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Dear Esteemed Readers, Authors, and Colleagues,

I hope this letter finds you in good health and high spirits. It is my distinct pleasure to address you as the Editor-in-Chief of Integrative Omics and Applied Biotechnology (IIOAB) Journal, a multidisciplinary scientific journal that has always placed a profound emphasis on nurturing the involvement of young scientists and championing the significance of an interdisciplinary approach.

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I would like to extend my gratitude to our authors, reviewers, editorial board members, and readers for their unwavering support. Your dedication is what makes IIOAB Journal the thriving scientific community it is today. Together, we will continue to explore the frontiers of knowledge and pioneer new approaches to solving the world's most complex problems.

Thank you for being a part of our journey, and for your commitment to advancing science through the pages of IIOAB Journal.

Yours sincerely,

Vasco Azevedo

Vasco Azevedo, Editor-in-Chief
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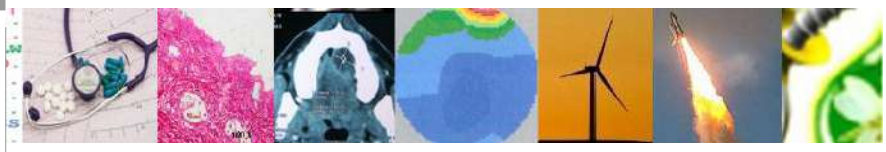

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RESEARCH: PLANT GENOMICS

IDENTIFICATION OF RAPD (RANDOM AMPLIFIED POLYMORPHIC DNA) MARKERS FOR ETHIOPIAN WILD *COFFEA ARABICA* L. GENETIC RESOURCES CONSERVED IN INDIA

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ABSTRACT

Ethiopian coffee (Coffea arabica L.) genetic resources conserved in India have not been subjected to molecular analysis. In the present study, fifteen wild coffee genetic resources representing eight populations and three cultivated varieties of Ethiopian origin were subjected to RAPD analysis. A total of 77 polymorphic RAPD bands were generated by seventeen random primers. The number of polymorphic bands detected with each primer ranged from 2 to 10 with a mean of 6.2 bands per primer. The amount of genetic variation among populations estimated by Shannon diversity indices were in the range of 4.02 to 4.3 indicating a moderate to relatively high level of diversity in the wild populations. Cluster analysis indicated geographical groupings for a few populations without any duplications among the germplasm studied. Present study adds new knowledge on Ethiopian Arabica coffee germplasm conserved in India that is of potential importance in developing a more comprehensive breeding programme for C. arabica and Gene Bank maintenance.

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Keywords: *Coffea; DNA markers; genetic diversity; germplasm; RAPD*

[I] INTRODUCTION

Coffee is one of the world's most valuable commodities, contributing largely to the economy of more than 50 countries of Asia, Latin America and Oceania. Of approximately 100 taxa of the genus Coffea (Family: Rubiaceae), only two species are economically important i.e., Coffea arabica L. and Coffea canephora Pierre ex Froehner, accounting respectively for about 70 percent and 30 percent of the world coffee production. In India the area under coffee cultivation is around 3,48,995 ha of which arabica and robusta account for 48 percent and 52 percent respectively[1].

Genetic consistency within varieties is essential for quality assurance of any agricultural product. Traditionally genetic diversity of genotypes was assessed based on differences in a range of expressions of morphological and agronomical characters. Currently, a variety of molecular techniques are available for measuring genetic diversity. The most common techniques are RAPD, RFLP, AFLP and SSR. All of them detect polymorphism by assaying subsets of the total amount of DNA sequence variation in a genome. However, they differ in principle, application, type and amount of polymorphism detected and cost and time requirements [2]. DNA polymorphisms are important in understanding the genetic variations in natural populations and can be used to explore issues of genetic diversity.

Cultivated varietal resources, utilized in coffee improvement programme posses a narrow genetic base. Thus, there is a need for widening of the existing genetic base by having more introductions especially from the centre of diversity [3]. Lack of variability may lead to vulnerability [4]. if, for example, new virulent pathogen races evolve. It is also important to understand that the adapted, elite germplasm does not contain all the most desirable alleles available in a

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species. Thus, utilization of the available germplasm in the gene banks assumes importance and their characterization is an urgent priority.

Development of coffee varieties of commercial importance in India followed the path of introduction, selection and hybridization. Exotic germplasm conserved in the Gene Bank should be characterized morphologically, biochemically and by molecular markers for further selection and integration of these wild progenitors in the main breeding program. Information about the genetic make-up of accessions helps decision making for conservation activities, which range from collecting and managing through identifying genes to adding value to genetic resources. The information from molecular markers or DNA sequences offers a good basis for better conservation approaches. In combination with morphological and biochemical characterization several DNA marker technologies have been developed and are available for genetic diversity studies [5]. Information on genetic diversity is also needed for the optimal design of plant breeding programmes, influencing the choice of genotypes to be crossed for the development of new plant varieties.

RAPD is the most commonly used marker system and is mainly used in studies assessing genetic diversity in natural plant populations. Adavantages of this technique include requirement of small amounts of template DNA; does not require sequence information for primer construction; is easy and quick to assay, has low cost, has random distribution throughout the genome and generates multiple fragments per reaction [6]. However, it is a dominant marker technique and has a problem of reproducibility of amplification [7]. and requires stringent optimization [8].

The specific objective of this study is to assess the genetic diversity among various accessions from different geographical locations of Ethiopia, maintained in CCRI Gene Bank so as to avoid maintenance of duplicate germplasm in the Gene Bank and possible utilization of the RAPD markers generated for future use in genetic improvement of arabica coffee.

[II] MATERIALS AND METHODS

2.1. Plant material

Fifteen accessions of coffee genetic resources with the entry numbers S.2440, S.2441, S.2600, S.2601, S.2602, S.2615, S.2604, S.2608, S.2642, S.2644, S.2649, S.2650, S.2707, S.2709 and S.2708 in the planting register of Botany Division, Central Coffee Research Institute (CCRI), growing in the Germplasm block of CCRI (Balehonnur, Chikmagalur District, Karnataka) were used as experimental material for RAPD studies. For DNA extraction young leaf tissue sample was used. The materials S.2440 and S.2441 were collected from Abyssinia during 1955 and the rest were from different geographical locations of Ethiopia collected in the wild during an F.A.O. sponsored expedition to Ethiopia in 1964 for exploration of coffee germplasm and the establishment of World Coffee Collections [9] **[Figure–1]**. Accessions S.2600 and S.2601 represent Harar province; S.2602 and S.2615 represent Shoa province; S.2604 and S.2608 represent Sidamo province; S.2642 and S.2644 represent Kaffa province; S.2649 and S.2650 represent Illubabor province; S.2707 and S.2709 represent Gojjam province and S.2708 represents Eritrea province **[Figure–1]**. The three cultivated types of Ethiopian arabica coffee *viz.,* Agaro, Cioccie and Tafarikela were included as comparative standards. At the time of leaf harvesting, plant materials of Abyssinian collections were 42 years old and rest of the collections including the comparative standards were 33 years old; all the genetic resources are healthy with good yield and vegetative growth.

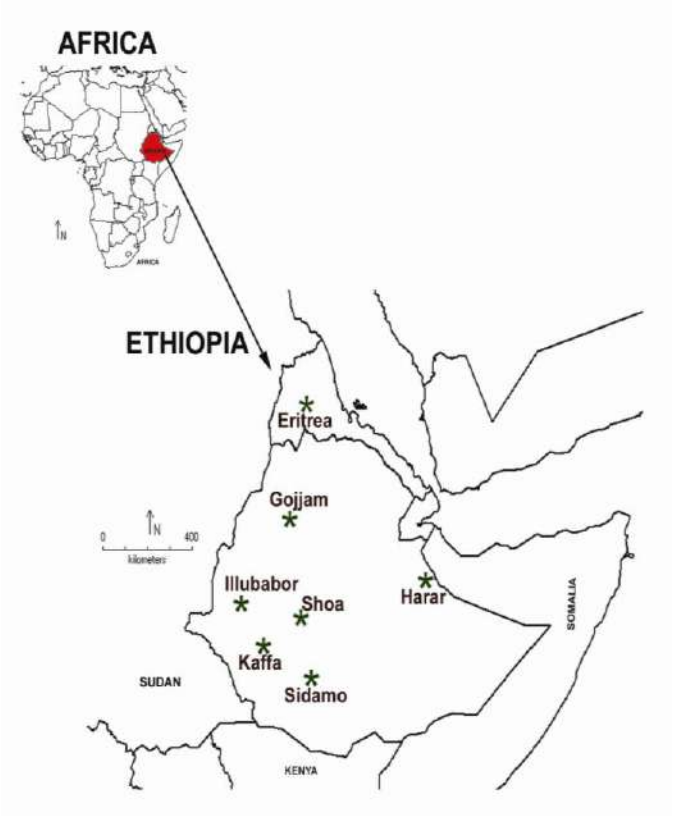

Fig: 1. Germplasm collection localities in Ethiopia during 1964

2.2. DNA extraction and purification

Total DNA was extracted from fresh young leaf tissue following the CTAB method with modifications suggested by Porebski et al. [10]. The protocol involves high salt precipitation, PVP, RNAse treatment followed by Phenol: Chloroform wash. Extracted DNA was purified by phenol extraction [11]. DNA quality was checked by electrophoresis in a 1 % agarose gel and the concentration was estimated in relation to the concentration of co-migration of □-phage DNA and by repeated measurements with spectrophotometer (Shimadzu, Japan) at 260nm.

2.3. Screening of RAPD primers

An initial screening of 140 oligonucleotide primers from Operon Technologies (CA, USA) was carried out, to identify primers that detect polymorphisms; only seventeen primers produced reproducible variation and were used in further analyses **[Table-1]**.The reproducibility of the banding patterns of each primer was tested with respect to the template DNA and magnesium chloride concentration. Optimal conditions were determined for each specific primer, the conditions were strictly followed.

IIOAB ISSN: 0976-3104

Table: 1. Primers (Operon Technologies, Ca, USA) used for RAPD analysis and proportion of polymorphic bands, monomorphic bands, total number of bands and molecular size in different Ethiopian arabica germplasm

Primer	Primer sequence 5' to 3'	Total number of bands scored	Number of polymorphic bands	Number of monomorphic bands	Molecular size range (bp)
OPB07	GGTGACGCAG	5	4	1	800 - 1400
OPB09	TGGGGGACTC	6	3	3	300 - 1280
OPB12	CCTTGACGCA	2	1	1	820 - 1150
OPB18	CCACAGCAGT	3	3	0	740 - 1500
OPC02	GTGAGGCGTC	8	7	1	250 - 1700
OPE04	GTGACATGCC	4	6	1	240 - 800
OPE14	TGCGGCTGAG	7	3	3	870 - 1300
OPE15	ACGCACAACC	4	4	3	300 - 1800
OPE18	GGACTGCAGA	10	1	4	400 - 1400
OPF06	GGGAATTCGG	9	9	2	90 - 1300
OPF14	TGCTGCAGGT	10	7	1	500 - 1200
OPG08	TCACGTCCAC	2	1	1	800 - 1500
OPG11	TGCCCGTCGT	5	4	1	800 - 1400
OPG12	CAGCTCACGA	8	7	1	150 - 1700
OPG13	CTCTCCGCCA	6	3	3	430 - 1750
OPK05	TCTGTCGAGG	10	8	2	275 - 1250
OPP19	GTGGTCCGCA	8	6	2	250 - 1400
Total		107	77	30	--
Range		2-10	1 - 9	1-4	90 - 1800
Average		6.2	4.52	1.76	--

2.4. PCR amplification

The DNA amplification reactions were performed in a total volume of 25 µl containing 1x reaction buffer (75 mM Tris-HCl, pH 8.8, 20 mM (NH4)2SO4, 0.01 % (v/v) Tween 20), 1.5 mM MgCl2, 5 pmoles primer,

1.5 mM dNTPs (10 mM each of dATP, dCTP, dGTP and dTTP), 3U μl^{-1} of Taq polymerase (Bangalore Genei), and 25 to 30 ng of sample DNA. A master mix was prepared for each primer to minimize measurement deviation. The reaction mixtures were overlaid with two drops of light mineral oil.

Amplification was carried out in a Thermal cycler (Eppendorf, Germany) using programme profile consisting of an initial template denaturation at 95ºC for 5 minutes, followed by 42 cycles of one minute denaturation at 94ºC, one minute annealing at 36ºC and 2 minutes extension at 72ºC, with a final extension step of five minutes at 72ºC resulting in an exponential accumulation of specific fragments.

The PCR amplification products were separated on 1.4 % agarose gels containing 0.5 µg ml^{-1} ethidium bromide, and run in 1x TAE buffer (40 mM Tris acetate pH 8.0, 1 mM EDTA) at 90 volts.

2.5. Data scoring and analysis

Each amplified DNA fragment was considered as an independent character (locus), and scored as present (1) or absent (0). Those fragments that were monomorphic, not reproducible, appear in the control reaction or too difficult to score with certainty were excluded from the data analysis. Each amplified product was named by the code of the primer followed by its size in base pairs. Since RAPD markers are dominant, a locus was considered to be polymorphic if present in some and absent in other individuals of the population, and monomorphic if the bands were present in all individuals. No distinction was made between fragments of the same molecular size that varied in intensity. Estimation of genetic variation between the accessions was calculated from Shannon's diversity data. Shannon's diversity index is frequently used in RAPD data analysis because the index is insensitive to bias that may be introduced into data due to undetectable heterozygosity [12, 13]. The similarity matrices computed for each pair of populations were subjected to cluster analysis using STATISTICA for windows (Stat Soft Inc.1999, September 6).

[III] RESULTS

Of the 140 primers initially screened, RAPD patterns from seventeen primers were found to be reproducible and suitable for investigation **[Table–1, Figure–2]**. The seventeen oligonucleotide primers generated a total of 107 countable bands across 18 accessions representing 8 populations and three cultivated varieties. Of which 77 (71.9%) bands were stable (reproducible) polymorphic bands and 30 (28.1%) bands were monomorphic. The number of bands per primer varied from two (OPB12 and OPG08) to ten (OPE18, OPF14 and OPK05) with an average of 6.2 per primer, and the estimated molecular

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size was in the range of 90 to 1800 base pairs [Table–1]. Figure–2 depict the representative picture of the electrophoretic pattern of PCR amplified DNA fragments obtained during the analysis using these primers. The mean value for similarity indices represented by the eighteen coffee germplasm accessions ranged from 0.63 to 0.95 [Table–1] with an overall mean of 0.81, which means that the individuals from each coffee germplasm share, on average, 81 % of their RAPD fragments.

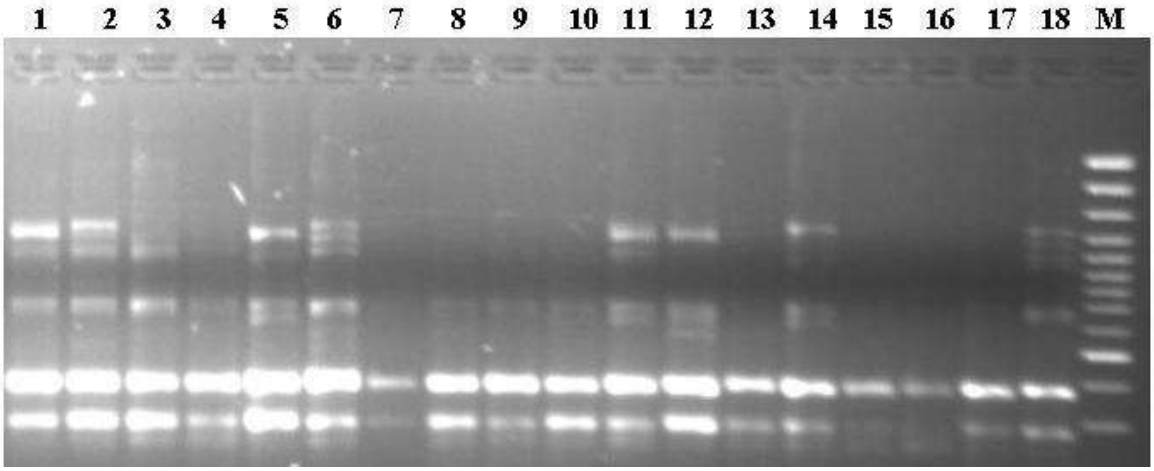

Fig: 2. Example of electrophoretic pattern of PCR amplified DNA fragments of wild coffee genetic resources produced by RAPD primer OPK5. (1- Agaro; 2-Cioccie; 3- Tafarikela; 4-S.2440; 5-S.2441; 6-S.2600; 7-S.2601; 8-S.2602; 9-S.2604; 10-S.2608; 11-S.2615; 12-S.2642; 13-S.2644; 14-S.2649; 15-S.2650; 16-S.2708; 17-S.2709; 18-S.2707; M-marker)

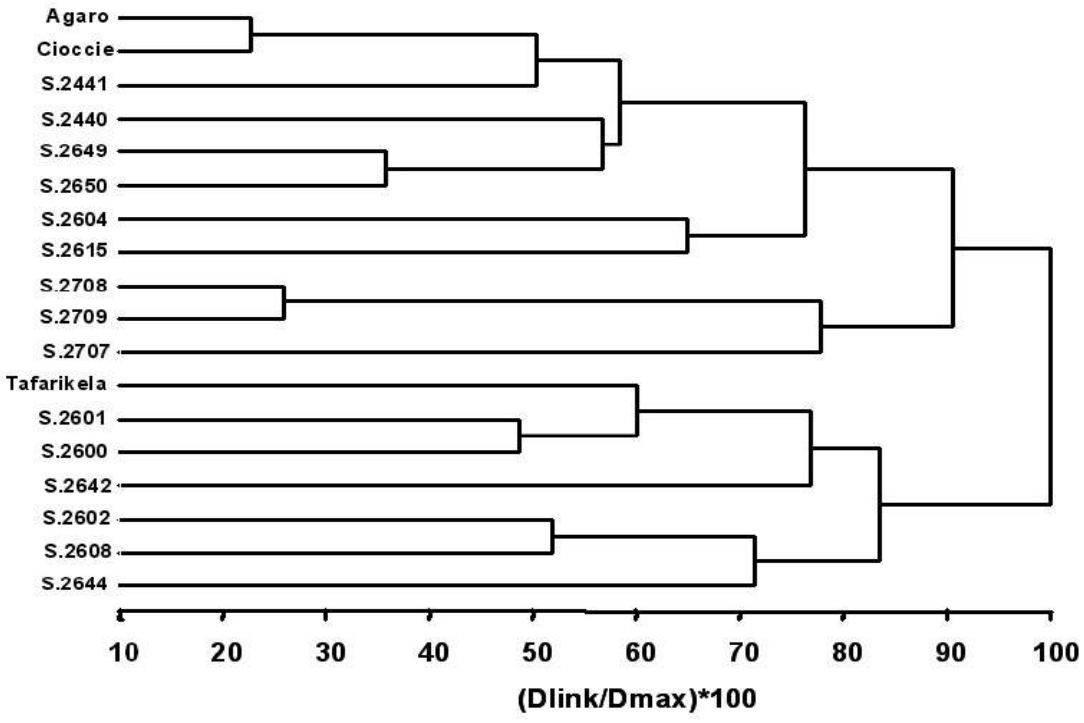

Fig: 3. Dendrogram generated by multivariate paired group Euclidean similarity measure by cluster analysis based on RAPD profiles in the Ethiopian germplasm

IIOAB ISSN: 0976-3104

Table: 2. Dice coefficient of similarity between the 18 germplasm from 5 different geographic locations of Ethiopia

	Agaro*	Cioccie *	Tafarikela *	S.2440	S.2441	S.2600	S.2601	S.2602	S.2604	S.2608	S.2615	S.2642	S.2644	S.2649	S.2650	S.2708	S.2709	S.2707
Agaro *	1																	
Cioccie *	0.95	1																
Tafarikela *	0.80	0.81	1															
S.2440	0.85	0.89	0.80	1														
S.2441	0.85	0.83	0.83	0.84	1													
S.2600	0.88	0.89	0.86	0.84	0.88	1												
S.2601	0.89	0.89	0.79	0.87	0.85	0.82	1											
S.2602	0.76	0.75	0.73	0.79	0.85	0.80	0.77	1										
S.2604	0.84	0.86	0.76	0.86	0.81	0.80	0.88	0.80	1									
S.2608	0.76	0.77	0.81	0.82	0.84	0.84	0.79	0.87	0.86	1								
S.2615	0.82	0.83	0.78	0.80	0.82	0.86	0.82	0.81	0.87	0.82	1							
S.2642	0.75	0.74	0.77	0.77	0.83	0.80	0.77	0.78	0.75	0.82	0.79	1						
S.2644	0.72	0.75	0.70	0.80	0.77	0.80	0.75	0.84	0.80	0.82	0.80	0.79	1					
S.2649	0.85	0.89	0.75	0.88	0.78	0.85	0.86	0.76	0.86	0.77	0.87	0.73	0.82	1				
S.2650	0.86	0.90	0.76	0.87	0.81	0.82	0.90	0.75	0.83	0.73	0.81	0.71	0.77	0.92	1			
S.2708	0.77	0.82	0.70	0.83	0.73	0.75	0.82	0.73	0.78	0.71	0.73	0.65	0.77	0.83	0.86	1		
S.2709	0.78	0.82	0.68	0.81	0.72	0.73	0.83	0.75	0.80	0.71	0.76	0.63	0.77	0.85	0.88	0.94	1	
S.2707	0.82	0.84	0.67	0.78	0.75	0.75	0.81	0.74	0.77	0.67	0.75	0.68	0.75	0.80	0.84	0.81	0.85	1

*Comparative standard

3.1. *Cluster analysis*

The result of the cluster analysis obtained using all the 18 germplasm produced two main clusters **[Figure–3]** of 11 and 7 accessions each. Cluster one consisted of Agaro, Cioccie, the material considered as standard for the study with S.2440, S.2441, S.2649, S.2650, S.2604, S.2615, S.2708, S.2709 and S.2707. Geographical groupings were noted for S.2440 and S.2441 from Abyssinia and S.2707 and S.2709 from Gojjam province in the sub-clusters of the first main cluster but not in pairs. Within the cluster one direct geographical pairing was observed only for S.2649 and S.2650 from Illubabor. Cluster two composed Tafarikela, the material considered as standard for the study with S.2601, S.2600, S.2602, S.2642, S.2644 and S.2608. Geographical groupings were noted only for S.2600 and S.2601 from Harar province sub-clustering with Tafarikela in the cluster two. No duplications were noted among the germplasm studied.

[IV] DISCUSSION

Extent of distribution, areas of sampling and plant characteristics, breeding behavior and generation time are some of the important parameters that determine the level of genetic variability in a species. Because *C. arabica* is an allotetraploid and predominantly self-pollinated species, a high degree of genetic uniformity is expected [3, 14]. Molecular markers revealed the loss of genetic diversity due to the history of *C. arabica* cultivation, the self-pollination and selection by farmers [15, 16].

In coffee, there have been no alternatives to *ex situ* field collections for long-term germplasm conservation, due to recalcitrant seeds. If conventional methods of seed storage are used, the *C. arabica* seeds are known to be viable for a maximum of two to three years [17]. In *ex situ* field collections

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there is a risk of losing valuable germplasm due to diseases, pests and natural disasters as well as to poor adaptation to the local environment. Dulloo *et al.* [18]. have described strategies for *in situ* conservation of *Coffea* species, the *in situ* approach of plant genetic conservation has been recently emphasized [19]. *Ex situ* and *in situ* conservation strategies are complementary and should not be viewed as antagonistic [20]. The *in situ* method allows continuing evolution of the species in its natural habitat in order to allow perpetuation and integration of co-adapted gene complexes [19]. The *ex situ* approach, on the other hand, safeguards the species genetic diversity in case of possible habitat destruction and represent a readily available source of germplasm for research and breeding.

Table: 3. Shannon diversity indices for RAPD markers studied in the Ethiopian coffee germplasm

CCRI accession number	Province details	Taxa (S)	Shannon diversity index (H')
S.2440	Abyssinian province	66	4.19
S.2441		64	4.15
S.2600	Harar province	69	4.23
S.2601		72	4.27
S.2602	Shoa province	67	4.20
S.2615		73	4.29
S.2604	Sidamo province	57	4.04
S.2608		81	4.39
S.2642	Kaffa province	60	4.09
S.2644		71	4.26
S.2649	Illubabor province	79	4.36
S.2650		74	4.30
S.2707	Gojjam province	69	4.23
S.2709		69	4.23
S.2708	Eritrea province	80	4.38
Agaro*	----	76	4.33
Cioccie*	----	77	4.34
Tafarikela*	----	56	4.02
Mean±sd	----	70±7.4	4.23±0.10

* Comparative standard; Standard deviation (sd)

In order to quantify the level of polymorphism between wild coffee germplasm, the Dice coefficient of similarity was used to generate a similarity matrix **[Table–2]**. The genetic distances at the accession level fell in the range of 0.63 to 0.95 indicating a high level of polymorphism among the accessions. Lashermes *et al.* [14] reported a comparable level of molecular polymorphism in wild accessions of *C. arabica*. Anthony *et al.* [3] also reported a similar level of polymorphism among 80 accessions of *C. arabica* derived from spontaneous and sub-spontaneous trees in Ethiopia. In the present study Shannon diversity indices **[Table–3]** were in the range of 4.02 to 4.3, suggesting a relatively higher level of diversity in the wild populations, this holds good since the materials used in the study are collected in the wild without any bias towards the agro morphological characters. Present study has demonstrated that the RAPD technique could be applied for measuring the degree of variability in the wild coffee genetic resources from Ethiopia conserved at CCRI gene bank, India. The RAPD profiles generated in the present study showed moderate to high levels of DNA polymorphism which are in agreement with the morphological studies as per the IPGRI format by Dinesh *et al.,* [1–.2] Thus, for further improvement of coffee in India, we suggest the maintenance of Ethiopian germplasm showing good genetic variability in the gene bank.

ISSN: 0976-3104

In the present study, relatively large differences were observed among the germplasm accessions collected in the Harar and Kaffa provinces (south west highlands of Ethiopia) in comparison to the other accessions from Illubabor, Gojjam and Eritrea and no duplications were noticed from the different collection localities of Ethiopia. Similar observations have been reported from agro morphological data [1, 22].

Since coffee plant is not indigenous to India any further improvement of existing coffee varieties or development of new varieties follows the path of hybridization with the plant of desired character. Results of the present study and earlier studies on morphological parameters [1, 21] are of potential importance in developing a more comprehensive breeding programme for *C. arabica* and Gene Bank maintenance in India as molecular profiles and morphological character profiles become parallel and collateral. Interspecific hybrids can be used as bridge genotypes to transfer desired traits from wild germplasm [1, 21] to cultivated forms and can further augment the crop improvement programme in coffee.

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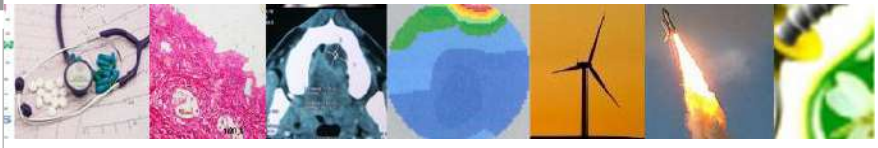

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REVIEW: IMMUNOMICS AND VACCINOLOGY

THE REVERSE VACCINOLOGY – A CONTEXTUAL OVERVIEW

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ABSTRACT

In recent years, the wide availability of complete genome sequences has changed the way we think about vaccine targets. From a few dozen potential targets we can now count on hundreds of targets per organism. This candidate vaccine is an extensively scrutinized plethora based on the concept of reverse vaccinology (RV) with special attention reserved for exported targets, generating promising results for various organisms. However it should be borne in mind that we still lack effective vaccines for organisms sequenced within a decade, a period much longer than expected for producing an effective vaccine by RV. This consideration leads to the reflection that, in the research on a vaccine, other variables may be as important as choosing a target exported. Attention is paid to the fact that the universe of possibilities for an effective vaccine can be exponential in the order of 2^n where n is the number of variables. This review compiles results of some key research using the concept of RV and raises some potential issues that may be hindering the efficient use of this technique to attain attractive and promising targets for vaccine research.

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Keywords: *reverse vaccinology; vaccine variables chances; exported proteins; exponential function; vaccine candidates*

[I] INTRODUCTION

A decade has passed since the term *Reverse Vaccinology (RV)* was first introduced [1]. RV starts from the genomic sequence of a pathogen, which is an expected codified sequence for all the possible genes expressed in the life cycle of the pathogen. All Open Reading Frames (ORF's) derived from the genome sequence can be evaluated with a computer program in order to determine their ability to be vaccine candidates. Special attention is given to exported proteins because they are essential in host pathogen interaction. Examples of this interaction can be cited: (i) adherence to host cells, (ii) the invasion of the cell to which there was compliance, (iii) damage to host tissues, (iv) resistance to environmental stresses from machinery defense of the cell being infected and finally, (v) mechanisms for subversion of host immune response [2-5].

The word 'Reverse' from RV can be explained by the reverse genetics (RG) technique. Before the dawn of genomics, there have been attempts to discover the responsible genes from one phenotype. With Crick's Central Dogma (DNA → RNA → Protein) the research path was reversed. In possession of the likely gene sequence, several techniques were used to identify changes in the phenotype of an organism derived from sequence changes in genes. The principle of the Crick's dogma is also used by RV, in which possession of a gene sequence is searched for the possibility of a probable protein encoded by this sequence to be an antigen capable of stimulating an immune response in a host organism.

Long before the creation of the term RV, a number of approaches had been considered to meet the demand of exported proteins in order to move to the next step of the production of a subunit vaccine [6]. For example, the research using exported proteins was motivated as alternative to subunit vaccines based on polysaccharide capsule of meningococci. Vaccines produced with such antigens have low capacity to induce a satisfactory immune response. This research effort on exported proteins dates back to almost two decades of work searching for a

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vaccine against meningococcal serogroup B, and now it produces good results. This vaccine currently is the best RV research results in the production of a subunit vaccine for *Neisseria meningitidis* serogroup B. Meningitis caused by serogroup B (Men B) is responsible for approximately half of the worldwide incidence of the disease [6] and this research result for targeted vaccination is commonly used as a reference card for the RV due to its excellent results. Currently a subunit vaccine against Men B created with antigens targeted by RV is selected in clinical trials of phase 2 [7, 8]. The advantages of RV are still attractive, enabling vaccine research for organisms whose cultivation in the laboratory remains difficult or impossible. However, reducing the time of selection by target proteins is feasibly usable in different species or strains at the same time and allows selecting vaccine candidates with possibility of eliciting adaptive immune responses. To achieve these benefits all we need is to have a sequenced genome, a personal computer and core software widely known by the scientific community. These conditions show another advantage of using RV, the low cost. What we agreeably call the core software is a set of tools for identifying well-known motifs such as, for example, SignalP, TMHMM, LipoP, and HMMSEARCH. In the use of core software there is still room for innovation when it determined that the choice can be directed to the identification of vaccine candidates specific to an organism such as in the case of gram-negative (bilayer) or gram positive (monolayer) or also placed according to the type of heuristic for selection of vaccine candidates with specific characteristics. For example, membrane or exported to the extracellular environment [9-12].

The concept of RV was adapted to fit a new reality of widespread availability of genomic data [13]. Instead of doing the research for vaccine targets in a single strain or subspecies of an organism, we can do it simultaneously in dozens of genomes, exploring potential joint antigens or exclusive to multiple genomes [14]. The possibility of having a large number of genomes available to implement RV leads to the emergence of the concept of Pan Genomics RV (PGRV) [8]. PGRV can also apply the concepts of core, extended, and character genomes. The core genome in PGRV is composed of exported genes (genes that transcribes for exported proteins) that are common to all strains, genes that could be candidates for a universal vaccine, while the extended genome consists of genes that are absent in at least one of the strains of the studied species and the character genome consists of genes that are specific to a strain [14]. From the standpoint of vaccine, the core and character genomes would be good candidates to compose a vaccine that is suitable for all strains studied, without losing sight of the particularities of specific genes in each strain.

[II] SYSTEMATICAL ANALYZES OF VARIABLES

Considering the motion that many studies using RV are yet to produce effective vaccines [15], an evidence that the limiting factors of RV still have considerable strength despite the enormous advances in genome sequencing has been created herein. Such limiting factors are insignificant amount of currently known antigens and the RV inability to detect non-protein antigens as polysaccharides and glycolipids [16]. These major drawbacks could be minimized with introduction of glycomics and lipidomics studies combined with genomics, proteomics, and peptidomics approaches in vaccine research that would culminate in knowledge and discovery of a wider range of antigens for *in silico* comparisons as new antigens from a survey of RV. More so, core software could also be created to identify patterns in polysaccharides and glycolipids, increasing the repertoire of antigens of an organism.

A limiting factor to the success of RV is the belief that identifying a set of exported proteins is the solution to the lack of production of an effective subunit vaccine against pathogen. Therefore, there are many possibilities of failure and only one chance of success; raising three hypothetical questions in planning a vaccine: **(A)** "Is the set of antigens suitable?" [17], **(E)** "Are antigens expressed in a critical stage of infection?" [10, 18] and (V) "What is the use of a DNA vaccine?" [19-20]. Supposing that initially, each of these three questions could have a TRUE or FALSE answer. In this case we can relate the questions A, E and V into a set of eight (2^3) possibilities, as shown in Table 1. It is the end result that matters, **(R)** "Will the vaccine be effective? The response is "YES" only if the three questions are answered with an assertive TRUE; otherwise the response will invariably be "NO".

Table-1 shows that there are possibilities, as earlier mentioned, of choosing a set of antigens sufficient to confer immunogenicity. In other words, there are chances of choosing a set of antigens effective in conferring immunogenicity, for example, for only one bacterium strain, or the selection of antigens not expressed in an important stage of infection or even the simple act of trying a subunit vaccine instead of DNA vaccine, though the set of selected antigens are adequate and expressed.

The planning of a hypothetical vaccine as shown in **Table-1** still leaves room for doubts by not taking the type of immune response most appropriate to a certain pathogen into consideration. Supposing, for example, a humoral response is not the most suitable for the pathogen of this hypothetical vaccine. Thus, even though **(A)**, **(E)**, and **(V)** are answered as TRUE, yet the vaccine could not induce protective immunity because the most appropriate response lies in the cellular immunity. So after including a fourth question being a variable in **Table-1**, **(C)** "Does vaccine generate immune response?" [21], a set of 16 possibilities was obtained (2^4) among which there are 15 possibilities of failure and only one possibility that matters the most.

This hypothetical example of planning a vaccine in **Table-1** may explain why only the selection of a set of suitable candidates still, leaving a lot of variables that can lead to failure of a vaccine approach. In planning a hypothetical vaccine for these four questions, even if the question **(A)** holds, there still remain seven other possibilities for failure to be adequately answered.

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Table: 1. Possible vaccine results considering only three variables: The result shows seven failure possibilities and a record of just one success which matters the most.

No	(A) "Is the Set of antigens suitable?"	(E) "Are antigens expressed in a critical stage of infection?"	(V) "Use of a DNA vaccine?"	(R) "Will the vaccine be effective?"
1	FALSE	FALSE	FALSE	NOT
2	FALSE	FALSE	TRUE	NOT
3	FALSE	TRUE	FALSE	NOT
4	FALSE	TRUE	TRUE	NOT
5	TRUE	FALSE	FALSE	NOT
6	TRUE	FALSE	TRUE	NOT
7	TRUE	TRUE	FALSE	NOT
8	TRUE	TRUE	TRUE	YES

[III] DISCUSSION

The popularization of new technologies of genome sequencing has led to a substantial increase in the number of complete genomes for use in PGRV [14]. Given the particularities of the operating mode of each of various pathogen results and strategies, these can be used in the search for vaccine targets. Below are some of the pathogens for which RV has been used, starting with initial pathogens in the paper described the concept of RV [1] and as a result, we continue with other pathogens which do not necessarily affect humans.

3.1. Tuberculosis (TB)

Despite the prediction of decline in the world TB cases, its incidence continues to grow with more than 10 million cases reported only in 2010, keeping it among the diseases with the highest incidence worldwide [22]. Also, despite the vast amount of research for vaccine against TB, an efficient vaccine against this global scourge is still a promise. The first *Mycobacterium tuberculosis* genome sequence has been released over a decade [23, 24], but still insufficient to bring about a promising vaccine against TB. Considering the availability of complete M. tuberculosis genome sequences, the global urgency of a final solution against the scourge and the facility to conduct *in silico* research, it is inferable that in the search for vaccines understanding the wide range of research involving TB comes easier. A simple search for the term "tuberculosis" in the last three years using the PubMed database generated over 20 thousands published works that are directly or indirectly related to TB. RV was applied over *M. tuberculosis H37Rv* genome aimed at detecting secreted proteins, generating evidence of seven proteins as exoproteome properties that are possible targets for a vaccine [25]. Three secreted proteins belonging to the cutinase-like protein family (Culp) was tested and the Culp6 eliciting a strong cellular response was found [26]. It is the first cellular response recognized in patients affected by TB. These are examples of studies that fit the question **(A)** "Is the set of antigens suitable?" and the last example also characterizes the question **(C)** "Does vaccine generate immune response?" Although many of these studies did not explicitly cite the term RV, many fit the concept and try to get more information about the functional genome released by special attention to exported proteins. Questions of type **(C)** "Does vaccine generate immune response?" from our hypothetical vaccine shall be answered by researches for more effective antigens. The hypothetical protein Rv2626c was found capable of induction of adaptive and humoral immune responses [27]. However, using the concept of epitope density was to create a list of proteins with "hot spots" of the affinity of MHC class II molecules [28]. A hypothetical protein with high affinity to the promoter of genes fbp (Ag85 complex) was the result from search for over expressed factors in proteins from this antigen complex, a protein that belongs to the protein family of transcriptional regulators Mars [29].

Hypothetical membrane proteins were tested and evidenced that Rv0679c protein is expressed in only three strains of *M. tuberculosis*, although 26 strains of bacteria that possessed the gene for this protein were used [18]. Research like this show attempts to answer questions such as **(E)**, from **Table-1** for the planning of a vaccine, being crucially important as much as the question of identification of a secreted protein. Another variable that could be added to the planning table of vaccines would be **(D)** "How low is genetic diversity of selected antigens?" [30]. Included this variable, our universe of possibilities of failure would increase to 31 (2^5 - 1). For example, it was showed that classical vaccine candidates like genes such as esx, fbpB and Esat-6 would not be affected by genetic diversity in 88 strains of *M. tuberculosis* [31]. In this case the answer is "TRUE", increasing the chances of these candidates in our hypothetical screening of candidates. Under the aspect of PGRV, a set of character genes of *M. tuberculosis H37Rv* characterized as important for invasion and survival of the pathogen in the host was found by Al-Attiyah *et al*. 2010 [21]. Among these genes RD1504 was able to induce a strong

IIOAB ISSN: 0976-3104

immune response T-helper (Th) type 1 and may be an important candidate for vaccine target.

3.2. Group B meningococcus

With rates of 16.9/100,000 for bacterial meningitis and 8.9/100,000 for *Neisseria meningitidis* and high number of fatalities in children, meningococcal disease remains a concern and compounded when considering the short period between infection and death, which can possibly be only one day [32]. The experience gained by *in silico* research for candidate vaccine against Men B [33] was a major factor that led to the creation of the term RV. In this work most of the antigens selected in silico and successfully expressed in *Escherichia coli* were exported proteins, including lipoproteins, OMP's, periplasm and membrane proteins. A list of five selected antigens of this study were tested with the adjuvant aluminum hydroxide, CpG oligonucleotides or MF59, achieving antibodies against more than 90% of 85 strains of meningococci representative of the global population diversity [7]. Research by adjuvants can also be included as a requirement to produce an effective vaccine that could be an additional variable in our planning of a hypothetical vaccine **[Table-1]**. It was showed that the amount of factor H, an important regulator of the complement pathway, is correlated with the level of expression of GNA1870, suggesting the inclusion of this protein in the set of antigens of Men B [34]. This research is useful in trying to answer questions of type **(E)** "Are antigens expressed in a critical stage of infection?" [35]. Although there are two vaccines in development for incorporating the Men B protein named Factor H-binding protein or fHbp [36]; it has not been possible to produce a comprehensive vaccine based on this antigen due to its wide antigenic variety [30, 37]. This variety motivated the establishment of a nomenclature to categorize this diversity [38], a study that answers questions such as **(D)** "How low is genetic diversity of selected antigens?". The discovery that convalescent patients develop long-term protective immunity against *N. meningitidis* motivated the search for antigens capable of eliciting such immune response [15]. Contrary to the RV concept, most of the antigens were found cytoplasmic and were not able to produce a satisfactory immune response in guinea pigs. There is also the protein RplY proven to belong to the cell surface of the pathogen. This result makes it a little more confusing to answer the question **(A)** "Is the set of antigens suitable? ", since most of candidates would not be exported. After a decade of the first results of RV on *N. meningitidis*, suggested antigens continue to be researched. It was discovered recently that GNA2132 known as a protein capable of inducing a bactericidal antibody in mice, is also capable of inducing protective immunity in humans. This protein is recognized by serum of convalescent patients, and has been renamed Neisserial Heparin Binding Antigen (NHBA), which is one of the most promising in the search for a vaccine against the pathogen [39] and helping to answer questions of type (C) "Does vaccine generate cellular immune response?".

3.3. Staphylococcus aureus

Staphylococcus aureus, a gram positive bacterium remains one of the major human pathogens and a major cause of nosocomial infections worldwide. Failure of antibiotic therapy to eradicate infection is frequently described in literatures and the rate of resistance to clinically relevant antibiotics, such as methicillin, is increasing. Furthermore, there has been an increase in the number of methicillin-resistant S. aureus community-acquired infections [40]. The high prevalence of infections is confounded by the ability of the pathogen to readily acquire genetic elements that confer resistance to antibiotics [41]. The first *S. aureus* complete genome was available on the Gene Bank databases and the Broad Institute since 2007 [42], followed by other 14 different strains at NCBI. There is need for decoding the sequences of complete genome of *S. aureus* that could offer the possibility for comprehensive screening to identify the targets for effective vaccine development [43]. So, it could be interesting to try the answer **(D)** "How low is genetic diversity of selected antigens?" when considering vaccine candidates. Clinical trials with monovalent traditional vaccines already failed to protect against the disease. Now the need is to shift from monovalent vaccine development towards the potential use of multivalent formulations, therapeutic antibodies, and more systematic and rapid identification of optimal antigens by applying *in silico* tools [44]. The RV concept is most suitable for the *S. aureus* to meet the research needs and there are case studies applying it. For example, there are at least 153 individual antigens characterized with the immunome of *S. aureus* [45], despite their subcelular location, which help to answer the question of type **(C)** "Does vaccine generate immune response?". Antibody responses produced against those antigens are accessible to B cells in vivo, most likely extracellular and cell wall-associated proteins, but also non-protein antigens, such as wall teichoic acids (WTA) lipoteichoic acids (LTA), and peptido glycans (PGN) [45]. Surface protein antigens IsdA, IsdB, SdrD, and SdrE were tested for its vaccine efficacy in a combination and individually, the serum IgG titers of immunized mice were almost the same [46]. Immunodominant antigen B (IsaB) is a surface protein believed to be a virulence factor, although its biological functions are not well defined. Its nucleic acid-binding activity is being observed. IsaB has greater affinity for dsDNA than it has for ssDNA or RNA, there is need to evaluate and understand the role of IsaB and its nucleic acid-binding activity which are important in establishment and/or progression of *S. aureus* infection [47] and to answer questions like **(A)** and **(C)** from our hypothetical vaccine planning. Immune dominance of extracellular and surface-exposed proteins has indeed been observed with an *S. aureus* genomic expression library, ribosome display, and 2D-IB. Also most surface associated genes in the core variable genome as well as a large amount of virulence and resistance factors, and many are encoded on mobile genetic elements [45], helping to answer questions like **(A)** and **(D)** from our hypothetical vaccine planning.

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3.4. Corynebacterium diphtheriae

Corynebacterium diphtheriae bacteria are responsible for Diphtheria. The pathogen produces a toxin that can harm or destroy body tissues and organs, diphtheria toxin (DT). The disease primarily affects mucous membranes of the respiratory tract (Respiratory Diphtheria), although it can also affect the skin (Cutaneous Diphtheria). The bacteria were identified for the first time in the 1880's. In the 1890's, the first antitoxin were developed, and the first vaccine in 1921 [48]. The vaccine is made of inactive forms of the toxin. According to the World Health Organization (WHO), diphtheria affects people of all ages, but it is more frequent in non-immunized child. In 2004, 5000 deaths owing to the disease were reported worldwide [49]. The most effective treatment consists the administration of diphtheria antitoxin (DAT) associated with the elimination of the microorganism using appropriate antibiotics. Since the beginning of the 20th century, many countries produced their own antitoxin preparation from horses. However many factors led to fall of this traditional stocks. Among this factors it is possible to exemplify the lost of economic viability, once the incidence of the disease has fallen a lot, and public objections to the use of horses as donors [50]. In the 1990's, the lack of DAT and vaccines were the two major causes behind the outbreak of diphtheria in the former Union of Soviet Socialist Republics (USSR) states. Between 1990 and 1998, more than 157,000 cases and 5,000 deaths were registered, which represented 80% of diphtheria reports worldwide [51]. This was the major diphtheria epidemic since the 1950's, when the spread of immunization had began. The first genome annotation of *C. diphtheriae* was released in 2003 [52]. With the genome published, Hansmeier *et al.* mapped and analyzed the extracellular and membrane surface of the C7s(-)tox-lineage. This work identified unambiguously ~32% (85/263) of the protein previously described as being extracellular. There were 107 extracellular proteins and 53 of the cell surface, representing a total of 85 different proteins [53]. The importance of this study is to identify secreted proteins, once they can be involved in important interactions between bacteria and host, helping to plan for a vaccine according to the variables in table 1, more specifically question **(A)** "Is the set of antigens suitable?", characterizing a RV research.

Another possible question is **(P)** "Is the antigen in a Pathogenicity Island (PAI)?" [54]. In order to try answer such question for the model *C. diphtheriae,* a comparative genomic hybridization of different strains was made against the specie reference strain [54]. Now, our hypothetical vaccine planning could become more complicated, reaching a total of 63 (2^6 -1) failure possibilities. C7(-) strain was suggested to lack 11 PAIs among 13, while strain PW8, isolated earlier than the C7(-) strain, were lacking only 3 regions related to PAIs.

Additionally, a large genomic diversity among various *C. diphtheriae* strains and clinical isolates were observed. Although the difference between C7(-) strain was higher than the PW8, the adhesion of C7(-) were comparable to the reference strain, while PW8 showed reduce adherence compared to the other strains [54]. This is some odd result considering that adhesions of *C. diphtheriae* to human epithelial cells followed by internalization are signs of pathogenicity.

Searching the literature we can also find studies about the pilli, a very important structure in the adherence of the bacteria and the host [55, 56]. These structures are often involved in the initial adhesion of the bacteria to host tissues during colonization and so helps answer the question **(A)** "Is the set of antigens suitable?" Besides having a structural importance, there are some reports in the literature stating that it might be an important vaccine target, as it is critical in the invasion process [57, 58]. This also helps answer the question **(E)** "Are antigens expressed in a critical stage of infection?"

Compared to the research of vaccine targets of other pathogens in this review, the research on targets of *C. diphtheriae* is less intensive. This apparent calm may be associated with the impression that diphtheria is controlled and that existing vaccines, mostly based on only one antigen, is sufficient to control the disease. However, a feature that makes the development of a vaccine against the bacteria very interesting is the raise of the nontoxigenic strains. Although they don't release the DT in the organism, they are capable of causing morbidity and death [59]. They were isolated from injection drug users in Switzerland [60], homeless alcoholics in France [61], and from poor populations of Vancouver, Canada [59]. In addition, an increasing proportion of strains isolated in the United Kingdom are nontoxigenic [62]. For such non toxic *C. diphtheriae* strains it is possible that the search for adequate variables also can make the difference between a vaccine success or failure.

[V] CONCLUSION

Despite the extensive use of the initial concept of RV and advanced rated results in the search for vaccines against certain pathogens, in general its best and most practical results are still expected. This conclusion is based on fact that the genome sequences of some of the major human pathogens are known longer than the average time required for RV application and promising vaccine against these pathogens still seems far away. Also despite the limitations of RV, this is a low cost technique, fully feasible of use into the plethora of genomic data being generated. It is justifiable to use it in a broader range of pathogens. It is possible that for some of the major human and animal pathogens we can find an appropriate combination of antigens enabling the creation of effective vaccines capable of improving the people's quality of live directly through prevention of diseases or indirectly by improving the economic conditions dependent on breeding. However, as shown in the hypothetical example of planning a vaccine, the discovery of suitable antigens could be a small part of the problem of producing an effective vaccine, but never the less important.

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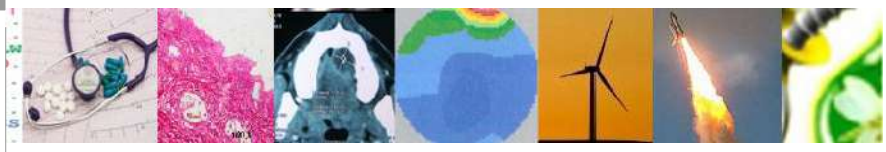

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RESEARCH: TISSUE ENGINEERING

APPLICATION OF DEXTROSCOPE VIRTUAL REALITY SYSTEM IN ANATOMICAL RESEARCH OF INNER STRUCTURES IN PETROSAL BONE

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ABSTRACT

***OBJECTIVE:** To evaluate the application of virtual reality technology in anatomical study of inner structures of petrosal bones, the comparison of three dimensional (3-D) virtual microanatomy and actual microanatomy of petrosal bone was carried out. **METHODS:** The experiment was divided into two groups, virtual group and corpus group, each group with 20 cases. In the virtual group, images data of cadaver heads were loaded into Dextroscope workstation and dissecting of the virtual petrosal bone was simulated. In the corpus group, actual dissecting of petrosal bone on the cadaver heads was examined under microscope correspondingly. Compare the data of locating internal auditory meatus, cochlear and internal carotid artery in each group. **RESULTS:** The distances from the projecting point on petrosal crest of anterior margin of internal auditory orifice to the intersection of arcuate eminence and petrosal crest, and the midpoint of superior margin of external auditory orifice were 24.23 ± 2.88mm and 40.65±4.48mm in the virtual group respectively, 23.62±2.82mm and 39.35 ± 4.83mm in the corpus group respectively (P＞0.05). The distances from the anterior margin of cochlea to the root of zygomatic arch and geniculate part of internal carotid artery were 27.15±3.25mm and 4.15±0.52 mm in the virtual group; 28.35±4.05mm and 4.50±0.54mm in the corpus group respectively (P＞0.05). The distances from the petrosal crest to the anterior margin of the three sub-segments were 12.20±1.42mm, 8.63±0.94mm, and 5.42±0.63mm in the virtual group respectively; 10.68±1.24mm, 8.62±0.92mm and 5.69± 0.61 mm in the corpus group respectively (P＞0.05). **CONCLUSIONS:** The data measured in the virtual group was highly coincided with those data in the corpus group. Virtual anatomy of petrosal bone realized by virtual reality technology is reliable.*

Key words: *virtual reality; internal auditory meatus; semicircular canal; cochlea; petrosal segment of internal carotid artery*

Abbreviations: **CT,** computerized tomography; **EAO,** external auditory meatus; **IAO,** internal auditory orifice; **ICA,** internal carotid artery; **MRI,** magnetic resonance image; **VR,** virtual reality

[I] INTRODUCTION

With continuous improvement of computer image processing technology and corpus specimen processing technique, visible human project (VHP) have undergone from data collection at initial stage to education in medicine school with virtual three dimensional (3-D) specimen reconstructed from volumetric data [1-9], and some reports on anatomical structures of petrous bone based on the VHP data set were seen on some journals [10-15]. However, it seemed there was no report which virtual reality (VR) technique was used to research the individual anatomy of inner structures in petrous bones in skull base operation approach. This study was to explore the liability and value of VR technology in individual labyrinthine anatomy studies.

ISSN: 0976-3104

[II] MATERIALS AND METHODS

Perfusion of adult head specimens 20 set, microsurgical anatomy of equipment set, Dextroscope Workstation (Volume Interactions Co. Ltd., Singapore), software Version 1.01R; 3.0-T whole-body MRI scanner (General Electric Medical Systems, GE Signa VH/i); SOMATOM Sensation 64-slices CT scanner (Siemens AG).

2.1. Groups and topical anatomy

The experiments were carried out in the two groups respectively, virtual group with 20 clinical cases (40 hemispheres) whose lesions did not involve the petrosal bone and corpus group with 20 sets head specimen (40hemispheres). Micro topical anatomical dissections including internal auditory meatus, petrosal internal carotid artery and cochlear were done on the Dextroscope workstation in clinical patients' data. Actual anatomical dissections were correspondingly done the specimen. The total process of virtual dissection of petrous bone were taken by inner camera in blue red forma in virtual groups and measurements results were compared with data from actual anatomical dissection under microscope.

2.2. Image data acquisition

The scanning of patients were scheduled 1-3 days before operation, images data of were transferred to Dextroscope laboratory by optic disc. CTA was performed on the 64-slices CT scanner following the intravenous contrast agent injection. CTA were obtained contiguous as axial, 1-mm slices (FOV=240mm×240mm; matrix size=256×256), Horizontal scanning range from 2nd cervical vertebrate body to the cranial top.

VR Anatomy In Dextroscope, co-registration of cranium segmented from head CT and internal carotid arteries from enhanced MR was done. The inner structures such as internal auditory meatus, cochlear and semicircular canals were showed by regulating threshold. Find the landmarkers and measure the distances corresponding to topical anatomy described in above text.

2.3. Statistical analyses

SPSS12.0 software was applied in the late stage. Paired T test checked quantitative data. RIDIT analysis checks numeration data. If value of P was less than 0.05, it demonstrated significant difference in the test.

Table: 1. The related measurement of internal auditory orifice and its surrounding structures

Groups	Virtual group (n=40)	Corpus group (n=40)	*P*– value
Dis. From Projecting point on the crest of Ante. Mar. of IAO to intersection of AE & crest	24.23±2.88mm	23.62±2.82mm	0.62
Dis. Bet. Projecting points on crest of Ante. Mar. of IAO & midpoint of ante. Mar. of EAO	40.65±4.48mm	42.15±5.13mm	0.75
Length of ante. Wall of IAO	11.68±1.54mm	11.55±1.30mm	0.65
Angle bet. Ante. wall of IAO & axon of the crest	43.56±4.62°	45.68±5.51	0.70

Dis, distance; Ante, anterior; Mar, margin; IAO, internal auditory meatus; EAO, external auditory meatus

[III] RESULTS

3.1. Measurements of internal auditory meatus

The Results of related measurement of internal auditory orifice and its surrounding structures were referred to **[Table–1]**. There were no significant differences between the measurements in two groups.

3.2. Measurement of cochlear

The cochlear is deeply sited in petrous bone. On the level of the crest of internal auditory meatus, the distances between anterior margin of cochlear and the root of zygomatic arch root and middle points of superior margin of external auditory meatus were 28.54±3.85mm and 27.15±3.25mm respectively **[Figure–1]**. The results of related measurements were referred to **Table–2**. There were no significant differences between the measurements in two groups.

3.3. Measurement of petrosal segment of internal carotid artery (ICA)

The petrosal segment of ICA was divided into three subsegments according to its relationship with trigeminal semi-ganglion, lateral segments of trigeminal ganglion (S1), segments of trigeminal ganglion (S2), and internal segments of trigeminal ganglion **[Figure–2]**. Distance between each subsegments of petrosal ICA and crest and thickness of bones on the petrosal ICA **[Table–3]**. There were no significant differences between the measurements in two groups.

ISSN: 0976-3104

Tab: 2. The distance from cochlear and its surrounding structures

Group	Virtual group (n=40)	Corpus group (n=40)	*P*– value
Angle Bet. the line of Internal Mar. of Co. to Ante. Mar. of IAO and crest	59.48±7.18°	61.56±7.24°	058
Dis.Bet.fundi of Co. and midpoint of superior Mar. of EAO	27.15±3.25mm	28.35±4.05mm	0.62
Dis.Bet. internal Mar. of Co. and geniculate part of ICA	4.15±0.52mm	4.50±0.54mm	0.60

Dis, Distance; Bet, Between; Mar, margin; IAO, internal auditory meatus; EAO,external auditory meatus; Co. cochlear

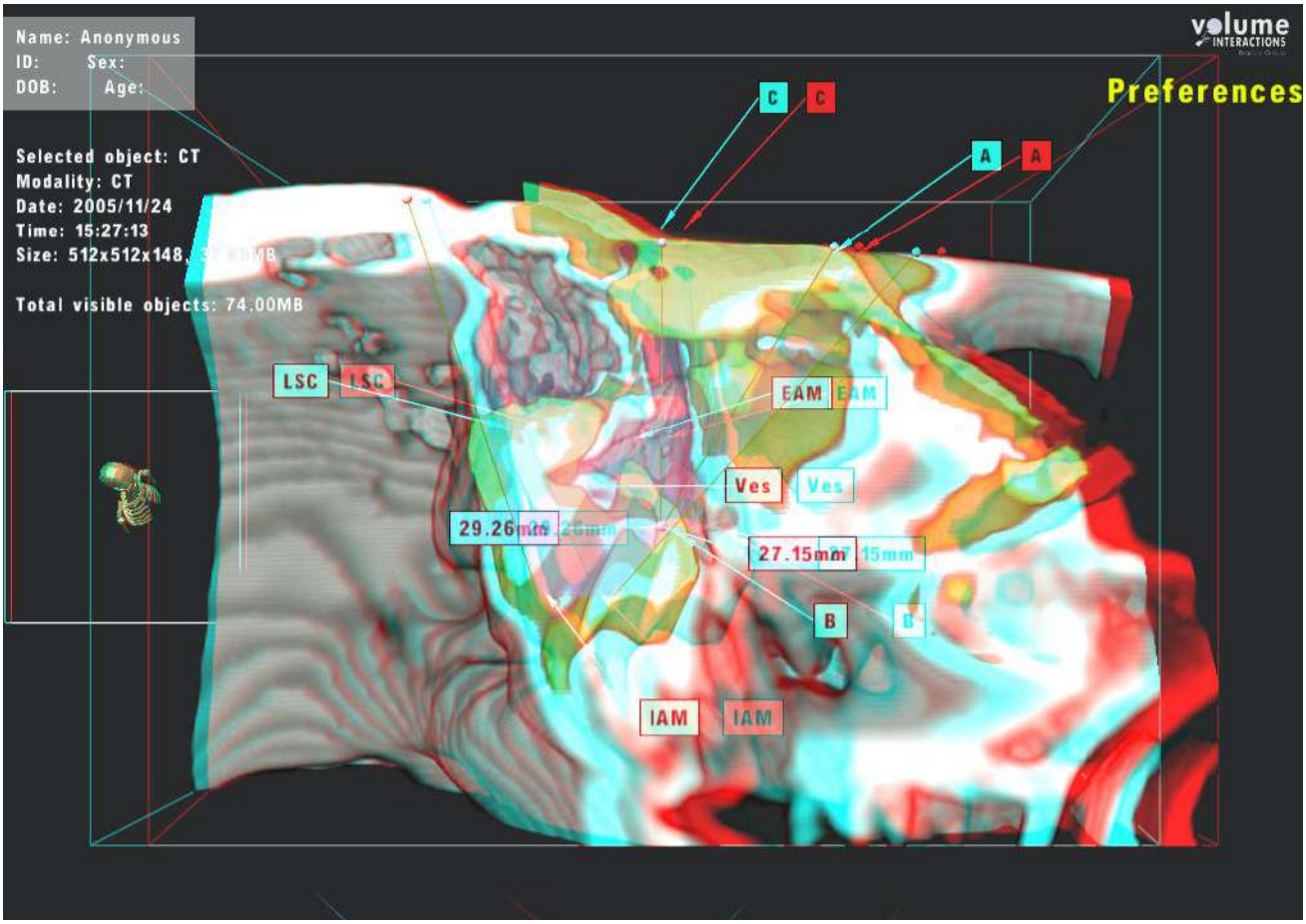

Fig: 1. The demonstration and related measurements of internal auditory meatus and cochlear A: the root of zygomatic arch; B: Lateral point of cochlear; C: midpoint of superior margin of external auditory orifice; IAM: internal auditory meatus; LSC: lateral semicircular canal; EAM: external auditory meatus; VES: vestibule; Line AB showed the interior boundary of cochlear.

ISSN: 0976-3104

Table: 3. Distance between each subsegments of petrosal ICA and crest and thickness of bones on the petrosal ICA

Group	Virtual Group (n=40)	Corpus group (n=40)	*P* –value
Dis. from S1 midpoint to crest	12.20±1.42mm	10.68±1.24 mm	0.55
Dis. from S2 midpoint to crest	8.63±0.94 mm	8.62±0.92 mm	0.58
Dis. from S3 midpoint to crest	5.42±0.63 mm	5.69±0.61 mm	0.60
T. of bone on Ante. Mar. of S1	3.22±0.37 mm	3.16±0.33 mm	0.62
T. of bone on Ante. Mar. of S2	3.05±0.31 mm	3.09±0.32 mm	0.54
T. of bone on Ante. Mar. of S3	2.28±0.23 mm	2.30±0.25 mm	0.67
T. of bone on Post. Mar. of S1	5.63±0.58 mm	5.82±0.61 mm	0.78
T. of bone on Post. Mar. of S2	3.58±0.41 mm	3.52±0.38 mm	0.69
T. of bone on Post. Mar. of S3	3.10±0.31 mm	3.23±0.35 mm	0.66

T, Thickness; Post, Posterior; Ante, anterior; Mar, margin; Dis, distance.

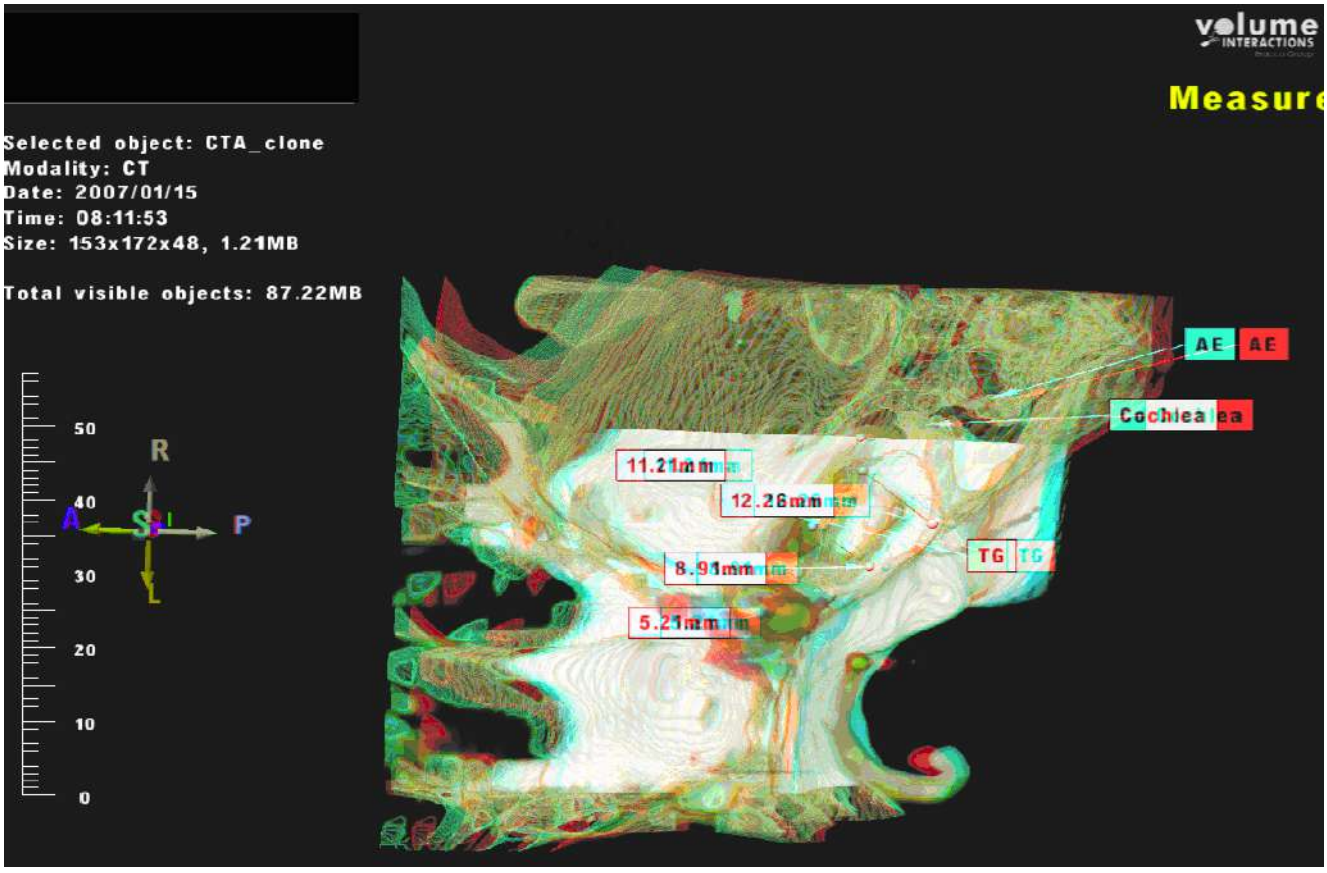

Fig: 2. The measurements of each sub segments of petrosal ICA. S1, S2, and S3 stand for three Sub segments of petrosal ICA from outwards to inwards.TG, trigeminal ganglion; AE, Arcuate eminence.

IIOAB ISSN: 0976-3104

[IV]DISCUSSION

Virtual Reality (VR) technology is a comprehensive synthesized technology which can produce three dimensional virtual realistic interfaces in which people can interact with complex data in immerse environment by proper instruments. This has been used in anatomical research and education in medicine, such as the USA visible human project (VHP), voxel man reconstructured from it and reconstructed research on temporal bone from visible Chinese Man data base [1-5, 8, 9, 16-18]. However, due to lack the changes of intensity of CT and MRI signals, the quality of reconstruction images were not very desirable and visible man researches were confined in normal corpus study which just could be used in medicine education, rather not extend to guide an actual individual procedures.

Dextroscope VR workstation provided a strong tool for individual anatomical study and clinical individual procedures approach [19-24]. The study of individual anatomy of inner structures in petrous bone by Dextroscope is to explore the reliability of virtual reality technology in anatomical researches and provide more evidences for clinical applications.

Precisely understanding the anatomy of petrous bone is crucial importance in dealing with tumors involved the petrous bones. When the approach of transtemporal transtenterium was used dealing with meningioma on the apex of petrous bone, especially the tumor on the posterior fossa, grinding the apex became one of keys to total removal of tumor. Therefore, there were many reports about methods on how to grind the apex in 90s [25-30]. The basic method depends on the landmarks of inner structures in petrous bone in middle cranial fossa, but they were all roughly and lack of accuracy in location. The safety way to grind petrosal apex away included advanced driller and skillful manipulation of driller et al, the most basic point is to how to acquire the individual data about petrous bone to help locate the important inner structures such as cochlear, internal auditory meatus, semicircular canal and petrosal segment of internal carotid artery because there were great variation of structures in this area.

4.1. Location of IAM and its courses

There were two ways to locate the position of IAM. One way is according to the bisector of the angle between inferior major petrosal nerve and arcuate eminence which is roughly course of IAM. Another way is to draw a 45-degree angle along the crest from the projection point on the petrosal crest of anterior margin of IAO. Another edge of the angle is the course of internal auditory meatus. The first way can be used in epidural approach, not for subdural approach. The second way sometime failed to variation of the angles. In the experiment, we found that there was great variance in the angle between IAM and the crest. If this method was used in each patient whose apex would be ground away in operation, it probably result the damage of cochlear or IAM. It is very essential to gain the individual data of the position of inner structures in petrous bone before operation. Actually, we used virtual reality technology to measure the individual data of the angle and design individual procedure protocol to avoid damaging of important inner structures.

4.2. Location of cochlear

It is very difficult to precisely locate the position of cochlear due to its deeply situated in petrous bone. The position of cochlear roughly used to be localized by the triangle determined by anatomical landmark on the middle fossa in past anatomical researches. Day and Fukshima[25] firstly used the anterior triangle of internal auditory meatus to locate cochlear, which is composed of anterior margin of IAM, geniculate part of ICA and geniculate ganglion where cochlear deeply situated laterally to the triangle. liu Jun[31] modified the triangle by replacement of geniculate part of ICA with midpoint of line from geniculate ganglion to spinal foramen to where cochlear deeply situated laterally. Above methods mentioned, it was feasible to locate cochlear by anatomical landmark, however lack of accuracy and individuality.

The magnified cochlear by virtual reality technology we found that cochlear situated cross the mark line of the IAM **[Figure–1].** If grinding apex went along the line, it would be great possible to destruct the cochlear. So if precise location of cochlear before operation was made, the map to grind petrous bone apex away would be more feasible. Our study demonstrated that at the level under superior wall of IAM 2mm, the cochlear sits medially to the fundi of IAM. The distance from internal auditory orifice to cochlear is 13.56±1.65mm or so and the distance to geniculate part of ICA 4.15±0.52mm. The angle between the line from anterior margin of IAM to anterior margin of cochlear and the crest is 59.48± 7.18°. Individual data such as distances between the fundi of cochlear and the root of zygomatic arch, from internal margin of cochlear to anterior margin of internal auditory orifice and geniculate part of ICA can be acquired by virtual reality without any damage to those structures. In operation, these data help us to locate the inner structures position precisely to reduce hearing loss resulted from imprecise location of cochlear.

4.3. Location of petrosal segment of ICA

The location of petrosal segment of ICA is very critical in operation which involved in middle fossa. The judgments of its position used to be drawn from the distance between the groove superior of petrosal nerve (GSPN) and the crest. It was reported that the distance was usually about 12mm or so. However, the actual situation is not entirely the case. The data about the distance we gained on workstation sometimes quite different from above mentioned. So we investigate the thickness of bone on the artery and also its distances. According to the position relationship between petrosal segment of ICA and trigeminal ganglion, the petrosal segment was in divided into three subsegments: lateral segments of trigeminal ganglion (S1), segments of trigeminal ganglion (S2), and internal segments of trigeminal ganglion. In virtual group, the thickness of bone

IIOAB ISSN: 0976-3104

covered on the arteries became thinner from posterior to anterior as it is similar in corpus group (P>0.05). There was also no significant difference in the distance from the midpoint of each subsegment of ICA to the crest between the two groups. The results mentioned above can show as followed: 1 the data measured in virtual group was not quite same to that in corpus group; however there was no significant difference after statistical treatment. So the data in virtual group can demonstrate actual situation, and they are also individually actual situation. 2 Petrous bone on the ICA shaped tribody whose posterolateral part is thicker and anteromedial part is thinner. When the petrous bone especially medial apex to the trigeminal was being ground away, operator should be careful of the changes of thickness of bone covered on the ICA in case of the damage arteries.

In summary, VR technology can demonstrate the anatomical structure intuitively and individually in three dimensional. We have published papers on its clinical applications of virtual temporal bone in dealing with cerebrospinal fluid leakage [19,32,33]. However, there was some error in segmentation of anatomical structures. It is one of study keys to develop more advanced artificial intelligence software to lower the difficulty of segmentation and shorten manipulation time in future. If biomechanics feedback were added to make simulation of dissection more closed to actual anatomy under microscope, it will provide neurosurgeons more tools to study neurotomia.

AUTHOR CONTRIBUTIONS

Delin Yang, Xiaoming Che, and Meiqing Lou have contributed equally to this work.

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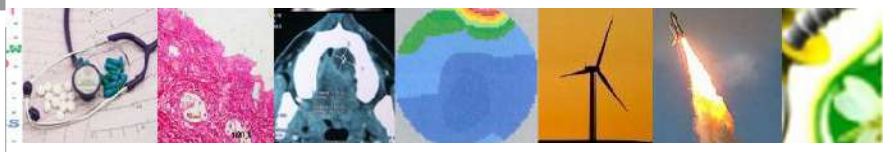

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RESEARCH: BIOINFORMTICS

AMINO ACID FREQUENCY DISTRIBUTION AT ENZYMATIC ACTIVE SITE

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ABSTRACT

Enzyme's active site sequence is crucial to execute its function. The present study is based on the analysis of comparative frequency distribution of enzymes catalytic residues belongs to different organisms and localizations. The frequency distribution of enzymes catalytic residues was computed using the number of amino acids of each type and the total number of residues. The percentage composition of catalytic residue indicates the occurrence of 13 amino acids (out of 20 total amino acids) at enzymatic active site. These 13 catalytic residues are cumulatively constituted by five charged (E, D, H, K, R), six polar (C, Y, T, S, Q, N) and two hydrophobic residues (A/F/W, G). Viral, Prokaryotic and Eukaryotic enzymes (VEs, pEs and eEs) commonly show preference for four charged residues (H, D, E, R) and a single polar residue 'S' at their active site. The residues 'R>A' are predominantly distributed at VEs active site, while in pEs and eEs the order of preference of catalytic residues is H>D>K>E>R>Y>S. The analysis further indicate that both Prokaryotic and Eukaryotic membrane and non-membrane enzymes (MEs and nMEs) show high degree of similarity in the overall percentage distribution of charged and polar residues at their active site. In addition, VEs are significantly similar to nMEs in the frequency distribution of charged and polar residues. The catalytic residues 'H, D, S, Y, T, F' play crucial role in the catalysis of Prokaryotic membrane enzymes (pMEs), while the residues 'H, K, Y, S, C' are important for Eukaryotic membrane enzymes (eMEs) catalysis. However, in non-membrane enzymes (nMEs) five charged residues (E, D, H, K, R) are important for their catalytic function, while the polar residues have supportive function. The knowledge of amino acid frequency distribution at the active site can be exploited in designing novel enzyme active sites as well as the specific inhibitors and novel peptide based drugs.

Key words: *enzyme catalytic residues; frequency distribution; viral enzymes; prokaryotic enzymes; eukaryotic enzymes*

[I] INTRODUCTION

At each step, the metabolic processes involve the versatile catalytic molecules termed as enzymes [1]. Along with biochemical information, the three dimension structure of enzymes in complex with substrate has been used extensively to determine their catalytic mechanism. In general, the amino acids at the enzyme active site act as acid-base, nucleophiles or electrophiles and also make hydrogen bond either with the substrate or with other residues of an enzyme to stabilize the transition state [2].

Most of the enzymes are unique and specific in catalysing a particular biochemical reaction. The amino acid residues involved in making the active site of an enzyme are not sequentially arranged rather distributed all over the protein sequence. These residues come together during folding to make the enzymatic active site. Bartlett et al. (2002) used a dataset of 178 enzymes for determining the frequency distribution of enzyme's catalytic residues [3]. Since then the number of enzyme structures in Protein Data Bank (PDB) has been increased six fold. Furthermore, the comparative data for the frequency distribution of catalytic residues of enzymes of different origin and cellular localization is not available. This study is an attempt to find out either the substantial similarities

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or significant differences among frequency distribution of catalytic residues of enzymes of Viral (VEs), Prokaryotic and Eukaryotic (pEs and eEs) origin. In addition, the Prokaryotic and Eukaryotic enzymes dataset was segregated for analysis into membrane and non-membrane enzymes (MEs and nMEs) depending upon their cellular localization. The improved understanding of the distribution of catalytic residues at the active site will be helpful in enzyme engineering and in designing novel inhibitors.

[II] MATERIALS AND METHODS

A dataset of 838 non-redundant enzymes was manually curated from the experimentally annotated entries of enzyme active site database called as 'Catalytic Site Atlas' (CSA) [4]. Out of 26,846 total entries, CSA (version - 2.3.11) possess 968 experimentally annotated entries of enzyme active site with a label 'literature reference'. For the purpose of this study, the whole dataset was divided in three categories namely Viral (VEs; 9), Prokaryotic (pEs; 481) and Eukaryotic (eEs; 348) enzymes based on the source organism. While preparing the database, repetitive CSA entries were excluded. Additionally, depending upon the cellular localization of the enzyme, the Prokaryotic and Eukaryotic dataset is further subdivided into membrane (pME and eME) and non-membrane (pnME and enME) enzymes by using the concerned journals and UNIPROT database [5]. Entire dataset was prepared in a fasta format by listing the amino acid residues present at the enzymatic active site in an ascending order. Residues constitute more than one active site of an enzyme are treated separately while preparing the dataset. The Viral protein dataset is small because till date only 72 viral protein structures, having 30% identity, are reported in PDB and only nine are viral enzymes. The viral protein dataset represents different type of proteins and categories of viruses such as chikunguniya, west nile virus, dengue, encephalitis, pancreatic necrosis virus etc.

The frequency distribution of 20 amino acid residues at enzymatic active site was computed using the number of amino acids of each type and the total number of residues. It is defined as Residue composition

$$(\%)\ (r) = \sum nr/N \times 100 \quad (1)$$

where 'r' stands for one of the 20 amino acid residues. ∑nr is the sum total of residue of each type and N is the total number of residues in the dataset. The prepared dataset contains 2683 catalytic residues in total. The catalytic residues in sub-datasets are Viral (27), Prokaryotic membrane (76) and non-membrane (1493) and Eukaryotic membrane (72) and non-membrane (1015) respectively.
The probability of each amino acid occurrence as a catalytic residue at the enzymatic active site was calculated as follows:

Amino acid probability = Sum total of an amino acid at the active site /Sum total of an amino acid in the whole enzyme……(2)

[III] RESULTS

3.1. General

The composition of catalytic residues is calculated by using equation 1. The percentage composition of catalytic residue indicates the occurrence of only 13 amino acid residues (out of 20 total amino acids) at enzymatic active site **[Table-1]**. Five charged residues (E, D, H, K, R), six polar residues (C, Y, T, S, Q, N) and two hydrophobic residues (A/F/W, G) constitute the total 13 catalytic residues. Depending upon the organism, the charged, polar and hydrophobic catalytic residues show percentage frequency distribution range of 55-65%, 22-40%, and 8-11% respectively **[Table-2]**.

In contrast to nMEs, MEs show higher percentage frequency of polar catalytic residues ~10-15%; [**Table–2**]. In MEs, two polar catalytic residues 'S' and 'Y' possess higher percentage frequency and responsible for creating the difference between MEs and nMEs. In general, 'S' forms hydrogen bonds with water and other neighbouring molecules and helps in the stabilization of transmembrane domains, while some of the membrane proteins such as ion-channels are regulated via phosphorylation and dephosphorylation of 'Y' residue [6].

The comparison of frequency distribution of catalytic residues among VEs, pEs and eEs revealed that these enzymes possess preference for four charged residues (H, D, E, R) and a single polar residue 'S' at their active site **[Table–3]**. This implies that at least five catalytic residues i.e. four charged (H, D, E, R) and one polar 'S', are extremely favoured at the active site of enzymes of different origin irrespective of their cellular localization. Furthermore, pEs and eEs also show preferential occurrence of another charged 'K' and a polar residue 'Y' at their active site. Therefore, the active site of pEs and eEs prefer five charged and two polar residues with their frequency distribution order of H>D>K>E>R and Y>S respectively. It can be concluded that the seven catalytic residues (H>D>K>E>R>Y>S) constitute the functional elements of Prokaryotic and Eukaryotic enzymes and mutation of any of these residues might affect the enzyme kinetics most significantly [7–9]. The probability occurrence calculation revealed that 'H' residue has highest chance of occurrence as catalytic residue among all the 13 frequently distributed amino acids. 'D' residue has almost equal probability of occurrence except eMEs (eq. 2). 'E' has exactly half the chances of occurrence in pMEs in comparison to other enzymes. Comparatively, the amino acid 'K' has least possibility of occurrence in VEs. 'R' strongly favours the VEs active site, while 'S' best occupy the MEs active site. 'Y' has the highest probability of occurrence at the active site of eMEs **[Table–1].**

3.2. Frequency distribution

3.2.1. Viral enzymes (VEs)

The frequency distribution comparison of active site residues revealed unique features among enzymes of different origin. In VEs, order of charged residue distribution is R>H≥D>E>K (66.65% of total), polar residue order is S>Y≥T≥C≥Q (22.21% of total). In addition, VEs also possess high frequency of a hydrophobic residue 'A' (7.41% of total). VEs are significantly similar to nMEs in the overall frequency distribution of charged and polar residues probably due to the usage of host cell machinery for their production. VEs show high dependency on four charged catalytic residues R>H≥D>E for catalysis. Interestingly, except 'S', frequency distribution indicates that the rest of the polar residues are equally contributing to the

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catalytic activity of VEs. VEs uniquely possess 'A' residue at their active site, while pMEs and eMEs totally lack it. pnMEs and enMEs have in-significant distribution of 'A' at their active site. Among charged residues 'R' have highest percentage frequency distribution (22.22%), which is almost double over pEs and eEs (8%-10%).

Table: 1. Percentage frequency distribution of catalytic residues of enzymes belongs to different organisms. The probability occurrence of active site amino acids is shown in bracket.

Amino acid residues	Viral enzymes (VEs)	Prokaryotic Membrane enzymes (pMEs)	Eukaryotic Membrane enzymes (eMEs)	Prokaryotic non-Membrane enzymes (pnMEs)	Eukaryotic non-Membrane enzymes (enMEs)
L	0%	0%	0%	0.59%	0.65%
I	0%	0%	1.16% (0.01)	0.39%	0.47%
F	0%	4.23% (0.04)	2.33% (0.03)	1.84% (0.01)	1.49% (0.02)
W	0%	0%	0%	1.90% (0.02)	1.40% (0.02)
V	0%	0%	0%	0.26%	0.19%
M	0%	1.40% (0.01)	0%	0.85% (0.01)	0.28%
A	7.41% (0.08)	0%	0%	1.97% (0.02)	1.02% (0.01)
G	3.70% (0.04)	2.82% (0.03)	4.65% (0.04)	2.95% (0.03)	3.63% (0.05)
P	0%	0%	0%	0.41% (0.004)	0.19% (0.002)
C	3.73% (0.04)	2.82% (0.03)	8.14% (0.09)	5.38% (0.06)	3.72% (0.04)
Y	3.70% (0.04)	9.86% (0.08)	13.95% (0.12)	5.77% (0.05)	6.42% (0.06)
T	3.70% (0.04)	7.04% (0.07)	3.49% (0.04)	2.69% (0.03)	3.72% (0.04)
E	11.11% (0.11)	5.63% (0.06)	9.30% (0.11)	11.42% (0.13)	13.58% (0.14)
S	7.41% (0.07)	11.27% (0.11)	11.63% (0.13)	5.31% (0.05)	6.42% (0.06)
Q	3.70% (0.04)	0%	2.33% (0.03)	1.64% (0.02)	2.42% (0.02)
D	14.81% (0.15)	14.08% (0.14)	5.81% (0.07)	15.94% (0.16)	15.81% (0.02)
H	14.81% (0.15)	18.31% (0.18)	15.12% (0.17)	15.16% (0.14)	19.06% (0.19)
N	0%	4.23% (0.04)	1.16% (0.02)	4.13% (0.04)	3.81% (0.04)
K	3.70% (0.04)	9.86% (0.10)	11.63% (0.13)	10.70% (0.11)	7.53% (0.07)
R	22.22% (0.22)	8.45% (0.08)	9.30% (0.11)	10.70% (0.11)	8.19% (0.08)

Table: 2. Percentage frequency distribution of three broad classes of catalytic residues of enzymes of different origin and cellular localization. Polar 'H' is categorised under charged group of residues due to its pK_a alteration in a protein.

Enzyme origin	Catalytic residue type (%)		
	Charged (E, D, H, K, R)	Polar (C, Y, T, S, Q, N)	Hydrophobic
VE	66.65	22.24	11.11
pME	56.33	35.22	8.45
eME	51.16	40.70	8.14
pnME	63.92	24.92	11.16
enME	64.17	26.51	09.32

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Table: 3. Comparison of most frequently occurring catalytic residues (highlighted in bold) of enzymes of different origin and cellular localization.

Enzyme origin	Enzyme's catalytic residues having highest frequency of occurrence										
VE	A		**D**	**E**		**H**		**R**	**S**		
pME			**D**	**E**	F	**H**	**K**	**R**	**S**	T	**Y**
eME		C	**D**	**E**		**H**	**K**	**R**	**S**		**Y**
pnME			**D**	**E**		**H**	**K**	**R**	**S**		**Y**
enME			**D**	**E**		**H**	**K**	**R**	**S**		**Y**

3.2.2. Membrane enzymes (MEs)

The comparison of catalytic residue frequency distribution of MEs in Prokaryotes and Eukaryotes show the definite preferences. The order of charged residues distribution is H>D>K>R>E and H>K>R≥E>D in pMEs (56.33% of total) and eMEs (50.96% of total), while polar residues are distributed in order S>Y>T>N>C and Y>S>C>T>Q>N in pMEs (35.22% of total) and eMEs (40.7% of total) respectively. The comparison revealed that the type and overall proportion of charged catalytic residues is similar in both pMEs and eMEs. The noteworthy feature is the distinct preference of 'D' and 'E' charged catalytic residues by pMEs and eMEs. The respective composition of 'D' in pMEs and eMEs is 14.08% and 5.81%, while the composition of 'E' in pMEs and eMEs is 5.63% and 9.30% respectively. The 'D' residue is a strong nucleophilic as well as electrophilic agent normally involved in the activation of molecules such as water and also forms metal co-ordination complexes [10]. The charged catalytic residues distribution indicates that 'H' (18.31%) and 'D' (14.08%) plays crucial role in the catalysis of pMEs, while 'H' (15.12%) and 'K' (11.63%) are important for eMEs catalysis. Also, the polar residues distribution indicates that 'S' (11.27%), 'Y' (9.86%) and 'T' (7.04%) plays crucial role in the catalysis of pMEs, while 'Y' (13.95%), 'S' (11.63%) and 'C' (8.14%) are important for eMEs catalysis **[Table-1]**. Among polar residues, pMEs prefer 'T' instead of 'C' probably because hydroxyl moiety of 'T' side chain acts as a potent nucleophile for binding and catalyzing the substrate [11]. The reactive hydroxyl and thiol group in side chain of 'Y' and 'C' have the ability to de-protonate and re-protonate for stabilizing the enzyme-substrate transition state complex [12]. The main chain atoms of 'Y' also involve in the formation of hydrogen bonds for stabilization of active site during catalysis [13]. As the probability occurrence of 'Y' (0.12) is higher than 'D' (0.07) in eMEs active site, it is possible that 'Y' can partly contribute in activation of substrate molecule as it is a weaker nucleophile than 'D'. The amino acid 'S' is equally distributed in both pMEs (11.27%) and eMEs (11.63%). Comparatively, MEs have almost double distribution of 'S' with respect to VEs and nMEs. The short side chain of 'S' may help in maintaining the stability of the active site while its reactive hydroxyl group may function like 'Y' in acid-base catalysis, formation of transition state complex [14]. In contrast to other class of enzymes, pMEs exclusively possess high percentage of one hydrophobic 'F' (4.23%) amino acid at their active site. The presence of 'F' at the active site helps in stabilizing the ligand and enzyme interaction [15]. Depending upon the frequency distribution of catalytic residues, we can conclude that the 'H, D, S, Y, T, F' and 'H, K, Y, S, C' residues are most important in pMEs and eMEs catalysis.

3.2.3. Non-membrane enzymes (nMEs)

Comparison of composition of catalytic residues of nMEs in Prokaryotes and Eukaryotes revealed that the charged residues are distributed in order of D>H>E>R≥K and H>D>E>R>K in pMEs (63.92% of total) and eMEs (64.18% of total), while polar residues are distributed in order Y>C>S>N>T>Q and Y≥S>N>C≥T>Q in pMEs (24.92% of total) and eMEs (26.51% of total) respectively **[Table-1]**. In contrast to MEs, nMEs have dominance of charged catalytic residues at their active site as evident by their percentage composition. This implies that all the five charged residues are critical for the function of nMEs, while the polar residues having supportive functions like stabilization of the substrate/intermediate molecule. In pnMEs, three polar catalytic residues (Y, C and S) are equally important for catalytic function, while in enMEs 'Y and S' are the two key residues having broader spectrum of distribution and possibly more suitable for supportive catalytic functions in comparison to other polar residues.

3.2.4. Comparison of MEs and nMEs

The comparison of catalytic residue composition and distribution between pMEs and pnMEs and eMEs and enMEs was carried out separately **[Figure-2].** In pnMEs the role of 'H' is partly shared by 'D' and 'E' residues, while in pMEs the polar catalytic residues (Y, S and T) have greater importance. Interestingly, both pMEs and pnMEs have more or less equal distribution of 'K, R and N'. The conserve distribution of these three residues indicates that the terminal hydrogen atom of these

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residues necessary for the stabilization of the intermediate via hydrogen bonding and keeping the intermediate at the active site during catalysis. The high proportion of 'C' in pnMEs shows that the terminal sulphur atom 'C' plays a key role in attacking the substrate and initiating the catalytic reaction [16]. The absence of 'Q' amino acid in pMEs and its less significant proportion in pnMEs indicate that this polar residue is not vital for executing the catalytic function of enzymes. Overall, pMEs depends more or less equally on charged (56.33%) and polar (35.22%) residues for catalysis, while pnMEs primarily dependent on charged residues (63.92%) for catalysis and polar residue (24.92%) support the catalytic function.

The comparison of eMEs and enMEs revealed that enMEs show marked dependency on 'H, D' and 'E' charged residues for their catalytic activity, while eMEs preferentially dependent on 'H and K' residues for their catalytic activity and least dependent on 'D' residue. It is difficult to understand the significant compositional difference for 'D' residue between eMEs and enMEs, at least this difference does not reflect the biased dataset. The three polar catalytic residues 'Y, S and C' contribute actively in eMEs catalysis; their proportion is more or less half in enMEs. It is possibly that these three residues are involved in executing certain specific catalytic function in eMEs which are not carried out by enMEs. With respect to eMEs, enMEs have significantly high frequency of 'N' residue, possible it is related to the size of the substrate. eMEs depends equally on charged (50.96%) and polar (40.7%) residues for catalysis, while enMEs show high dependence on charged residues (64.18%) and polar residue (26.51%) support the catalytic function. In total, both Prokaryotic and Eukaryotic MEs and nMEs show high degree of similarity in the percentage distribution of charged and polar residues at their active site.

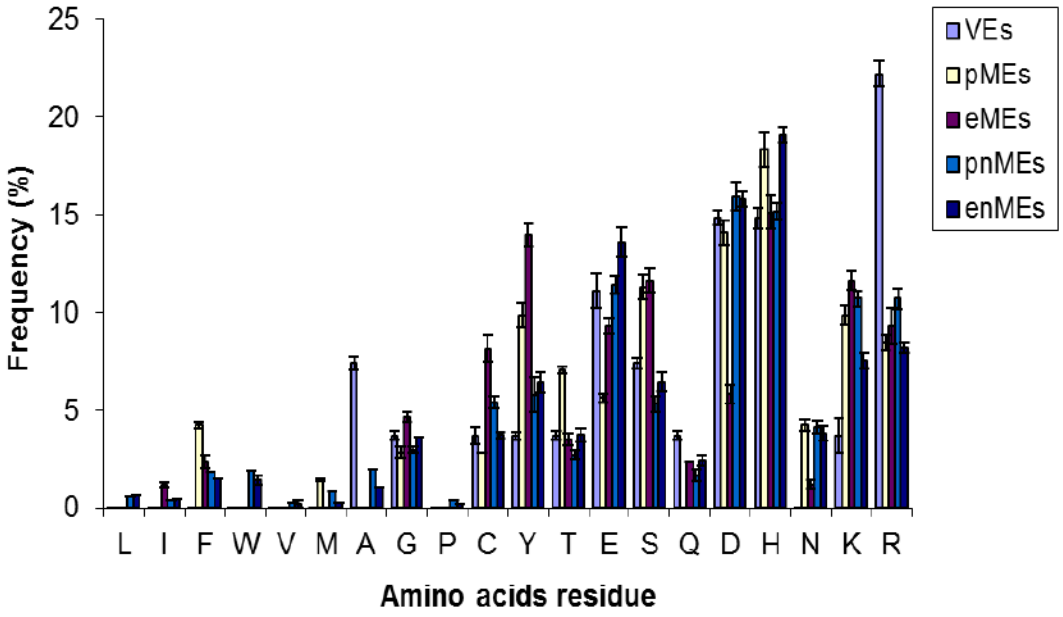

Fig: 1. Amino acid frequency distribution at the enzymatic active site of different organisms. The amino acids are arranged in decreasing order of hydrophobicity. The standard deviation for frequency distribution of a particular catalytic active site residue of an enzyme belongs belong to different organisms is represented separately as a bar. VE: Blue bar; pME: brown bar; eME: yellow bar; pnME: purple bar; enME: black bar.

[IV] DISCUSSION

The study is based on the comparative frequency distribution of catalytic residues occurring in enzymes belongs to different organism and cellular localization **[Table−1 and Figure−1],** which is different from the earlier study where the frequency distribution of enzyme catalytic residues was reported irrespective of their origin and cellular localization (Charged - 65%; Polar - 27%; Hydrophobic - 8%) [3]. In general, the significant occurrence of charged and polar residues at the enzymatic active site is expected as they provide the electrostatic force necessary for the movement of electrons and protons during catalysis. The results indicate that the catalytic residues of membrane enzymes (MEs) show significant variation in the distribution in contrast to the composition reported by Bartlett et al. (2002). In addition, the slight deviation of catalytic residues frequency distribution in non-membrane enzymes (nMEs) is probably due to the large dataset used in this study in comparison to 178 proteins (from undefined origin) included in the dataset of Bartlett et al.

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(2002).

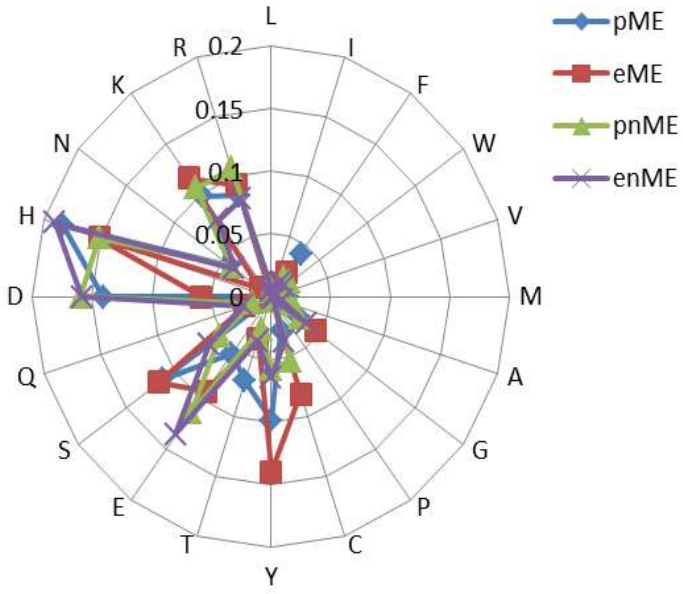

Fig: 2. Radar projection highlighting the difference between amino acid frequency distribution of membrane and non-membrane enzyme active site.

The highest probability occurrence of 'H' as catalytic residue among all the 13 frequently distributed amino acids indicates its multiple roles at enzymatic active site such as nucleophilic and electrophilic agent, acid and base, and stabilization of enzyme-substrate transition state complex. It has been recently reported that the shape of 'H' is also important in enzyme catalysis [17]. 'D' residue has almost equal probability of occurrence at enzymes active site except in eMEs **[Table–1]**. It is possible that not all the eMEs possess 'D' residues at their active site except those belong to particular family [18]. 'E' has exactly half the chances of occurrence in pMEs and also shows low distribution in eMEs in comparison to other enzymes. This pattern of frequency distribution of 'E' may be due to the occurrence of less number of membrane enzymes in general and its role in selected category of membrane proteins [19]. Comparatively, the amino acid 'K' has least possibility of occurrence in VEs. 'R' strongly favors the VEs active site, while 'S' best occupy the MEs active site. 'Y' has the highest probability of occurrence at the active site of eMEs **[Table–1]**.

The Viral dataset used in this study is relatively smaller in comparison to Prokaryotes and Eukaryotes because only nine viral enzyme (VEs) structures, having 30% sequence identity, are currently available in PDB. Furthermore, the viral enzyme homologues active site is identified with the help of multiple sequence alignment by using viral enzymes, having solved structure in PDB, as reference [4]. Though the viral dataset used here is small, it is a representative of protein mosaic (e.g. Protease, Helicase, RNA polymerase etc.) belongs to different types of viruses. The VEs show distinct high occurrence of 'R and A' catalytic residues in comparison to prokaryotic and eukaryotic enzymes. The replication and production of viral particles inside the host cell require viral enzymes to catalyse exponentially the replication and other reactions. Probably three nitrogen atoms in the side chain of 'R' help viral enzymes to make stronger and stable electrostatic interactions with the substrate to facilitate the enzymatic reactions at an exponential rate. Also 'R' side-chain has a good geometry to stabilize a pair of oxygen atoms on a phosphate group, which facilitate the interaction of Viral enzymes with host DNA/RNA molecules [20]. The significantly high occurrence of an aliphatic and short side chain 'A' residue probably helps in assisting the proper positioning of the substrate at the enzyme active site and also keeps the intermediate ions/substrate stable.

The high occurrence of polar catalytic residues in MEs play an essential role in functioning of transmembrane helices via mediating and stabilizing helical interactions [21]. In addition, enzyme frequently uses polar and non-polar groups to make weak interactions with the substrate in their specificity sub-site of the active site [22].

[V] CONCLUSION

Enzymes of different origin have distinct preference of charged and polar amino acids at their active site. Certainly, high probability occurrence of these amino acids does not indicate their conservation at the active site. The knowledge of amino acid frequency distribution at the active site can be exploited in designing novel enzymes active site as well as their specific inhibitors.

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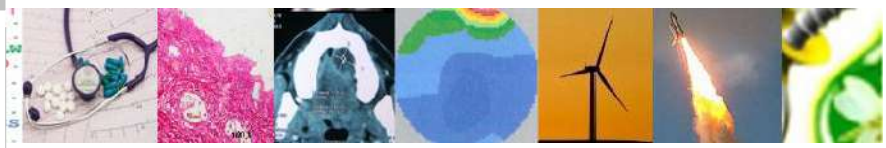

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RESEARCH: MOLECULAR BIOLOGY

DNA SYNTHESIS IN THE PANCREATIC ACINAR CELLS OF AGING MICE AS REVEALED BY ELECTRON MICROSCOPIC RADIOAUTOGRAPHY

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ABSTRACT

For the purpose of studying the aging changes of macromolecular synthesis in animal cells, we studied 10 groups of aging mice during development and aging from fetal day 19 to postnatal month 24. They were injected with ^{3}H-thymidine, a precursor for DNA synthesis, sacrificed and the pancreatic tissues were taken out, fixed and processed for light and electron microscopic radioautography. On many radioautograms the localization of silver grains demonstrating DNA synthesis incorporating ^{3}H-thymidine in the pancreatic acinar cells in respective aging groups were analyzed. The number of silver grains and the number of cell organelles in each cell in respective aging groups were analyzed quantitatively in relation to the aging of animals. The results revealed that the DNA synthesis as expressed by the number of silver grains in cell nuclei, cell organelles, changed with the aging of animals. The number of mitochondria, the number of labeled mitochondria and the mitochondrial labeling index labeled with silver grains were counted in each pancreatic acinar cell. It was demonstrated that the number of mitochondria increased from embryonic day 19 to postnatal newborn day 1, 3, 9, 14, adult month 1, 2 and 6, reaching the maxima, then decreased to senile year 1 to 2.. On the other hand, the number of labeled mitochondria and the labeling indices showing DNA synthesis at various ages increased from embryonic day 19 to postnatal newborn day 1, 3, 9, 14, reaching the maxima and decreased to adult month 1, 2 and 6, to senile year 1 to 2, indicating the aging changes. These results demonstrated that intramitochondrial DNA synthesis in the pancreatic acinar cells increased and decreased due to aging of individual animals depending upon the cellular activities at respective aging stages. Based upon our findings, available literatures on macromolecular synthesis in mitochondria of various cells are reviewed.

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Keywords: *DNA synthesis; mitochondria; pancreatic acinar cells; aging; mice*

[I] INTRODUCTION

The pancreas is a large gland in animals and men, next to the liver, among the digestive glands connected to the intestines. It consists of exocrine and endocrine portions and takes the shape of a compound acinous gland. The exocrine portion is composed of ductal epithelial cells, centro-acinar cells, acinar cells and connective tissue cells, while the endocrine portion, designated as the islet of Langerhans, is composed of 3 types of endocrine cells, A, B, C cells and connective tissue cells.

We have studied the macromolecular synthesis of the aging mouse pancreas at various ages by means of light and electron microscopic radioautography. We first studied the DNA synthesis of mouse pancreas by LM and EM RAG using ^{3}H-thymidine [1, 2, 3]. Light and electron microscopic radioautograms (LM and EM RAG) of the pancreas revealed that the nuclei of pancreatic acinar cells, centro-acinar cells, ductal epithelial cells, and endocrine cells were labeled with ^{3}H-thymidine demonstrating DNA synthesis **[Figures–1-10]**. The number of labeled cells were counted and the labeling indices of these cells in 10 groups of litter mate mice, fetal day 19, postnatal day 1, 3, 7, 14, and month 1, 6, 12 (year 1) and 24 (2 years) were analyzed. The labeling indices of these cells

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reached the maxima at day 1 after birth and decreased gradually from postnatsl day 1 **[Figure–1]** to day 7 **[Figure–2],** day 14 **[Figures– 3-5]**, month 1 **[Figure-6],** month 2 **[Figure–7]**, month 6 and month 24 or 2 years **[Figure-10]**. The maximum of the labeling indices in the acinar cells proceeded to the ductal and centro-acinar cells, suggesting that the acinar cells completed their development earlier than the ductal and centro-acinar cells [1, 2, 3].

On the other hand, LM and EM RAG of pancreas of mouse injected with ^{3}H-uridine into the aging mice from embryo to postnatal year 2, demonstrated its incorporation into exocrine and then in endocrine cells, and more in pancreatic acinar cells than in ductal or centro-acinar cells [1, 2, 3]. Among the acinar cells, the number of silver grains increased after birth to day 7 and day 14, then decreased to month 1, 2, 6, 12, and 24 with aging. Quantification of silver grains in the nucleoli, chromatin, and cell body were carried out by X-ray microanalysis [5, 6], which verified the results obtained by visual grain counting. In EM RAG obtained from the pancreas of fetal day 19 embryos, newborn day 1 and newborn day 14 mice labeled with ^{3}H-uridine, demonstrating RNA synthesis, the number of silver grains in the nucleoli, nuclear chromatin and cytoplasm increased [6, 7]. In order to quantify the silver contents of grains observed over the nucleoli, nuclei and cytoplasm, X-ray spectra were recorded by energy dispersive X-ray microanalysis (JEM-4000EX TN5400), demonstrating Ag-Kα peaks at higher energies.

On the other hand, in contrast to the DNA and RNA syntheses in nuclei and nucleoli in various cells of aging mice, we also found the silver grains due to DNA and RNA synthesis in mitochondria of various isolated cells such as the livers and kidneys in vitro showing intramitochondrial DNA and RNA syntheses [8]. We later found that the activities of DNA and RNA syntheses in mitochondria of various cells changed due to aging of individual animals [8, 9, 10, 11].

Thus, we have recently concentrated to clarify the intramitochondiral DNA and RNA as well as protein synthesis in various cells of aging mice [12], especially in hepatocytes which contained many mitochondria [13]. This paper deals with the intramitochondrial DNA synthesis in pancreatic acinar cells of aging ddY mice at various ages in 10 groups from prenatal embryos to postnatal 2 years at senescence.

[II] MATERIALS AND METHODS

2.1. The experimental animals

The pancreatic tissues were obtained from 10 groups of aging normal ddY strain mice, each consisting of 3 litter mates of both sexes, total 30, from prenatal embryo day 19 to newborn postnatal day 1, 3, 7, 14, adult at month 1, 2, 6, 12 (year 1) to month 24 (year 2). All the animals were housed under conventional conditions and bred with normal diet (mouse chow Clea EC2, Clea Co., Tokyo, Japan) with access to water ad libitum in our laboratory. They were administered with ^{3}H-thymidine, a DNA precursor, and the pancreatic tissues were taken out, fixed and processed for electron microscopic radioautography. All the procedures used in this study concerning the animal experiments were in accordance with the guidelines of the animal research committee of Shinshu University School of Medicine as well as the principles of laboratory animal care in NIH publication No. 86-23 (revised 1985).

2.2. Procedures of electron microscopic radioautography

All the animals were injected intraperitoneally with ^{3}H-4-thymidine (Amersham, England, specific activity 877 GBq/mM) in saline, at 9 a.m., one hour before sacrifices. The dosage of injections was 370 KBq/gm body weight. The animals were perfused at 10 a.m., one hour after the injection, via the left ventricles of the hearts with 0.1 M cacodylate-buffered 2.5% glutaraldehyde under Nembutal (Abbott Laboratories, Chicago, ILL, USA) anesthesia. The right end of the pancreatic gland was taken out from each animal, excised and 3 small pieces of the pancreatic tissues (size 1mm x 1mm x 1mm) were immersed in the same fixative at 4˚C for 1 hr., followed by postfixation in 1% osmium tetroxide in the same buffer at 4˚C for 1 hr., dehydrated in graded series of ethanol and acetone, and embedded in epoxy resin Epok 812 (Oken, Tokyo, Japan).

For electron microscopic radioautography, semithin sections at 0.2μm thickness, thicker than conventional ultrathin sections in order to shorten the exposure time, were cut in sequence on a Porter-Blum MT-2B ultramicrotome (Dupont-Sorvall, Newtown, MA, USA) using glass knives. The sections were collected on collodion coated copper grid meshes (VECO, Eerbeek, Netherlands), coated with Konica NR-H2 radioautographic emulsion (Konica, Tokyo, Japan) by a wire-loop method [5, 6, 7]. They were stored in dark boxes containing silica gel (desiccant) at 4˚C for exposure. After the exposure for 10 months, the specimens were processed for development in freshly prepared gold latensification solution for 30 sec at 16˚C and then in fresh phenidon developer for 1 min at 16˚C in a water bath, rinsed in distilled water and dried in an oven at 37˚C overnight, stained with lead citrate solution for 3 min, coated with carbon for electron microscopy. The electron microscopic (EM) radioautograms were examined in a JEOL JEM-4000EX electron microscope (JEOL, Tokyo, Japan) at accelerating voltages of 400 kV for observing thick specimens.

2.3. Quantitative analysis of electron micrographs

For quantitative analysis of electron micrographs, twenty EM radioautograms showing cross sections of pancreatic acinar cells from each group, based on the electron microscopic photographs taken after observation on at least 100 pancreatic acinar cells from respective animals were analyzed to calculate the total number of mitochondria in each cell, and the number of labeled mitochondria covered with silver grains by visual grain counting.

On the other hand, the number of silver grains in the same area size as a mitochondrion outside cells was also calculated in respective specimens as background fog, which resulted in less than 1 silver grain (0.02/mitochondrial area) almost zero. Therefore, the grain count in each specimen was not corrected with background fog. From all the data thus obtained the averages and standard deviations in respective aging groups were computed with a personal computer (Macintosh type 8100/100, Apple Computer, Tokyo, Japan). The data were stochastically analyzed using variance and Student's t-test. The differences were considered to be significant at P value <0.01.

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[III] RESULTS

3.1. Morphological observations

The pancreatic tissues obtained from ddY strain mice at various ages from embryo day 19 to postnatal month 24, consisted of 2 portions, the exocrine portion **[Figures–1–4, 6–10]** and the endocrine portion or designated as the islets of Langerhans **[Figure– 5]**. The exocrine portion is consisted of several cell types, the pancreatic acinar cells **[Figures–1-3, 6-10]**, the centroacinar cells **[Figure– 4]**, ductal cells and fibroblasts, as observed by electron microscopy. The acinar cells are main components of the exocrine portions which contained well developed endoplasmic reticulum, zymogen granules, and many mitochondria in the cytoplasm. Because the number of mitochondria in the pancreatic acinar cells were relatively much more than the other cells, only the pancreatic acinar cells were analyzed in this study.

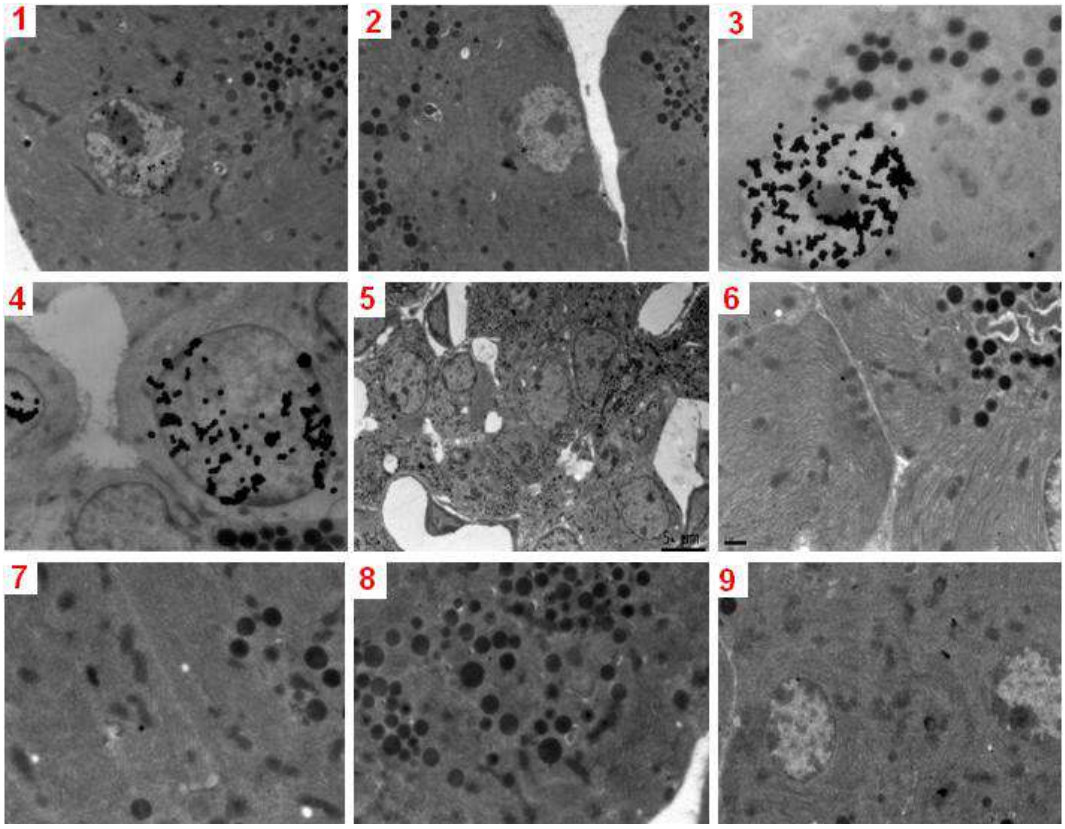

Fig: 1. EM RAG of a pancreatic acinar cell of a postnatal day 1 mouse labeled with ^{3}H-thymidine. x3,000 **Fig: 2.** EM RAG of 2 pancreatic acinar cells of a postnatal day 7 mouse labeled with ^{3}H-thymidine. x3,000 **Fig: 3.** EM RAG of a pancreatic acinar cell of a postnatal day 14 mouse labeled with ^{3}H-thymidine. x5,000 **Fig: 4.** EM RAG of 2 pancreatic centro-acinar cells of a postnatal day 14 mouse labeled with ^{3}H-thymidine. x5,000 **Fig: 5.** EM RAG of endocrine portion of a postnatal day 14 mouse labeled with ^{3}H-thymidine. x3,000 **Fig: 6.** EM RAG of 3 pancreatic acinar cells of a postnatal month 1 mouse labeled with ^{3}H-thymidine. x3,000 **Fig: 7.** EM RAG of a pancreatic acinar cell of a postnatal month 2 mouse labeled with ^{3}H-thymidine. x5,000 **Fig: 8**. EM RAG of a pancreatic acinar cell of a postnatal month 6 mouse labeled with ^{3}H-thymidine. x5,000 **Fig: 9.** EM RAG of 2 pancreatic acinar cells of a postnatal month 12 mouse labeled with ^{3}H-thymidine. x3,000

3.2. Radioautographic observations

Observing electron microscopic radioautograms, the silver grains were found over the nuclei of some pancreatic acinar cells **[Figures–1, 3, 4]**, labeled with ^{3}H-thymidine, demonstrating DNA synthesis mainly in perinatal stages at embryonic day 19, postnatal day 1 **[Figure–1]** and day 3, increased to day 7 and day 14 **[Figures–3, 4]**, reaching the maximum showing labeled mitochondria around 0.8-0.9/cell, then decreased to adult stage at month 1, month 2 and 6, showing labeled mitochondria around 0.4-0.5/cell.

The localizations of silver grains over the mitochondria were mainly on the mitochondrial matrices similarly to other cells such as in the livers [13] or the adrenal glands [14]

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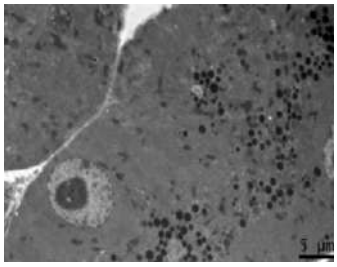

Fig: 10. EM RAG of 3 pancreatic acinar cells of a postnatal month 24 mouse labeled with ^{3}H-thymidine. x3,000

3.3.2. Mitochondrial DNA synthesis

The results of visual counting on the number of mitochondria labeled with silver grains obtained from 10 pancreatic acinar cells of each animal labeled with ^{3}H-thymidine demonstrating DNA synthesis in 10 aging groups at perinatal stages, from prenatal embryo day 19 (0.5/cell), postnatal day 1, 3, 7 and 14, to adult stages at month 1, 3, and 6, 12 and 24, increased gradually to day 7 and 14, reaching the maximum (0.8/cell), then decreased gradually to month 1, 2, 6, 12 and 24 (0.2/cell) as shown in **Figure– 12**. The data were stochastically analyzed using variance and Student's t-test. The increases of the numbers of labeled mitochondria from embryo day 19 to postnatal day 14, as well as the decreases from day 14 to month 24 were stochastically significant ($P < 0.01$).

3.3.3. The labeling index

Finally, the labeling indices of mitochondrial DNA synthesis in pancreatic acinar cells at respective aging stages were calculated from the number of labeled mitochondria **[Figure–11]** dividing by the number of total mitochondria per cell **[Figure– 10]** which were plotted in **Figure–13**.

The results showed that the labeling indices gradually increased from prenatal day 19 (6.4%) to postnatal newborn day 1 (6.1%), day 3 (5.6%), day 7 (6.1%), day 14 (5.6%), reaching the maximum, and decreased to adult stages at month 1 (3.4%), month 2 (3.1%), month 6 (2.8%), month 12 (2.1%) and 24 (1.4%) as shown in **Figure–13**. From the results, the increases of the mitochondrial labeling indices in pancreatic cells from embryo day 19 to postnatal day 14, as well as the decreases from day 14 to month 24 were stochastically significant ($P < 0.01$).

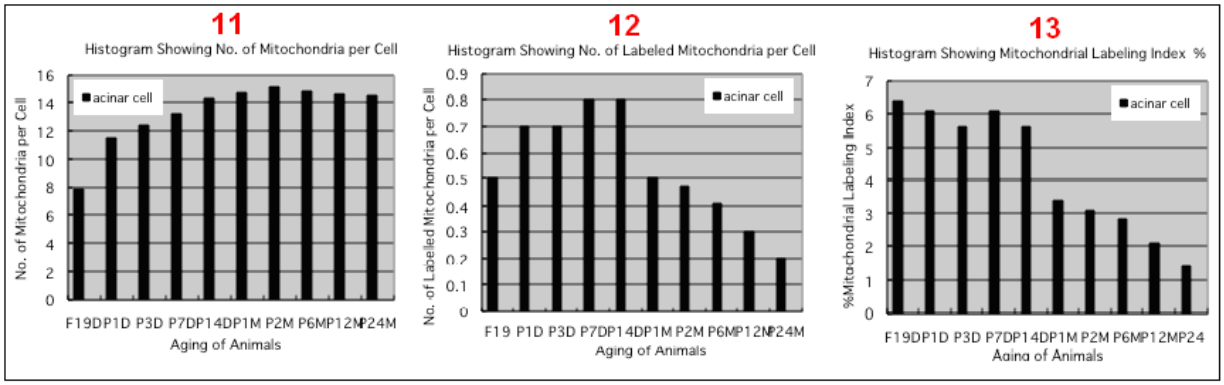

Fig: 11. Histogram showing average number of mitochondria per cell in a pancreatic acinar cell of aging mice at various ages from prenatal day 19 to postnatal month 24 **Fig: 12.** Histogram showing average number of labeled mitochondria per cell in a pancreatic acinar cell of aging mice at various ages from prenatal day 19 to postnatal month 24, labeled with ^{3}H-thymidine **Fig: 13.** Histogram showing labeling indices of labeled mitochondria per cell in aging mice at various ages from prenatal day 19 to postnatal month 24, labeled with ^{3}H-thymidine

[IV] DISCUSSION

From the results obtained in the present study on the pancreatic acinar cells of ddY aging mice at various ages in 10 groups from perinatal stages at embryo day 19, to newborn day 1, 3, 7, 14, and young adult at postnatal month 1, 2, 6 as well as the senescent adult at postnatal month 12 and 24, it was shown that intramitochondrial DNA synthesis was observed in the pancreatic acinar cells of all the aging stages from prenatal embryos to postnatal newborn, young juvenile and adult stages and the number of mitochondria per cell showed increases due to aging, while the number of labeled mitochondria per cell and the labeling indices showed increases and decreases due to aging. These results demonstrated that intramitochondrial DNA synthesis in the pancreatic acinar cells revealed variations due to aging of individual animals depending upon the cellular activities at respective aging stages.

With regards to the macromolecular synthesis in various cells in various organs of experimental animals observed by light and electron microscopic radioautography, it is well known that the silver grains due to radiolabeled ^{3}H-thymidine demonstrate DNA synthesis [1, 4, 6, 10, 12-18]. The previous results obtained from the studies on the hepatocytes of aging mice by

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light and electron microscopic radioautography revealed that silver grains indicating DNA synthesis incorporating ^{3}H-thymidine were observed over the nuclei of some hepatocytes at perinatal stages from postnatal day 1 to day 14 and decreased due to aging [15-18]. Then, we lately observed the intramitochondrial DNA synthesis in the various organs such as the livers [12, 13, 19-22] adreno-cortical [14, 23-26] and adreno-medullary cells [14, 27, 28], at various ages from fetal day 19 to postnatal newborn day 1, 3, 7, juvenile day 14 and to adult month 1, 2, 12 and 24. In the present study, further data obtained from the pancreatic acinar cells from prenatal to adult senescent animals at postnatal month 12 and 24 were added.

From these studies, the numbers of silver grains showing nuclear DNA synthesis resulting from the incorporations of ^{3}H-thymidine into mitochondria indicating mitochondrial DNA synthesis demonstrated the silver grain localization over the mitochondria independently from the nuclei whether the nuclei were labeled with silver grains or not in the pancreatic acinar cells from prenatal embryo day 19 to postnatal month 24 during the development and aging. The numbers of labeled mitochondria showing DNA synthesis as well as the labeling indices increased from perinatal embryonic day to postnatal newborn and juvenile stages at day 14, reaching the maxima, and then decreased to the adult stages at month 1, 2, 6, 12 and 24.

With regards to DNA in mitochondria in animal cells or plastids in plant cells, many studies have been reported in various cells of various plants and animals since 1960s [29-34]. Most of these authors observed DNA fibrils in mitochondria which were histochemically extracted by DN'ase. Electron microscopic observation of the DNA molecules isolated from the mitochondria revealed that they were circular in shape, with a circumference of 5-6 µm [35]. It was calculated that such a single molecule had a molecular weight of about 10^{7} daltons [36]. Mitochondria of various cells also contained a DNA polymerase, which was supposed to function in the replication of the mitochondrial DNA [37]. On the other hand, the incorporations of ^{3}H-thymidine into mitochondria demonstrating DNA synthesis were observed by means of electron microscopic radioautography in lower organism such as slime mold [38, 39], tetrahymena [40] or chicken fibroblasts in tissue culture under abnormal conditions [41]. However, these authors used old-fashioned developers consisting of methol and hydroquinone (MQ-developer) which produced coarse spiral silver grains resulting in inaccurate localization over cell organelles when observed by electron microscopy. All of these authors showed photographs of electron radioautographs with large spiral-formed silver grains (2-3 µm in diameter) localizing not only over the mitochondria but also outside the mitochondria. In order to obtain smaller silver grains, we first used elon-ascorbic acid developer after gold latensification [7, 15], which produced comma-shaped smaller silver grains (0.4-0.8 µm in diameter), then later we used phenidon developer after gold latensification, producing dot-like smaller silver grains (0.2-0.4 µm in diameter) localizing only inside the mitochondria showing ultrahigh resolution of radioautograms [1, 12, 13, 42, 43]. These papers were the first which demonstrated intramitochondrial DNA synthesis incorporating ^{3}H-thymidine with accurate intramitochondrial localization in avian and mammalian cells. With regards the resolution of electron microscopic radioautography, on the other hand, many authors discussed the sizes of silver grains under various conditions and calculated various values of resolutions [8, 10, 44-46]. Those authors who used the M-Q developers maintained the resolution to be 100-160 nm [44, 45], while those authors who used the elon-ascorbic acid developer [8, 10, 46] calculated it to be 25-50 nm. When we used phenidon developer at 16˚C for 1 min after gold latensification, we could produce very fine dot-shaped silver grains and obtained the resolution around 25 nm [1, 12, 13, 42, 43, 46]. For the analysis of electron radioautographs, Salpeter et al. [40] proposed to use the half-distance and very complicated calculations through which respective coarse spiral-shaped silver grains were judged to be attributable to the radioactive source in a certain territory within a resolution boundary circle. However, since we used phenidon developer after gold latensification to produce very fine dot-shaped silver grains, we judged only the silver grains which were located in the mitochondria which were dot-shaped very fine ones to be attributable to the mitochondria without any problem as was formerly discussed [8, 10, 12, 13, 42, 43].

Then we also demonstrated intramitochondrial DNA synthesis incorporating ^{3}H-thymidine in some other established cell lines originated from human being such as HeLa cells [8, 10] or mitochondrial fractions prepared from in vivo mammalian cells such as rat and mouse [9, 11]. It was later commonly found in various cells and tissues not only in vitro obtained from various organs in vivo such as the cultured human HeLa cells [47], cultured rat sarcoma cells [48], mouse liver and pancreas cells in vitro [48, 50, 51], but also in vivo cells obtained from various organs such as the salivary glands [52], the liver [53-64], the pancreas [65], the trachea [66], the lung [67], the kidneys [68], the testis [69,70], the uterus [71,72], the adrenal glands [73-75], the brains [76], and the retina [77-81] of mice, rats and chickens. Thus, it is clear that all the cells in various organs of various animals synthesize DNA not only in their nuclei but also in their mitochondria.

The relationship between the intramitochondrial DNA synthesis and cell cycle was formerly studied in synchronized cells and it was clarified that the intramitochondrial DNA synthesis was performed without nuclear involvement [8]. However, the relationship between the DNA synthesis and the aging of individual animals and men has not yet been fully clarified except a few papers published by Korr and associates on mouse brain [82-85]. They reported both nuclear DNA repair, measured as nuclear unscheduled DNA synthesis, and cytoplasmic DNA synthesis labeled with ^{3}H-thymidine in several types of cells in brains such as pyramidal cells, Purkinje cells, granular cells, glial cells, endothelial cells, ependymal cells, and epithelial cells as observed by light microscopic radioautography using paraffin sections. They observed silver

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grains over cytoplasm of these cells by light microscopy and maintained that it was reasonable to interpret these labeling as ^{3}H-DNA outside the nuclei, which theoretically belonged to mitochondrial DNA without observing the mitochondria by electron microscopy. From the results, they concluded that distinct types of neuronal cells showed a decline of both unscheduled DNA and mitochondrial DNA syntheses with age in contrast that other cell types, glial and endothelial cells, did not show such age-related changes without counting the number of mitochondria in respective cells nor counting the labeling indices at respective aging stages. Thus, their results from the statistics obtained from the cytoplasmic grain counting seems to be not accurate without observing mitochondria directly. To the contrary, we had studied DNA synthesis in the livers of aging mice [53-64] and clearly demonstrated that the number of mitochondria in each hepatocytes, especially mononucleate hepatocytes, increased with the ages of animals from the perinatal stages to adult and senescent stages, while the number of labeled mitochondria and the labeling indices increased from the perinatal stages, reaching a maximum at postnatal day 14, then decreased.

Our previous studies [59, 60] also clarified that the DNA synthesis and cell proliferation by mitosis were the most active in the nuclei of mononucleate hepatocytes at the perinatal stages in contrast that binucleate cells were less active at the perinatal stage but the number of binucleate hepatocytes increased at senescent stages and the results suggest the possibility that the mitochondria in mononucleate hepatocytes synthesized their DNA by themselves which peaked at postnatal day 14 in accordance with the proliferation of mononucleate hepatocytes while binucleate hepatocytes increased after the perinatal stage and did not divide but remained binucleate keeping many mitochondria in their cytoplasm which were more in number than mononucleate hepatocytes at the senescent stage.

Thus, our previous papers were the first which dealt with the relationship between the DNA synthesis and aging in hepatocytes of mice in vivo at various ages by means of electron microscopic radioautography observing the small dot-like silver grains, due to incorporations of ^{3}H-thymidine, which exactly localized inside the mitochondria.
Later we also studied intramitochondrial DNA synthesis in adreno-cortical cells from prenatal day 19 to postnatal day 1, 3, 9, 14, month 1, 2, 6, 12 and 24 (year 2) and found that the numbers of mitochondria in 3 zones, glomerulosa, fasciculate and reticularis, increased reaching the maxima at postnatal month 2 and which kept continued until senescence up to 24 months (2 years). To the contrary, the numbers of labeled mitochondria and the labeling indices increased to postnatal month 2, reaching the maxima, then decreased to month 24 [23-28].

The present results also revealed that an increase was observed by direct observation on mitochondria at electron microscopic level and obtaining accurate mitochondrial number and labeling indices in the pancreatic acinar cells in 10 groups of developing and aging mice. There was a discrepancy between our results from the hepatocytes [59, 60] and the adrenal cells [23-28] as well as the pancreatic acinar cells at present and the results from the several types of cells in the brains by Korr et al. [82-85]. The reason for this difference might be due to the difference between the cell types (hepatocytes, adrenal cells, pancreatic cells from our results and the brain cells from their results) or the difference between the observation by electron microscopy, i.e., direct observation of mitochondria in our results or light microscopy, i. e., indirect observation of mitochondria without observing any mitochondria directly by Korr et al. [82-85].

Anyway, the results obtained from the pancreartic acinar cells of aging mice at present should form a part of special cytochemistry [17] in cell biology, as well as a part of special radioautographology [12], i.e., the application of radioautography to the pancreas, as was recently reviewed by the present author including recent results dealing with various organs [86-90]. We expect that such special radioautographology and special cytochemistry should be further developed in all the organs in the future.

[V] CONCLUSION

From the results obtained at present, it was concluded that almost all the pancreatic acinar cells in the pancreatic exocrine portions of mice at various ages, from prenatal embryo day 19 to postnatal newborn, day 1, 3, 7 and 14, and to postnatal month 1, 2, 6, 12 and 24, were labeled with silver grains showing DNA synthesis with ^{3}H-thymidine in their mitochondria. Quantitative analysis on the number of mitochondria in pancreatic acinar cells resulted in increases from the prenatal day to postnatal day 1, 3, 9, 14, to month 1 and 2, reaching the maximum at postnatal month 2, then slightly decreased to month 6, 12 and 24. To the contrary, the numbers of labeled mitochondria with ^{3}H-thymidine showing DNA synthesis and the labeling indices also increased from prenatal day 19 to postnatal day 14, reaching the maximum at postnatal day 14, and then decreased to month 1, 2, 6, 12 and 24. These results demonstrated that the number of mitochondria in pancreatic acinar cells increased from perinatal stages to postnatal month 2, keeping the maximum up to month 24, while the activity of mitochondrial DNA synthesis increased to postnatal day 14, reaching the maximum, then decreased to month 24 due to aging of animals.

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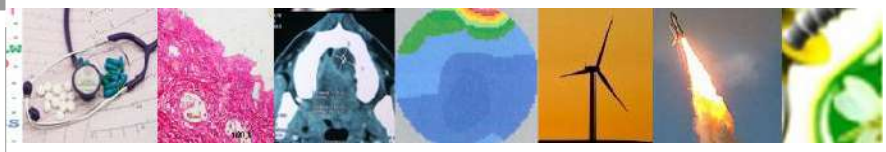

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RESEARCH ARTICLE

ASSESSMENT OF NUTRITIONAL STATUS OF CHILDREN LESS THAN 10 YEARS OLD IN RURAL WESTERN KORDOFAN

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ABSTRACT

This paper is based upon a field survey carried out in 2005 to assess the nutritional status of children less than 10 years old in rural western Kordofan. The main findings depicted an average contribution of each of the carbohydrates, protein and fat with 58.2%, 9.5%, and 32.3%, to the total energy with fat slightly higher and carbohydrates lower than the recommended values. Cereals products provided 45.7% of total consumption and 45.2% of total protein. About 58.3% of the children suffered from underweight and 72.8% were moderate underweight while 80.6% suffered from severe underweight. Wasting prevalence was 37.9% (19.6% as moderate and 18.3% as severe). Stunting prevalence was 23.7% (12.3% moderate and 11.4% as severe). Factors considered responsible for determining the nutritional status of these children were modeled and the paper recommended a strategy for promotion of the nutritional status in the study area.

Keywords: *rural setting; under–nutrition; malnutrition; stunting; wasting; poverty; food insecurity; nutrition security*

Abbreviations and acronyms: ***FAO,*** *Food and Agriculture Organization;* ***FSU,*** *Food Security Unit;* ***ILO,*** *International Labor Organization;* ***MOL,*** *Ministry of Labor;* ***PPS,*** *Probability Proportional to Size;* ***RDA,*** *Recommended Dietary Allowance;* ***SD,*** *Standard Deviation;* ***SDG,*** *Sudanese Gene;* ***SERISS,*** *The Sudan Emergency and Recovery Information and Surveillance;* ***SMCHS,*** *Sudan Maternity and Child Health Survey;* ***UNDP,*** *United Nations Development Program;* ***USD,*** *United States Dollars;* ***WFP,*** *World Food Program*

[I] INTRODUCTION

Although Sudan is rich in natural and human resources, 77.5% of the households surveyed in north Sudan were on or below the poverty line [1]. The study by the United Nations Development Program in 2005 reported that 75% of north Sudan population as poor and the majority (80%) is concentrating in rural areas where 30% of them suffered from extreme poverty [2]. Malnutrition is a real health problem in the country **[Table–1]**. Vulnerable groups to nutrition insecurity in Sudan are those whose food intake provides less than that recommended for refugees and internally displaced groups [3]. Camber and el Magboul's study excluded areas of armed conflict and identified areas nutritionally insecure in Sudan as to include rural areas of low crop and animal production; areas of low purchasing power and education and knowledge; areas of low access to health facilities and areas with low access to water especially during dry season [4]. Food insecure groups in Sudan were identified as those internally displaced people; vulnerable residents who were indirectly affected by the influx of internally displaced population in their communities and returnees numbering 4 million internally displaced population and 600,000 refugees almost all from south Sudan [5].

Nutrition security strongly connected with food security, and is achieved at the household level when its members' food intake provides the recommended levels of protein, vitamins, minerals and energy. Food security is "a situation exists when all people at all times have sufficient access to sufficiently safe and nutritious for a healthy and active life" [6]. Food insecurity is "a limited or uncertain access to foods of sufficient quality or quantity to sustain a healthy and active life" [7]. Moreover, Thomas and Metz differentiated between chronic and transitory food insecurity [8]. The first type occurs when individuals or groups suffer from food insecurity at all times.

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The second type associates with a temporary decline in access to food due to temporary adverse circumstances. Transitory food insecurity was further divided into temporary and seasonal types. The first one is unpredictable, e.g. drought, unemployment. The second one usually follows a regular pattern of inadequate accessibility to food, i.e. agricultural season. Food insecurity can be related to fluctuation of production and price affecting the food or non-food sector leading to fluctuations in real income producer within the community [9]. Natural disasters, armed conflicts and hunger are also responsible for food insecurity [10, 11].

Table: 1. Malnutrition in Sudan

Low weight/age		Wasting		Stunting		References
Prevalence	Severe	Prevalence	Severe	Prevalence	Severe	
-	-	14.1	1.7	32.1	12.6	[12]
34.0	11.3	13.0	3.0	33.0	17.0	[13]
26.6	-	9.8	-	32.3	-	[14]
41.0	15.0	-	-	41.0	15.0	[5]

Nutrition insecurity leads to protein – energy malnutrition and usually assessed by surveys. Nutrition status is measured directly by dietary surveys, biochemical data, and anthropometric and clinical examination methods. While food adequacy is necessary for a household to achieve nutrition security, but it is not in itself sufficient. This is because some other key contributors to good nutrition are also important, such as poverty reduction, female education and a healthy environment. However, some researchers view poverty as the main cause of malnutrition while some others believe in malnutrition eradication without reduction in poverty pointing to well nourished children living in very poor households. Female education is positively correlated with reduction in infant mortality rate [15, 16]. Environment health largely determines nutritional status either through infections, depletion of nutrients and illness or vice versa [17, 18, 19].

[II] MATERIALS AND METHODS

2.1. Data collection

Data was collected during August – October 2005 in rural western Kordofan [Figure–1] by selecting six villages randomly. The villages selected included Abu Serour; Rahad el Selik; Wad Gudiem; Um el Badri; el Karanik and Maryoud. The area was chosen due to its fragile environment with expected food problems. Population number is 92368 persons distributed in 14512 households [20]. Cluster sampling is used for selection of rural villages by using PPS, and secondly for selection of the households from the ever selected villages.

The sample size was calculated by the formula:
$n = t^2\, pq/d^2 \times deff.$

Where: n is the sample size, t = 1.96, q= 0.50, p=0.05, d =0.08 and Deff. =1.5 (design effect).
Therefore: $n = (1.96)^2 \times 0.05 \times 0.50 / (0.08)^2 \times 1.5 = 225$
Clusters = sample size/desired number of households in a cluster = 225/20= 11.25 (all clusters).

Since there are 92368 persons distributed in 14512 households, this gives 25431 households.

Then rural clusters= 14512/11.25/25431=ca.6. So rural households included = 20x6=120

The study covered dietary and anthropometric assessments of children less than 10 years old. Dietary assessments determined individual and household food consumptions. Anthropometric assessments involved physical measurements of the body such as weight, height, etc. Therefore, there are weight/height, weight for age, height for age and weight for height data, in addition to their sub-classifications [21, 22, 23]. Underweight, wasting, and stunting, in addition to measuring food frequency as consumption patterns during a week, were used to assess the nutritional status of children less than 10 years in the study area.

Underweight is a measure of wasting or stunting or both. Weight measurements, which were taken to the nearest 0.1 kg, were taken by electronic scale. Subjects were weighted barefooted wearing minimum clothing. Salter scale was used for children less than two years old who are unable to stand. Height of children less than 2 years old was measured while they are lying on their backs with stretched legs and heads up. A wooden scale is used for children whose ages were 2 to less than 10 years old.

Evaluation of nutritional status for children less than 10 years old was done by Z- score for the parameters of underweight (weight/age); wasting (weight/height); and stunting (height/age). The criteria used is normal (≥I SD) and undernourished (≤ - I SD). The (≤ - I SD) criterion is further divided into mild (-1 to - 2 SD); moderate (-2 to - 3 SD) and severe (≥ - 3 SD).

Measuring food frequency as consumption patterns during a week was recorded as consumption/day; every other day; twice/week; one/week; rarely or none. Household food intake (the 24-hour recall) was recorded in domestic measures and converted to weights. Nutrients intake were calculated using food composition tables [24, 25]. Individual food intake is calculated as an average per individual since all family members eat together, and it was evaluated as energy and protein intake. Recommended RDA calculation is based on FAO [26] RDA, and the figure for protein intake based on high fiber diet was applied since it represents the dietary pattern of the subjects covered by the study. An average figure was calculated from the RDA of household members above five years old to obtain the RDA per household for energy and protein. Adequacy intake based on households' RDA was calculated by: adequate ≥ 80% and inadequate ≤ 80%.

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2.2. The study area

The study area is part of north Kordufan state [Figure–1]. It has a semi arid environment with climatic fluctuations and average annual rainfall of 200 mm. Sand dunes and sandy soils are dominant and natural vegetation is sparsely. Children less than 10 years constituted 36.5% of the total population distributed as 17.8% males and 18.7% females. Average household size of 4-6 person included 50.8% of total households surveyed, while 7-10 person households represented 36.7%. Male headed households represented 44.1% of total households. Illiteracy is high by 75.9% among households surveyed, who are mainly peasants. They cultivate sorghum (Dura) and bulrush millet (Dukhn), sesame and groundnuts. Monthly income is distributed as 59.2% earns less than 200 SDG, 30.0% earns 200-300 SDG. They generally fall below poverty line as their incomes are far less than one USD per day. The so called higher income groups (301-500 and ≥ 500 SDG) totaled 10.8%.

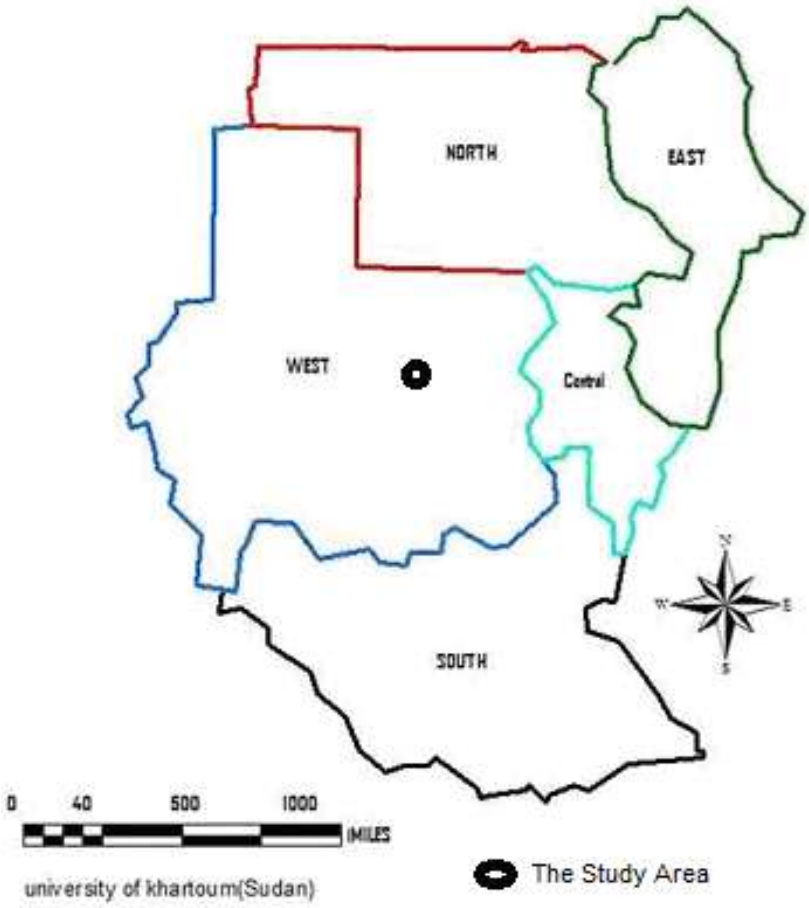

Fig: 1. Location of the study area in western Sudan

[III] RESULTS

Dietary and anthropometric evaluations are used to assess the nutritional status of children less than 10 years old in the study area. Factors considered responsible for the nutritional status are also presented.

3.1. Dietary evaluation (macro level)

Average contributions of carbohydrates, protein and fat to total energy were 56.2%, 9.3%, and 34.5% respectively [Table–2]. Less meat was consumed here but, the higher fat figure was due to consumption of more groundnuts and groundnuts oil as it is produced, pressed and refined locally. Cereals highly contribute to energy and protein intake in the study area. Frequency of daily cereals' product consumption was higher for sorghum porridge "Asida", which provides less energy due to its high moisture content. This seems the only viable explanation for higher cereal products in rural households but there is less energy and protein intake. Cereals products provided 42.7% of total energy and 45.2% of total protein [Table–2]. Animal protein sources such as meat and milk have less contribution since meat is consumed by 70.5% of the surveyed households. Moreover, nutritional adequacy can be roughly assessed from energy and protein content of the daily diet relative to the RDA. Inadequate energy intake means that the quantity of food consumed was below optimum need as the energy value is derived from all three macronutrients. The

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rural households of the study area consume diets low in quantity and intermediate in protein quality as the major source was plant protein which produced locally.

3.2. Anthropometric evaluations (micro level)

Table–3 shows anthropometric evaluations for children less than ten years old in rural western Kordofan. Underweight children represented 58.3% here. Wasting prevalence was 37.9% (19.6% as moderate and 18.3% as severe. Wasting was prevalent in the study area by 41.0%. Within each level of wasting, 74.4% is moderate, and 67.5% is severe. Since the survey was carried out during the pre- harvest season (August/October), a period of food scarcity in such rural areas, there will be less food intake and by so more incidence of wasting is expected as the index indicated to recent low food intake in addition to poverty.

A stunting is a measure of chronic under–nutrition. Stunting prevalence in the study area was 23.7% (12.3% moderate and 11.4% as severe). The prevalence of underweight was rather similar to those found for fewer children aged less than five years old in the study area. Under weight was prevalent by 54.1% and constituted the majority within the moderate (68.1%) and severe cases (81.8%).

Table: 2. Parameters of Nutrients' contribution (%) to total energy intake & recommended range (R).
Macronutrients' mean daily intake and Cereals contribution to total energy (kcal) and protein (g) intake in the study area

Nutrients' contribution (%) to total energy intake & recommended range (R)		
Carbohydrates	56.2	R= 55-60
Protein	9.3	R= 10-15
Fats	34,5	R= 25-30
(2) Macronutrients' mean daily intake		
Energy (kcal)	1663±383*	1803±449
Protein (g)	37.7±11*	42.0±14
Carbohydrates (g)	232.6±70*	261.4±80
Fat (g)	63.5±23**	64.5±25
Animal protein (g)	9.8±4.6*	13.1±6.3
Animal fat (g)	7.5±9*	12.2±11
(3) Cereals contribution to total energy (kcal) and protein (g) intake		
Energy	774.7±163*	824.3±172
% of total energy	46.5	45.7
Protein	16.1±3.9**	19.0±6.8
% of total energy	42.7	45.2

Source: Fieldwork [2005]

Wasting for all children was 55.9%, 32.2% moderate and 23.7% severe. Wasting was higher for children less than five years old at all the three levels especially severe wasting, which is probably an indication of even lower food intake at the fieldwork time. Wasting prevalence was higher (60.5%), and within these rural households, it was found to be higher within the moderate (67.6%) and the severe (74.0%) cases. Stunting prevalence was 11.4% (7.1% moderate and 4.3% severe). Generally, higher prevalence rates for moderate and severe stunting were recorded for these rural children.

3.3. Factors influencing nutritional status

Factors influencing the nutritional status of children less than 10 years old in the study area are modeled in Figure–2. Fieldwork results depicted higher level of protein intake implies consumption of better quality protein with increasing income. In these rural households, energy and protein intakes also increased with increasing income. The level of significance was lower for energy, probability 0.042, and was significant for protein with probability of 0.053. Comparison between lowest income group and highest one depicts an increase by 34.6% in energy and 41.3% in protein. Higher income groups (301-500 and ≥ 500 SDG) totaled 10.8%. By that way, 89.2% of the rural household did not benefit from increasing income that lead to increasing intake of energy and protein [Table–4].

The fieldwork results support the assumption that increasing income had positively increased energy intake, and therefore increased protein intake. Income was also positively correlated, probability of 0.000, with the nutritional status. Less income resulted in prevalence of under–nutrition in the study area. Thus decreasing income led to marginal or sub-optimal intakes of energy and protein resulting in more prevalence of under–nutrition [Table–4].

ISSN: 0976-3104

Table: 3. Nutritional status of children underten in rural western Kordufan

Parameter	Nutritional status	No.	%
Wt/age[a]	Normal	60	41.7
	Moderate	59	40.9
	Severe	25	17.4
P=0.194	**total**	144	100.0
Wt/height	Normal	85	59.0
	Moderate	32	22.2
	Severe	27	18.8
P=0.003	**total**	144	100.0
Height/age	Normal	100	69.4
	Moderate	20	13.9
	Severe	24	16.7
P=0.001	**total**	144	100.0
Wt/age[b]	Normal	63	45.9
	Moderate	47	34.3
	Severe	27	19.7
P=0.025	**total**	137	99.9
Wt/height[b]	Normal	54	39.5
	Moderate	46	33.5
	Severe	37	27.0
P=0.064	**total**	137	100.0
Height/age	Normal	118	86.1
	Moderate	12	8.7
	Severe	7	5.1
P=0.121	**total**	137	99.9

A=≤ 5, b= ≤ 10. Source: Fieldwork [2005]

Increased food expenditure had significantly increased energy and protein intakes in the study area for both energy, probability of 0.042, and for protein with probability of 0.025. In the later case, such an increase is not as important as that for those whose food expenditure amounted to ≥ 100 SDG, who constitutes 9.1% of the households, while for the remaining 90.9% there was practically no increase. A significant relationship exists between food expenditure and under-nutrition prevalence in the study area with probability of 0.004 **[Table–4]**.

As far as number of meals per day is concerned, it is expected that more energy and protein will positively correlate with three meals per day other than with two meals. Meals provided more energy was not detected in these rural villages where the probability was 0.104. Increase in protein intake was not significant for these rural households where the calculated probability was 0.145. The noticeable increase in energy here can be attributed to poverty, where 95.8% of the households earned ≤ 5 US$/ day **[Table–4]**. Number of meals per day influences energy and protein intakes, a positive relationship confirmed in the study area by a probability of 0.006 **[Table–4]**.

Energy intake reversibly decreased with increasing household size, where the calculated probability was 0.042 **[Table–4]**. The decrease in kcal was slightly low here (17.3%). Protein intake also decreased with increasing household size, probability was 0.000. Decrease in protein was high in these poor rural areas (26.7%) as they had big families. Positive relationship exists between household size and nutrition status in these rural areas and the calculated probability was 0.018.

However, other factors influencing nutrition status of children less than 10 years old in the study area might include mothers' level of education and environmental degradation as well as those factors which are work at the Country level, such as absence of social development programs, insufficient productive capital investment in agriculture and industry, inappropriate development policies neglecting rural development and armed conflicts in southern and western Sudan.

[IV] DISCUSSION

The general findings of this study depict low weight, prevalence of wasting and stunting among children less than 10 years old in rural western Kordofan. They are thoroughly affected by their households' income and size and high illiteracy where less animal protein, vitamins, minerals, and abundant cereal are consumed. In the study area, fat consumed was slightly higher and carbohydrates were lower than the recommended values [27]. Comparing macronutrients daily intake in the study area with the study by Ministry of Agriculture and Forestry of Sudan [28] puts the study area below by that there are less protein, carbohydrates and lower energy intakes (1803 kcal vs. 1962 kcal) and above as there is more fat intake. Energy obtained by higher protein and carbohydrates intakes was more than double the value obtained

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by excess fat intake in this study [28]. Cereals highly contribute to energy and protein intake in the study area, a situation similar to rural Philippines where 361g/person/day are consumed there [29]. Animal protein sources such as meat and milk provide less than the recommended value which is 55.3g [28].

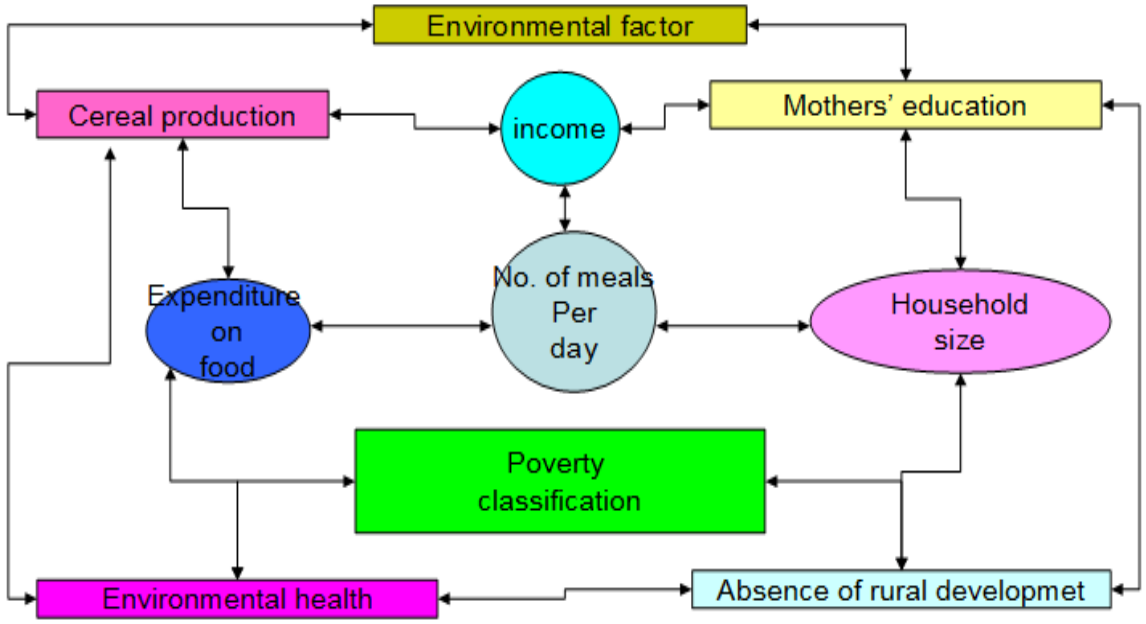

Fig: 2. Factors influencing nutritional status of children >10 years old in a rural environment of western kordofan. Adopted from Fieldwork [2005] and UNDP\FWP [2006]

Underweight children percent is almost similar to the 50.0% cited by FAO and WFP for pre-2001 studies for north Kordofan state [5]. However, it was higher than the most recent report *Sudan household health survey* of 42.9% (35.0% moderate and 7.9% severe) for north Kordofan state [30]. The result was also higher than all previous studies carried out in Sudan, although it is similar to that by Al Jaloudi for children less than five years old living in poor urban Khartoum state [31]. Yet, severe under nutrition was reported higher in west Kordofan than in north Kordofan [32]. In addition, the difference in prevalence rate is possibly due to geographic reasons. A drop from 50.0% to 42.9% indicated to better food intake and less infectious diseases [32], so probably this was not the case in our study area as 51.8% of the total households were food insecure.

Wasting prevalence rate in the study area is higher compared to north Kordofan state which was 16.0% as total and 13.5% as moderate and 2.5% as severe [30]. Figures obtained in this study were also higher than those obtained previously by Al Jaloudi [31] which were, 18.7% moderate and 2.2% severe. In all relevant studies severe wasting was ≤ 3% but, however our study shows higher level. Stunting prevalence in the study area is lower than all previous studies for total or severe cases [Table–1] which were 51.0% for north Kordofan and 47.7% for whole the Sudan [30]. It was even lower than the figure of Sub-Saharan Africa of 38.0% [33], or the 55.9% for rural Ethiopia [34]. However, one in every seven children was wasted and one is every three was stunted in north Sudan [12].

Environmental factors somehow determine food production, food availability and population affordability to buy food. The study area is environmentally fragile. It lies within "very high risk" zone of desertification designated by the United Nations [35]. Its average annual rainfall values decreased markedly since early sixties [36]. Natural vegetation is deteriorated and total biomass gets over-exploited by grazing and browsing animals. As a result, vegetation may still appear quite dense after heavy grazing, whereas in fact selective grazing has eaten out many of the palatable species and reduced the carrying capacity dramatically [37]. Resident population of the study area used to increase their cultivation area since coefficient of variation of the annual rainfall is about 30% the area cultivated and the productivity varies widely from one year to another [38]. They clear wide areas to grow crops and so compete with livestock for both land and water. In 1973, the Agricultural Conference recommended rainfed cultivation south of 300 mm rainfall line to stop environmental degradation. However, people are still cultivating areas north of that line [39], causing desertification [40] and deterioration of the food system where about half the population of sub-Saharan Africa is living below the poverty line, with both numbers and percentage on the increase [41].

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Table: 4. Factors influencing the nutritional status of children less than 10 years old in rural western Kordofan

Parameters	Rural	
	No.	%
1- **Income** (SDG)		
≤200	71	59.2
200-300	36	30.0
301-500	10	8.3
≥ 500	3	2.5
Total (P=0.000), P means probability	120	100.0
2- **Food expenditure** (SDG) / day P= 0.000		
≤ 5	29	24.2
5- ≤ 10	80	66.7
10- ≤ 15	10	8.3
≥ 15	1	0.8
Total	120	100.0
3- **Number of meals/day** P= 0.000		
Two	96	80.0
Three	24	20.0
Total	120	100.0
4- **Household size** P= 0.223		
1-3	8	6.7
4-6	61	50.8
7-10	44	36.7
≥ 10	7	5,8
Total	120	100.0
Poverty classification US$/ day		
≤ 5	115	95.8
5 - ≤ 10	-	-
10 - ≤ 15	5	4.2
≥ 15	120	100

Adopted from Fieldwork [2005]

Fieldwork results depicted higher level of protein intake implies consumption of better quality protein with increasing income. There is significant increase in energy and protein intakes with increasing incomes and a similar increase that was highly significant were recorded for protein [42]. Many studies in Sudan referred low weight, stunting and wasting among young children to unequal income distribution, vertically between incomes and horizontally between rural and urban areas [43]. Some researchers are convinced that increasing income leads to increasing food intake [44, 45], while some others believe that poor households spend their additional incomes on more expensive foods such as finer cereals, meat or dairy products which do not necessarily yield more energy. The fieldwork results support the first assumption that increasing income had positively increased energy intake, and therefore increased protein intake. Income was also positively correlated with the nutritional status. Less income resulted in prevalence of under-nutrition in the study area. Thus decreasing income led to marginal or sub-optimal intakes of energy and protein resulting in more prevalence of under-nutrition. Increased food expenditure had significantly increased energy and protein intakes in the study area for both energy and for protein.

A significant relationship exists between food expenditure and under-nutrition prevalence in the study area. This commensurate with the fact that food expenditure positively affected energy and protein intake and thus the energy status of the body. As far as number of meals per day is concerned, it is expected that more energy and protein will positively correlate with three meals per day other than with two meals. Energy intake reversibly decreased with increasing household size. Protein intake also decreased with increasing household size. Decrease in protein was high in these poor rural areas as they had big families. These factors point out to decreasing protein intake with increasing number of persons sharing the common dish which its protein content was originally low. Positive relationship exists between household size and nutrition status in these rural areas. Since energy and protein intakes were less and the households are big enough, it is expected to have positive relationship between household size and nutrition status.

Mothers' literacy positively effects low weight- for- age compared to illiterate mothers who have more stunted children in Sudan [5]. The study by Magboul, et al. [14] revealed that wasting was 11.3% in Khartoum and Omdurman towns, while it was 4.9% in Khartoum north, a result similar those by the

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Sudan emergency and recovery information and surveillance [12] and Sudan maternity and child health survey [13] where mothers' educational level was remarkably influential. However, other factors influencing nutrition status of children less than 10 years old in the study area might include those operating at the national level. They are absence of social development, insufficient productive capital investment in agriculture and industry [46], ill-conceived development policies neglecting rural development **[Figure–2]** and armed conflicts and drought [47].

[V] CONCLUSIONS AND RECOMMENDATIONS

The general conclusions of this study are as follows:

1. General nutritional status of households is below the recommended levels for population to remain healthy.
2. Children less than ten years old suffer malnutrition, underweight, wasting and stunting.
3. Poverty, big household size and illiteracy are as well as environmental factors highly influencing the nutritional status of this age group..
4. Promotion of community and child nutrition is a necessity in the study area.

Recommendations for promotion of the nutritional status of this vulnerable age group of the population in the study area require local and national collaboration and integration. This paper suggests a strategy of six integrated parts **[Figure–3]**. These are agricultural development; environmental conservation; community development; economic development; family development and village development.

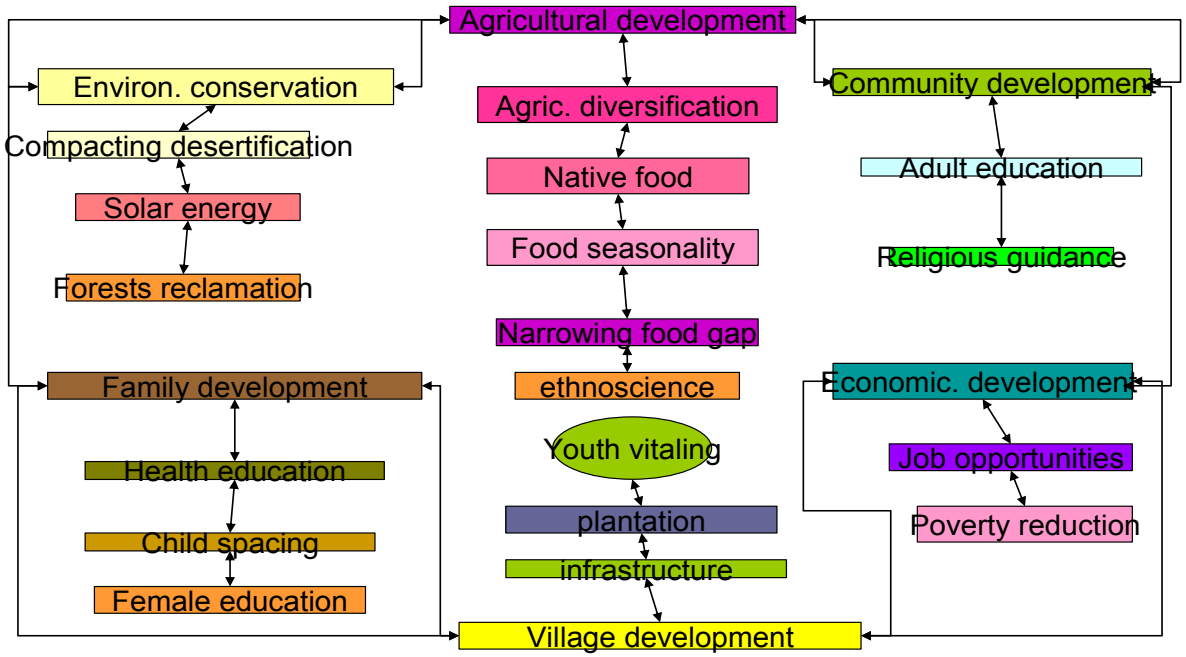

Fig: 3. A proposed model for the promotion of the nutritional status of children under 10 in rural western Kordofan. The application of this integrated strategy for promotion of child nutrition in the study area can work to assess nutrition in order to decide on appropriate methods through activation of the inputs, processes, and outputs outlined by the strategy with the ultimate result of reducing malnutrition in the study area and in Sudan. However, this strategy can also be applied for any other similar places facing similar problems of nutrition in Africa, Asia, and Latin America.

Agricultural development strategy adopted crop diversification to increase cash income among peasants; utilization of native food as a cheap and nutritive source for child nutrition and to benefit from food surplus during harvest as a reserve for times of shortage or deficiency and at times when household money reserve is depleted to the minimum. The strategy of enhancing traditional knowledge into agricultural practice and food preparation and conservation will help to avoid crop failure, combating insects and pesticides and improve households' food intake.

Environment conservation strategy will help to improve agriculture as it included combating desertification, reducing overgrazing and tree logging through the introduction of solar energy and reclamation of forests. Community development strategy can work to integrate family development strategy with female education, health education and child spacing to aware household on better use of food and for proper feeding of young children with qualitative food required at all stages of their growth. Economic development strategy will promote

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household incomes by creating job opportunities and poverty reduction through small credit finance and micro investment. Village development strategy can work towards enhancing capacity of local youth for social work and environmental awareness such as trees plantation around their settlements, awareness creation on nutrition and children feeding and educating of mothers on child feeding and nutrition. In addition, Basic infrastructure including schools, youth clubs, and medical dispensaries will promote social life and interaction while building roads and highways will link the study area with other parts of the Sudan.

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