase, such as Taq polymerase, an enzyme originally isolated from the bacterium Thermus aquaticus. This DNA polymerase enzymatically assembles a new DNA strand from DNA building blocks, the nucleotides, by using single-stranded DNA as a template and DNA oligonucleotides (also called DNA primers), which are required for initiation of DNA synthesis.(Hubscher *et al.,* 2002) The vast majority of PCR methods use thermal cycling, i.e., alternately heating and cooling the PCR sample to a defined series of temperature steps. These thermal cycling steps are necessary first to physically separate the two strands in a DNA double helix at a high temperature in a process called DNA melting. At a lower temperature, each strand is then used as the template in DNA synthesis by the DNA polymerase to selectively amplify the target DNA. The selectivity of PCR results from the use of primers that are complementary to the DNA region targeted for amplification under specific thermal cycling conditions.

PCR is now a common and often indispensable technique used in medical and biological research labs for a variety of applications. These include DNA cloning for

sequencing, DNA-based phylogeny, or functional analysis of genes; the diagnosis of hereditary diseases; the identification of genetic fingerprints (used in forensic sciences and paternity testing); and the detection and diagnosis of infectious diseases. In 1993, Mullis was awarded the Nobel Prize in Chemistry for his work on PCR.

# Principles of PCR

PCR is used to amplify a specific region of a DNA strand (the DNA target). Most PCR methods typically amplify DNA fragments of up to ~10 kilo base pairs (kb), although some techniques allow for amplification of fragments up to 40 kb in size (Barlet and Sterling 2003)

A basic PCR set up requires several components and reagents. These components include:

DNA template that contains the DNA region (target) to be amplified.

Two primers that are complementary to the 3' (three prime) ends of each of the sense and anti-sense strand of the DNA target.

Taq polymerase or another DNA polymerase with a temperature optimum at around 70

°C.

Deoxynucleoside triphosphates (dNTPs; also very commonly and erroneously called deoxynucleotide triphosphates), (Barlet and Sterling 2003) the building blocks from which the DNA polymerases synthesizes a new DNA strand.

Buffer solution, providing a suitable chemical environment for optimum activity and stability of the DNA polymerase.

Divalent cations, magnesium or manganese ions; generally Mg2+ is used, but Mn2+ can be utilized for PCR-mediated DNA mutagenesis, as higher

Mn2+ concentration increases the error rate during DNA synthesis Monovalent cation potassium ions.

The PCR is commonly carried out in a reaction volume of 10–200 µl in small reaction tubes (0.2–0.5 ml volumes) in a thermal cycler. The thermal cycler heats and cools the reaction tubes to achieve the temperatures required at each step of the reaction . Many modern thermal cyclers make use of the Peltier effect which permits both heating and cooling of the block holding the PCR tubes simply by reversing the electric current. Thin-walled reaction tubes permit favorable thermal conductivity to allow for rapid thermal equilibration. Most thermal cyclers have heated lids to prevent condensation at the top of the reaction tube. Older thermocyclers lacking a heated lid require a layer of oil on top of the reaction mixture or a ball of wax inside the tube.

# Application of PCR

# Selective DNA isolation

PCR allows isolation of DNA fragments from genomic DNA by selective amplification of a specific region of DNA. This use of PCR augments many methods, such as generating hybridization probes for Southern or northern hybridization and DNA cloning, which require larger amounts of DNA, representing a specific DNA region. PCR supplies these techniques with high amounts of pure DNA, enabling analysis of DNA samples even from very small amounts of starting material.

Other applications of PCR include DNA sequencing to determine unknown PCR- amplified sequences in which one of the amplification primers may be used in Sanger sequencing, isolation of a DNA sequence to expedite recombinant DNA technologies involving the insertion of a DNA sequence into a plasmid or the genetic material of another organism. Bacterial colonies (E.coli) can be rapidly screened by PCR for correct DNA vector constructs. PCR may also be used for genetic fingerprinting; a forensic technique used to identify a person or organism by comparing experimental DNAs through different PCR-based methods.

Some PCR 'fingerprints' methods have high discriminative power and can be used to identify genetic relationships between individuals, such as parent-child or between siblings, and are used in paternity testing. This technique may also be used to determine evolutionary relationships among organisms. (Barlet and Sterling 2003)

# Amplification and quantification of DNA

Because PCR amplifies the regions of DNA that it targets, PCR can be used to analyze extremely small amounts of sample. This is often critical for forensic analysis, when only a trace amount of DNA is available as evidence. PCR may also be used in the analysis of ancient DNA that is tens of thousands of years old. These PCR-based techniques have been successfully used on animals, such as a forty-thousand-year-old mammoth, and also on human DNA, in applications ranging from the analysis of Egyptian mummies to the identification of a Russian Tsar.

Quantitative PCR methods allow the estimation of the amount of a given sequence present in a sample – a technique often applied to quantitatively determine levels of

gene expression. Real-time PCR is an established tool for DNA quantification that measures the accumulation of DNA product after each round of PCR amplification.

* + - 1. **Uses of PCR** Mutation screening Genetic matching Detection of pathogens Pre-natal diagnosis DNA fingerprinting Gene therapy

Drug discovery Classification of organisms Genotyping

Molecular Archaeology Molecular Epidemiology Molecular Ecology Bioinformatics

Genomic cloning

Site-directed mutagenesis Gene expression studies

The speed and ease of use, sensitivity, specificity and robustness of PCR has revolutionised molecular biology and made PCR the most widely used and powerful technique with great spectrum of research and diagnostic application

# CHAPTER 3 MATERIALS AND METHOD

# MATERIALS

# Reagents

Tris Acetate EDTA (TAE) stock 50X Buffer Agarose powder

Ethidium Bromide (10mg/ml)

Loading buffer ( bromo thymol blue)

Primers (forward and reverse) 5'-GCGAATTCCGTTACTGGTAACCTGACC- TCTTC-3' and 5'-TCCTTAAGCTCCCACATACCTGCCA- TTGTC-3',

PCR and Sterile RNAse free water mix ;( inqaba biotech SA) DNA Ladder (1Kb)

Zymo DNA Extraction kit;( inqaba biotech SA) DNA Elution Buffer

Distilled Water

# Equipments

* + - * UV transluminator (TFX-35-M)
      * Electrophoretic tank (mini tank)HU10
      * Eppendorf pipette(RE43)
      * Rocking platform (Mini shaker) MS11P21
      * Eppendorf tube 1.5ml
      * Heating Mantle (Grant boekel type BBA2)
      * Micro Centrifuging machine (Eppendorf Concentrator 5301)
      * Weighing balance (QT 600)
      * Eppendorf Biophotometer (Eppendorf Ag 22331 HAMBURG)
      * PCR machine (Techne TC312)
      * Gel documentation unit(UVITEC P93E)
      * Microwave Oven (Mx310tcsl)

# METHOD

# Preparation of reagents

The primers were reconstituted using TE buffer, the forward primer was reconstituted with 371ml of TE while the reverse was reconstituted with 378ml of TE buffer.

# Extraction of DNA

39 frozen blood samples were thawed at room temperature, 80µl of each blood sample was measured with eppendorf pipette and transferred into an eppendorf micro centrifuge tube and made up to 100µl with distilled water. 95µl and 5µl of 2x Digestion Buffer and Proteinase K were added respectively into the tubes.

The blood and reagents were properly mixed and tubes incubated at 55ºC for 20 minutes on a hot mantle.

700µl of Genomic Lysis Buffer was added to each of the tubes and then thoroughly mixed by vortexing.

The mixtures were transferred into Zymo-Spin ΙΙC column in a collection tube and then centrifuged at 10,000g for one minute.

200µl of DNA Pre-Wash Buffer was added to the spin columns in a new collection tube and then centrifuged at 10,000g for one minute.

400µl of g-DNA Wash Buffer was added into the spin columns and centrifuged at 10,000g for one minute.

The spin columns were transferred into clean micro centrifuge tubes; 50µl of DNA Elution Buffer was added to the spin columns and incubated at room temperature for 2 - 5 minutes. The tubes were then centrifuge at 10,000g for 30 seconds to elute the DNA.

The eluted DNA was quantified using a biophotometer and characterized by Agarose gel Electrophoresis.

# Procedure of DNA Quantification

Elution buffer was used to blank the eppendorf biophotometer, 20µl of DNA sample was transferred to 1ml quartz cuvettes,

The readings for the absorbance at 260nm, 280nm, and the ratio of absorbance at 260/280 were recorded simultaneously.

This procedure was done for all samples and the readings were recorded for the calculation of DNA concentration (µg/ml).

DNA Concentration µg/ml = OD x Dilution factor x 50µgDNA/ul

\_\_\_ \_ \_ \_ \_ \_ \_ \_.

1 O D260 unit

# Protocol (Gel preparation)

0.8% Agarose was made by dissolving 0.8g of Agarose powder in 100ml of 1 x TAE buffer in a beaker, the mixture is homogenised by heating in a microwave oven for 2 minutes. Agarose mixture was allowed to cool to 60oC and 5ul of Ethidium bromide was added (this intercalates with the nucleic acid and when viewed under UV it fluoresce.)

The gel tray was placed inside the tank in opposite direction to prevent spillage when the gel was poured.

The well-forming comb was placed near the edge of gel to make sure that fingers of the comb are slightly above the plate, but not touching it.

The agarose solution was poured into gel tray to make gel.

While waiting for the gel to polymerise, 300ml of 1 x TAE buffer for electrophoresis tank was prepared and transferred into a clean electrophoresis tank.

The gel slab was placed horizontally in electrophoresis tank and the comb gently removes. The gel was totally submerged in the buffer.

5µl of gel loading buffer was added to each 15µl of sample (total 20µl) and the samples carefully loaded into individual well.

The power was turn on to run at 80volts for 45 minutes. (DNA moves from minus (negative) to plus (positive) electrode poles, alternatively, the gel can be run for a shorter time at higher voltage. Generally it can be run for a shorter time at higher voltage, up to 100volt depending upon the size of DNA)

When dye front has travelled approximately 80% of the gel length, the power was switch off and the gel tray was removed containing the gel.

The gel was viewed under UV and later documented in Gel documentation unit.

# PCR (Polymerase chain reaction)

The DNA samples were thawed at room temperature and each sample was treated as follows. 2µl of the DNA was taken into the PCR eppendorf tube , then 1.0µl of primer 1 (forward) was added followed by another 1.0µl of primer 2 (reverse). 21µl of RNAse

free water was the added followed by 25µl of PCR mix. This activities were carried out inside a bio safety cabinet and the resultant mixtures were kept on ice.

The mixture was vortex for 30 seconds to allow for proper mix, this procedure was followed for all the samples and then transferred to the thermocycler machine which has been programmed.

Optimisations of the PCR processes were carried out as follows after several rounds of trials:

Initial denaturation at 95oC for 5 minutes follows by denaturation at 95oC for 1 minute, Annealing at 48oC for 1 minute and Extension at 70oC for 2 minutes. Final extension at 72oC for 10 minutes and then final hold at 10oC for 30 minutes, the number of cycles used 30.

At the end of the process the samples are taken out from the thermocycler and loaded on already prepared agarose gel and allowed to run, then the agarose was viewed in the gel documentation unit. The results are as follows.

# CHAPTER 4 RESULTS

* 1. **THE GENOMIC DNA EXTRACTED FROM THE BLOOD OF PARENTS AND OFFSRINGS.**

The Genomic DNA Extracted from parents and their children (Rickets and non rickets children are shown in Figures 1a, 1b and 1c.

The intensity of the band is a function of the DNA concentration in the sample loaded to each lane.



F1M1 C1 R1 F2 M2 C2 R2 F3 M3 C3 R3 F4 M4 C4 R4

**Figure 4.1a.** Electrophoregraph of the genomic DNA extracted from the blood of parents and offspring.

**KEY**

F1 – Father family 1 M1 – Mother family 1

C1 – Child without rickets family 1 R1 – Child with rickets family 1 F2 – Father family 2

**Plate 1**

M2 – Mother family 2

C2 – Child without rickets family 2 R2 – Child with rickets family 2 F3 – Father family 3

M3 – Mother family 3

C3 – Child without rickets family 3 R3 – Child with rickets family 3 F4 – Father family 4

M4 – Mother family 4

C4 – Child without rickets family 4 39

R4 – Child with rickets family 4



C3 R3 F4 M4 C4 R4 F5 M5 C5 R5 F6 M6 C6 R6

**Figure 4.1b.** Electrophoregraph of the genomic DNA extracted from the blood of parents and offspring.

# Keys

C3 – Child without rickets family 3 R3 – Child with rickets family 3 F4 – Father family 4

M4 – Mother family 4

C4 – Child without rickets family 4 R4 – Child with rickets family 4 F5 – Father family 5

M5 -Mother family 5

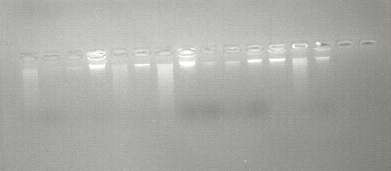
R5 – Child – Mother family 5

C5 – Child without rickets family 5 F6 – Father family 6

M6 – Mother family 6

R6– Child – Mother family 6

C6 – Child without rickets family 6



C7R7F8 M8C8 R8F9 M9C9 R9F10M10C10R10

**Figure 4.1c.** Electrophoregraph of the genomic DNA extracted from the blood of parents and offspring.

# Keys

C7 – Child without rickets family 7 R7 – Child with rickets family 7 F8 – Father family 8

M8 – Mother family 8

C8 – Child without rickets family 8 R8 – Child with rickets family 8 F9 – Father family 9

M9 – Mother family 9

C9 – Child without rickets family 9 R9 – Child with rickets family 9 F10 – Father family 10

M10 – Mother family 10

C10 – Child without rickets family 10 R10 - Child with rickets family 10

# DNA QUANTIFICATION OF PARENTS, RICKETS AND NON RICKETS CHILDREN

The quantification of genomic DNA extracted from parents and their children showing concentration in (µg/ml) and ratio of absorbance at 260/280nm are shown in Table 1 Lower concentration of DNA were observed for the rickets children.

Comparison of mean concentration (µg/ml) and absorbance between rickets and non rickets children are shown in Table 2. No significant differences (P>0.05) were observed for the comparison.

Table 1. Quantification of genomic DNA of parents and children.

|  |  |  |  |
| --- | --- | --- | --- |
| S/Number | Code | Concentration  µg/ml | Absorbance  260/280 |
| 1. | F1 | 2.9 | 1.04 |
| 2. | M1 | 22.4 | 1.27 |
| 3. | C1 | 12.8 | 1.20 |
| 4. | R1 | 6.7 | 1.18 |
| 5. | F2 | 15.2 | 1.15 |
| 6. | M2 | 13.0 | 1.13 |
| 7. | C2 | 17.9 | 1.56 |
| 8. | R2 | 8.8 | 1.07 |
| 9. | F3 | 27.0 | 1.20 |
| 10. | M3 | 36.9 | 1.02 |
| 11. | C3 | 3.5 | 1.25 |
| 12. | R3 | 1.8 | 1.04 |
| 13. | F4 | 5.6 | 1.08 |
| 14. | M4 | 4.8 | 1.11 |
| 15. | C4 | 3.6 | 1.03 |
| 16. | R4 | 1.9 | 1.07 |
| 17. | F5 | 28.5 | 0.65 |
| 18. | M5 | 22.0 | 1.06 |
| 19. | C5 | 3.0 | 1.06 |
| 20. | R5 | 1.3 | 1.02 |
| 21. | F6 | 3.5 | 1.43 |
| 22. | M6 | 4.5 | 1.24 |
| 23. | C6 | 1.3 | 1.14 |
| 24. | R6 | 6.2 | 1.3 |
| 25. | F7 | 4.8 | 0.95 |
| 26. | M7 | 7.3 | 1.29 |
| 27. | C7 | 6.8 | 0.81 |
| 28. | R7 | 3.8 | 1.18 |
| 29. | F8 | 9.8 | 1.11 |
| 30. | M6 | 11.3 | 1.14 |
| 31. | C8 | 5.9 | 1.36 |
| 32. | R8 | 3.4 | 1.42 |
| 33. | F9 | 20.4 | 1.09 |
| 34. | M9 | 33.2 | 1.05 |
| 35. | C9 | 7.2 | 1.04 |
| 36. | R9 | 2.4 | 1.07 |
| 37. | F10 | 19.0 | 1.06 |
| 38. | M10 | 19.3 | 1.06 |
| 39. | C10 | 16.8 | 1.02 |
| 40. | R10 | 9.1 | 1.10 |

Table 4.2. Comparison of genomic DNA concentrations of children with rickets and non rickets.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Rickets | Conc.  µg/ml | Absorbance  260/280 | Non  Rickets | Conc.  µg/ml | Absorbance260/280 |
| R1 | 6.7 | 1.18 | C1 | 12.8 | 1.20 |
| R2 | 8.8 | 1.07 | C2 | 17.9 | 1.56 |
| R3 | 1.8 | 1.04 | C3 | 3.5 | 1.25 |
| R4 | 1.9 | 1.07 | C4 | 3.6 | 1.03 |
| R5 | 1.3 | 1.02 | C5 | 3.0 | 1.06 |
| R6 | 6.2 | 1.3 | C6 | 1.3 | 1.14 |
| R7 | 3.8 | 1.18 | C7 | 6.8 | 0.81 |
| R8 | 3.4 | 1.42 | C8 | 5.9 | 1.36 |
| R9 | 2.4 | 1.07 | C9 | 7.2 | 1.04 |
| R10 | 9.1 | 1.10 | C10 | 16.8 | 1.02 |
| Total | 45.4 | 10.27 |  | 78.8 | 10.27 |
| Mean | 4.54 | 1.03 |  | 7.88 | 1.03 |

T test analysis gives 1.6 at 95% confidence interval (P>0.05)

# RESTRICTION FRAGMENT LENGTH POLYMORPHS OF DNA FROM PARENTS AND OFFSPRING’S.

Restriction fragment of DNA from parents and offspring produced using the enzymes

(hindi III) are shown in figure 2



1 2 3 4 5 6 7 8 9 10 11

**Figure 4.2.** Electrophoregraph of the restriction fragment length polymorphs of DNA from parents and offspring’s**.**

Key

* + 1. Genomic DNA from family 2 (M2)
    2. Restriction Digest of Genomic DNA M2
    3. Genomic DNA from Ricket Child Family 2 (R2)
    4. Restriction Digest of Genomic DNA R2
    5. Genonic DNA from Family 2 (F2)
    6. Restriction Digest from F2
    7. Overflow of well 6
    8. Genomic DNA from Family 3 (F3)
    9. Genomic DNA from Family 3 (M3)
    10. Restriction Digest of R3
    11. Genomic DNA from Family 3 (R3)

# POLYMERASE CHAIN REACTION

Amplified fragment length polymorphs from restriction fragment length polymorphs of parent and offspring’s. (Before optimisation) are shown in figure 4.3a. Only the primer front and no bands were observed.

The Amplified fragment length polymorphs from restriction fragment length polymorphs of parent and offspring’s (After optimisation) are shown in figure 4.3b. Intense amplified fragments at R2 and R3 while smear amplification at M2, F3 and C2 were observed.



**Figure 4.3a.** Electrophoregraph of amplified fragment length polymorphs from restriction fragment length polymorphs of parent and offspring’s. (Before optimisation)

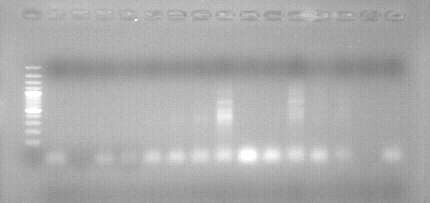
**KEY**

M – Molecular Ladder F1 – Father family 1 M1 – Mother family 1

C1 – Child without rickets family 1 R1 – Child with rickets family 1 F2 – Father family 2

M2 – Mother family 2

C2 – Child without rickets family 2 R2 – Child with rickets family 2



M F1 M1C1 R1 F2 M2 C2 R2 F3 M3C3R3 F4 M4 C4 R4

**Figure 4.3b**. Electrophoregraph of amplified fragment length polymorphs from restriction fragment length polymorphs of parent and offspring’s (after optimisation)

**KEY**

**M**- Molecular ladder F1 – Father family 1 M1 – Mother family 1

C1 – Child without rickets family 1 R1 – Child with rickets family 1 F2 – Father family 2

M2 – Mother family 2

C2 – Child without rickets family 2 R2 – Child with rickets family 2 F3 – Father family 3

M3 – Mother family 3

C3 – Child without rickets family 3 R3 – Child with rickets family 3 F4 – Father family 4

M4 – Mother family 4

C4 – Child without rickets family 4 R4 – Child with rickets family 4

Arrow indicating Amplified length polymorph Arrow indicating Amplified length polymorph

 Smear amplified length polymorph

# CHAPTER 5 DISCUSSION

* 1. **THE GENOMIC DNA EXTRACTED FROM THE BLOOD OF PARENTS AND OFFSRINGS.**

All the samples showed bands which indicated the presence of DNA. The differences observed in the intensity of the bands may be as a results of difference in the concentration of the nuclei acid in the samples, because the higher the intensity of the bands the higher the concentrations of the DNA.

It was observed from the electrophoregraph that the concentration of the DNA in rickets child was generally lower than other members of his/her family without the disorder, this could interpreted from the point of view that rickets is a long bone disease and blood formation occur in the long bone therefore the lesser concentration of nuclei acid the lesser the intensity of the bands in the rickets children.

# DNA QUANTIFICATION OF PARENTS, RICKETS AND NON RICKETS CHILDREN

All the samples tested were found to have different concentration of the DNA and there is no significant difference observed in comparing the mean concentration µg/ml and absorbance between rickets and non rickets using T-test with a calculated value of 1.6 as against 1.73 tabulated . (P>0.05)

# RESTRICTION FRAGMENT LENGTH POLYMORPHS OF DNA FROM PARENTS AND OFFSPRING.

Restriction fragment of DNA from parents and offspring, which were shown in fig. 2 were used in the polymerase chain reaction. Samples M2, R2, F2 and R3 were restricted while samples M3, F3, C2 and M1 were not restricted. The non restriction of these sample could be due to the limited number of restriction enzymes used.

# POLYMERASE CHAIN REACTION

The VDR gene responsible for HVDRR (hereditary vitamin D resistant rickets) was successfully amplified in R2 and R3. (Helena *et al.,* 1989). This implies that the hereditary influence of vitamin D receptor gene observed by Helena et al (1989) in a different study could also be responsible for rickets in kaso, jankasa and gonin gora areas of kaduna state Nigeria.

The slight bands observed for M2 and C2 in non rickets individuals could be evidence that the VDR gene is hereditary and that the mother pose the gene at an extremely low level which might be pronounced better at high PCR cycle.

# CHAPTER 6

**SUMMARY, CONCLUSION AND RECOMMENDATIONS**

# SUMMARY

Three families from Jankasa, Kaso and two families from Gonin Gora composing of father, mother, child without rickets and child with rickets each were used for the study. The DNA were extracted using zymo DNA extraction kits, they were quantified and purified using Biophotometer and gel electrophoresis respectively.

The isolated DNA from all blood samples were restricted, the restricted fragment length polymorph of DNA used in PCR showed that the vitamin D receptor (VDR) gene for hereditary vitamin D resistant rickets (HVDRR) was successfully amplified in rickets children and gave smear amplification in parents and children without rickets at the same PCR cycle.

Comparison of mean concentration (µg/ml) between rickets and non rickets children using T-test gives 1.6 calculated as opposed 1.73 tabulated suggesting no significant difference at 95% confidence interval.

# CONCLUSION

The prevalence of rickets in these communities ranges from 3 -7% of the population (Inuwa., 2011). Also Turaki (2007) and Inuwa (2011) in different studies detected low levels of calcium and high level of phosphorus in diet and blood of people living in these communities respectively. This work demonstrated that there could be evidence of hereditary link which explain the high incidence of Rickets in these communities.

# RECOMMENDATIONS

Further studies are needed to sequence and compare the amplified restriction fragment polymorph obtained from this study to ascertain the base sequence which could then establish this fact, however this work has taken a pertinent step towards sequencing the region of interest to confirm the hereditary link.

The following are also recommended for policy makers:

* + - The community based programmes on nutrition should be initiated at community’s level.
    - Advocacies group should commence IEC to Farmers in these settlements.
    - Consumption of calcium rich foods i.e. soya beans, nono, kindirmo should be encouraged in these areas.
    - Genotyping prior to marriages to help resolve the problem

# References

Anatoliotaki M, Tsilimigaki A, Tsekoura T, Schinaki A, Stefanaki S, Nikolaidou P**.** Sattur A (2003). Congenital rickets due to maternal vitamin D deficiency in a sunny island of Greece*. Acta Paediatr* 92:389–91

Atiq M, Suria A, Nizami S. Q, Ahmed I. (1998). Vitamin D status of breastfed Pakistani infants. ) *Acta Paediatr* 87:737–40.

Bartlett A, Stirling E, (2003) A Short History of the Polymerase Chain Reaction. In: Methods Mol Biol. 226: 3-6

Bhattacharyya A. K. (1992) Nutritional rickets in the tropics. In: Simopoulos A. P, ed.

Nutritional triggers for health and in disease*. World Rev Nutr Diet* 67:140-97.

Butler B. John M. (2001). Forensic DNA Typing. Elsevier. ISBN 978-0-12-147951-0.. pp. 14–15.

Chapuy M. C, Preziosi P, Maamer M, et al.(1997). Prevalence of vitamin D insufficiency in an adult normal population*. Osteoporos Int* 7:439–43

DeLucia M. C, Mitnick ME, Carpenter T. O–(2003). Nutritional rickets with normal circulating 25-hydroxyvitamin D: a call for reexamining the role of dietary calcium intake in North American infants. *J Clin Endocrinol Metab* 88:3539

DeLucia M. C, Mitnick M. E, Carpenter T. O. (2003). Nutritional rickets with normal circulating 25-hydroxyvitamin D: A call for reexamining the role of dietary calcium intake in North American infants*. J Clin Endocrinol Metab* 88:3539- 45.

Fischer P. R, Rahman A, Cimma J. P, (1998). Nutritional rickets without vitamin D deficiency in Bangladesh*. J Trop Pediatr*45:291-3.

Greer F. R, (2003).Vitamin D deficiency: it's more than rickets. . *J Pediatr* 143:422–3

Gregory S; Barlow, K. F; McLay, K. E; Kaul, R; Swarbreck, D; Dunham, A; Scott, C. E; Howe, K. L. (2006)."The DNA sequence and biological annotation of human chromosome 1 *". Nature* 441 (7091): 315–21.

Guillemant J, Cabrol S, Allemandou A, Peres G, Guillemant S.(1995) Vitamin D- dependent seasonal variation of PTH in growing male adolescents. *Bone* 17:513–6

Guillemant J, Le H. T, Maria A, Allemandou A, Peres G, Guillemant S.(2001).Wintertime vitamin D deficiency in male adolescents: effect on parathyroid function and response to vitamin D supplements. *Osteoporos Int* 12:875–9

Holick M. F. Shils M. E, Olson J. A, Shike M, Ross A. C, eds. (1999) Modern nutrition for health and disease. Baltimore: Williams and Wilkins,:329-45.

Hollis B. W, Roos B. A, Draper H. H, Lambert P. W. (1981) Vitamin D and its metabolites in human and bovine milk. *J Nutr*111:1240–8.*.*

Hubscher, U.; Maga, G.; Spadari, S. (2002). "Eukaryotic DNA polymerases". *Annual Review of Biochemistry* 71: 133.

Kessler C, Manta V (1990): "Specificity of restriction endonucleases and DNA modification methyltransferases a review (Edition 3)". 1–248. ).

Kokkonen J, Koivisto M, Kirkinen P (1983) Seasonal variation in serum-25-OH-D in mothers and newborn infants in northern Finland. . *Acta Paediatr Scand* 72:93– 6.

Ma N. S, Malloy P.J Specker B.L, Valanis B, Hertzberg V, Edwards N, Tsang R.C.(1985). Sunshine exposure and serum 25-hydroxyvitamin D concentrations in exclusively breast-fed infants*. J Pediatr* 107:372–6

Mandekarn M, Elias J, Eden D, Crothers D (1981). "The dimensions of DNA in solution *J Mol Biol* 152 (1): 153–61

Mohapatra A, Sankaranarayanan K, Kadam S.S, Binoy S, Kanbur W.A, Mondkar J.A (2003) Congenital rickets. . *J Trop Pediatr* 49:126–7.

Molla A.M, Badawi M.H, Al-Yaish S, Sharma P, El-Salam R.S, Molla A.M. (2000) Risk factors for nutritional rickets in children in Kuwait. *Pediatr Int* 42:280–4.

Oginni L.M, Sharp C.A, Badru O.S, Risteli J, Davie M.W.J, Worsfold M. (2003) Radiological and biochemical resolution of nutritional rickets with calcium. *Arch Dis Child* 88:812-7.

Olivieri M.B, Ladizesky M, Mautalen C.A, Alonso A, Martinez L. (1993) Seasonal variations of 25-hydroxyvitamin D and parathyroid hormone in Ushuaia (Argentina), the southernmost city of the world*. Bone Miner* 20:99–108.

Outila T.A, Karkkainen M.U, Lamberg-Allardt C.J.(2001) . Vitamin D status affects serum parathyroid hormone concentrations during winter in female adolescents: associations with forearm bone mineral density. *Am J Clin Nutr* 74:206–10.

Pettifor J.M, Ross P, Wang J, Moodley G, Couper-Smith J.(2004) Rickets in children of rural origin in South Africa: is low dietary calcium a factor *J Pediatr*

Pfitzner M.A, Thacher T.D, Pettifor J.M, Zoakah A.I, Lawson J.O, Isichei C.O, Fischer P.R.(1998) Absence of vitamin D deficiency in young Nigerian children *J Pediatr* 133:740-4.

Roberts R.J (1976). "Restriction endonucleases". CRC Crit. Rev. Biochem. 4 (2): 123–

64.

Saenger A.D, Wolfram C (1984). Principles of Nucleic Acid Structure. New York: Springer-Verlag. ISBN 0387907629.

Sattur J, Rudolf N, Tomashek M (2006) Respond to vitamin D in X linked hypophosphatamic rickets; Am J Clin Nutr 34: 231-3

Thacher T. D, Fischer P. R, Pettifor J. M, et al. (1999) A comparison of calcium, vitamin D, or both for nutritional rickets in Nigerian children. *N Engl J Med* 341:563-8.

Thacher T.D, Fischer P.R, Pettifor J.M, Lawson J.O, Isichei C.O, Chan G.M. (2000) Case-control study of factors associated with nutritional rickets in Nigerian children. . *J Pediatr* 137:367-73.

Thacher T.D, Ighogboja S.I, Fischer P.R. (1997) Rickets without vitamin D deficiency in Nigerian children*. Ambulatory Child Health* 3:56-64.

Thacher T.D. (2003) Calcium-deficiency rickets*. Endocr Dev* 6:105-25.

Tian H,(2000) . Evaluation of Silica Resins for Direct and Efficient Extration of DNA from Complex Biological Matrices Miniaturized Format. Analytical Biochemistry 283, 175-191 .

Wolfe, (2002). Toward a microchip-based solid-phase extraction method for isolation of nucleic acids. Electrophoresis 23, 727-733 .

Zeghoud F, Vervel C, Guillozo H, Walrant-Debray O, Boutignon H, Garabedian M. (1997) Subclinical vitamin D deficiency in neonates: definition and response to vitamin D supplements. . *Am J Clin Nutr* 65:771–8.

Keller N, Rudolf C, Eugste N*,* Helena H. R,. Tomashek M*,*(1999) Hereditary x linked hypophosphatemic rickets in Arab isreali family *Engl J Med* 310:63-5