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Highlights

- Efficacy of Halofantrine
- Direct Antisperm Antibody
- Effects of Tai Chi
- Social Welfare Services

The Blood Plasma

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Contents of the Volume

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Efficacy of Halofantrine in Mice Infected with Plasmodium Berghei

By Ologunde, C. A. , Olaoye, A. B. , Olofinjana, O.Akogun, O. O.
The Federal Polytechnic, Ado-Ekiti

Abstract - Drug adulteration is commonly reported in Nigeria. Halofantrine purchased in four different locations in Ondo and Ekiti state, Nigeria were tested in mice for their comparative efficacy. The average parasite clearance time (PCT) were 4.4±0.49, 4.2±0.40, 4.4±0.80 and 4.2±0.75 for Halofantrine coded HAL-AK, HAL-IO, HAL-AD and HAL-IK respectively. Also, average parasite clearance rate (PCR) were 8653±2557, 6895±1010, 5426±1850 and 6226±1850 respectively. The result shows that there was no significant difference in the PCR (P>0.05) between the drugs. All the halofantrine drugscompletely cleared the parasites within 4-5 days. The results of this study indicates that adulteration of Halofantrine is not presently in circulation in areas of Ondo and Ekiti states, South Western Nigeria.

Keywords : *Adulteration, halofantrine, parasite clearance, clearance rate*

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Efficacy of Halofantrine in Mice Infected With Plasmodium berghei

Ologunde, C. A. [α], Olaoye, A. B. [σ], Olofinjana, O.[ρ] & Akogun, O. O.[ω]

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Keywords : Adulteration, halofantrine, parasite clearance, clearance rate

I. Introduction

Malaria is a vector-borne infectious disease caused by protozoan parasites. It is widespread in tropical and subtropical regions, including parts of the Americas, Asia and Africa. Each year, there are approximately 350-500 million cases of malaria, killing between one and three million people, the majority of whom are young children in Sub-Saharan Africa (Snow *et al,* 2005). Ninety percent of malaria related death occur in Sub-Saharan Africa. Malaria is commonly associated with poverty, but is also a cause of poverty and major hindrance to economic development.

Halofantrine was developed in 1960s by the Walter Reed Army Institute of Research. Halonfantrine is used in the treatment of chloroquine resistant and multi-drug resistant, uncomplicated *P. falciparium* malaria therefore halofantrine is one of the choicest antimalaria drug in Nigeria. However, report from National Agency for Food and Drug Administration and Control (NAFDAC) shows that Nigeria is one of the haven of adulterated drugs in the world (FMH, Nigeria, 2004) but presently, only one brand of halofantrine is circulated and marketed in Nigeria, and no cases of adulteration of this drug has been reported. Although adulteration of another popular antimalaria (artesunate) had been detected in some part of the world (Lon *et al,* 2006), no cases of artesunate has been reported in Nigeria (Ologunde *et al*, 2008).

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This study is aimed at studying the therapeutic efficacy of halofantrine bought from different locations in Ondo and Ekiti states, Nigeria in mice infected with *Plasmodium berghei.*

II. Methodology

Swiss albino mice weighing between 12g and 17g were used in this study. The mice were kept in cages at room temperature where they were fed with standard mouse cubes manufactured by Ladokun Feeds Nigeria Limited, in Lagos state. They were randomly grouped into six groups of five mice each. The environmental conditions for all the mice throughout the period of the investigation were maintained the same.

Plasmodium berghei NK65 strain was obtained from malaria unit of Nigeria Institute of Medical Research Yaba Lagos state (NIMR) maintained by serial passage of blood collected from an infected donor animal to an uninfected mouse (Aina *et al*, 2006). Each mouse was inoculated with 1 x 10^6 paratisized red blood cells suspension blood in phosphate buffer saline (0.1) and were left for 3-7 days for the parasite to manifest. Two halofantrine tablets (250mg each) were dissolved in 10ml of water (which is equivalent to 50mg/ml) and 0.01ml of the prepared drug solution was administered into the mice following 24mg/kg body weight standard of oral halofantrine administration. The groups were coded according to the place of purchase of the drugs. The group that received halofantrine bought in Ikare-Akoko was coded HAL-IK, Akure was coded HAL-AK, Itaogbolu was coded HAL-IO and Ado-Ekiti was coded HAL-AD. However, two groups were not treated with the drug. One was given 0.5ml 0.9% normal saline (negative control) while the other was given chloroquine (positive control).

The blood sample of each mouse in each group was collected by tail snip with a sterile scissors. Thick blood film was made by smearing a drop of blood from tail snip on a microscopic slide and was dried at room temperature. These were viewed under oil immersion lens of light microscopy for the presence of the parasitemia. Responses of the mice to the drug were determined by the method of Ologunde *et al*, 2004, 2007.

III. Results

The results presented in figures 1- 4 show parasite responses to treatment in the four groups

treated with halofantrine, figure 5 show the parasite response to treatment in the negative control and figure 6 show the parasite response to treatment of chloroquine. All the animals in the negative control died after 10 days while those in other groups survived. Paired test was used for statistical significance. Table 1 show average parasite clearance rate (APCR) and average parasite clearance time (APCT). There was no significant difference in the average PCT between the halofantrine drugs ($P>0.05$) and also the average PCR shows no significant difference between the halofantrine drugs ($P>0.05$).

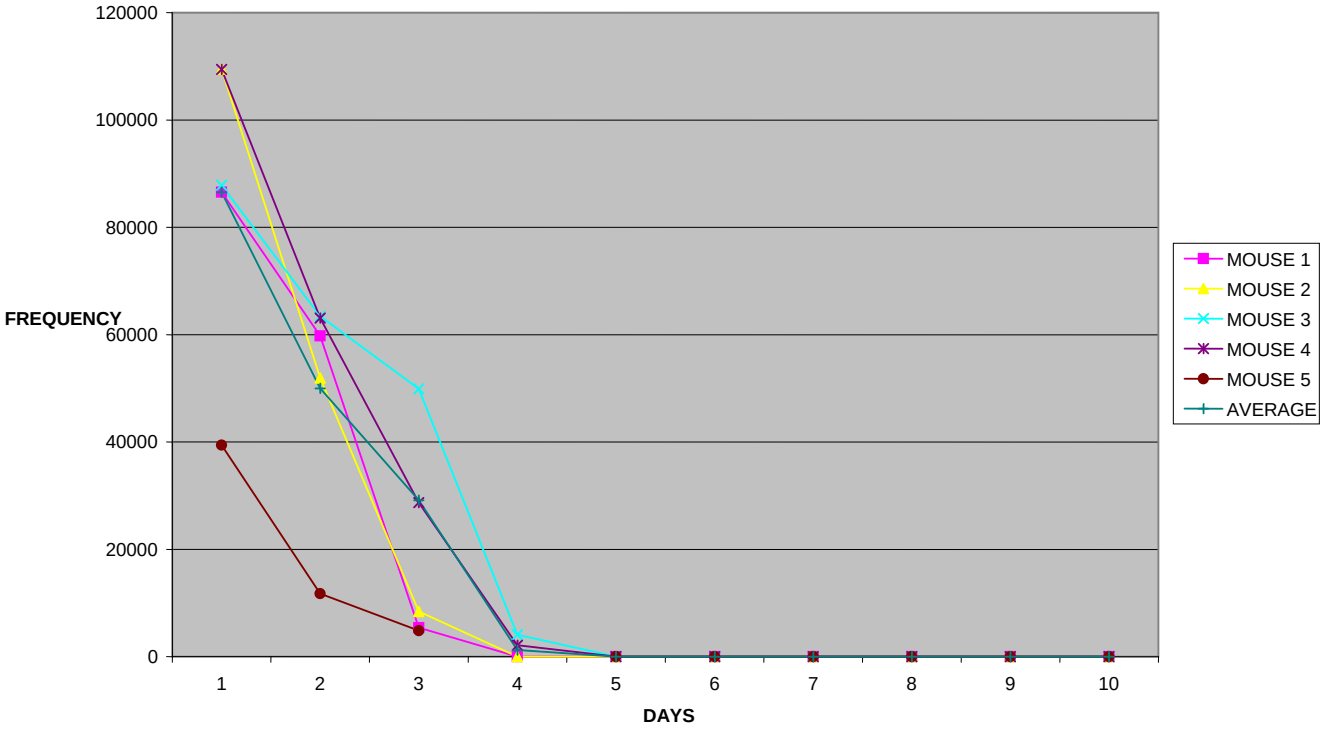

Figure 1 : Shows Parasite Response To Treatment (Hal-Ak).

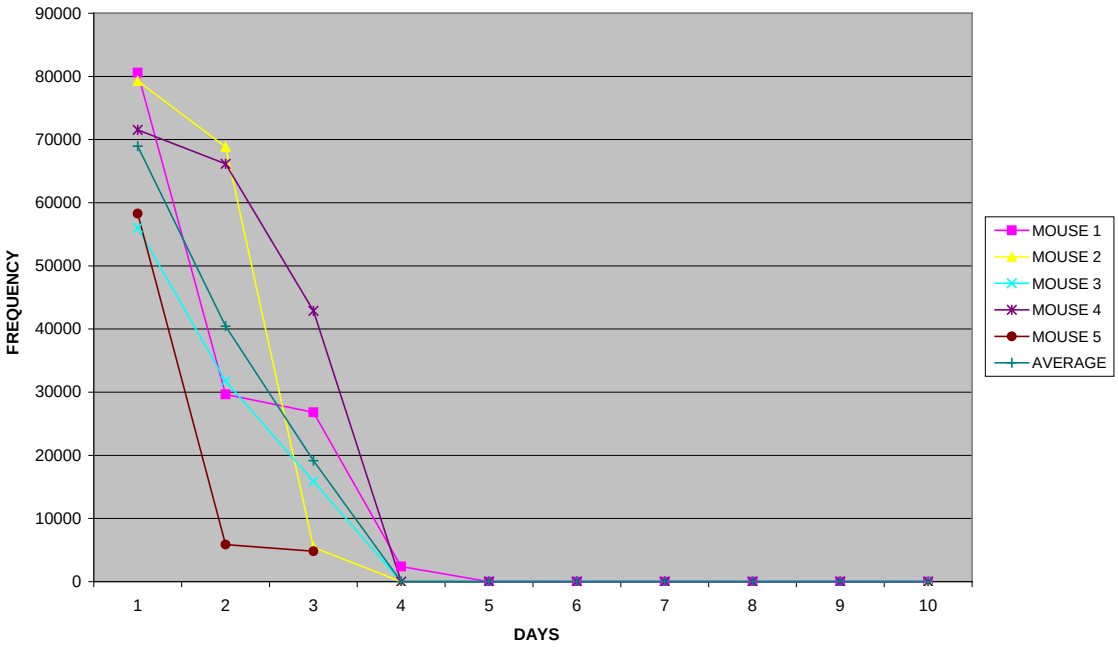

Figure 2 : Shows Parasite Response To Treatment(Hal-Io).

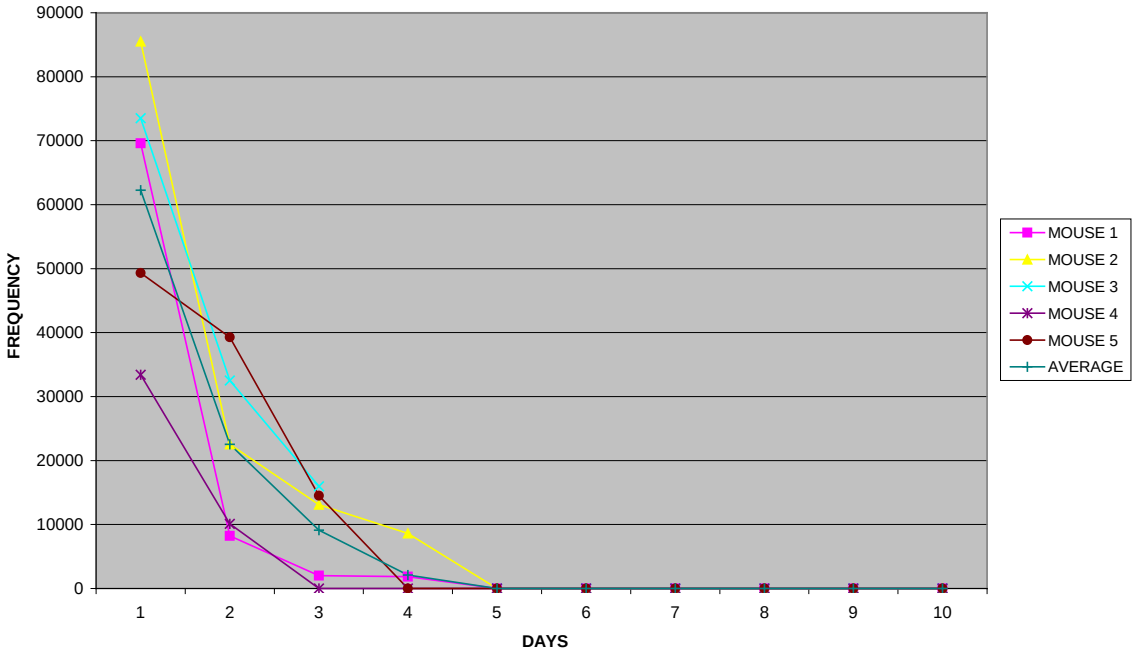

Figure 3 : Shows Parasite Response To Treatment (Hal-Ik).

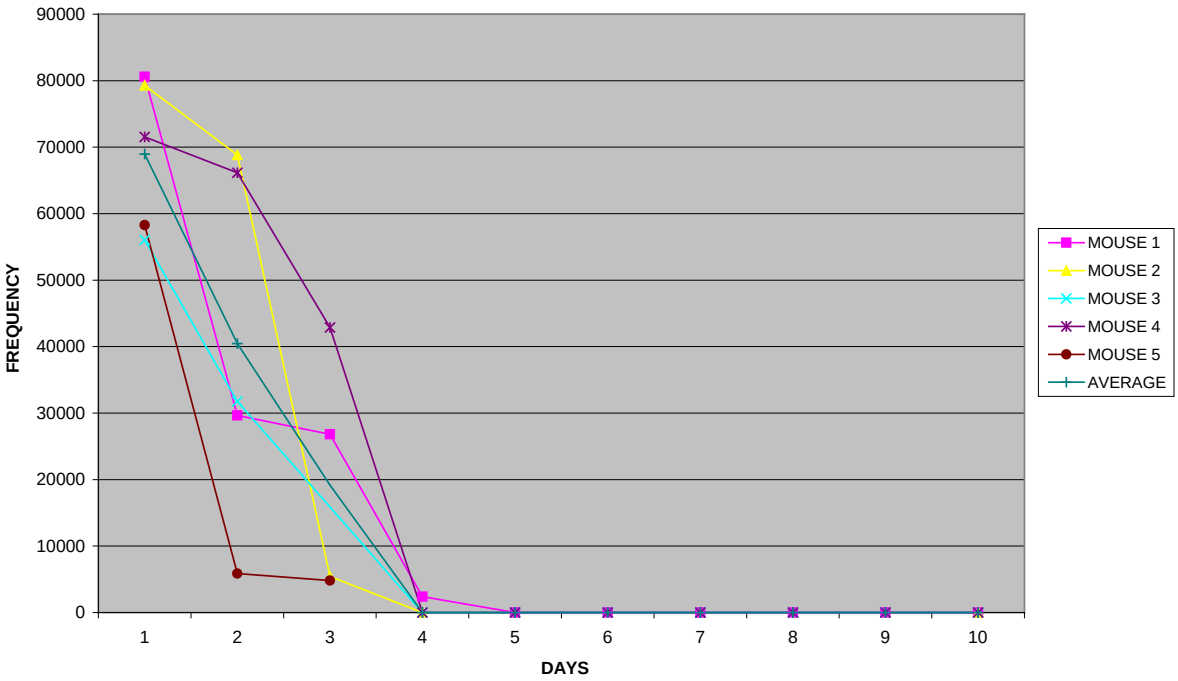

Figure 4 : Shows Parasite Response To Treatment(Hal-Ad).

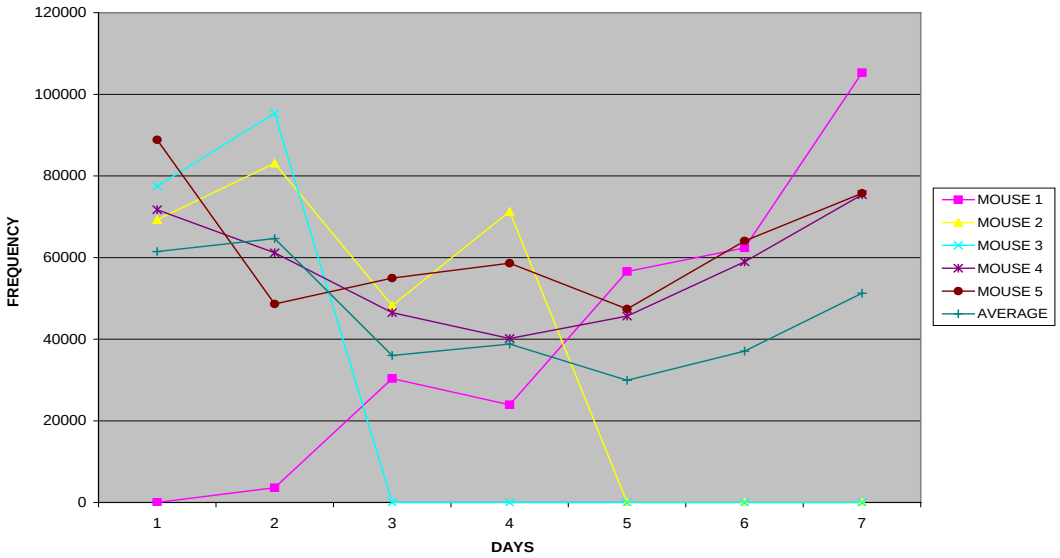

Figure 5 : Shows Parasite Response To Treatment (Negative Control).

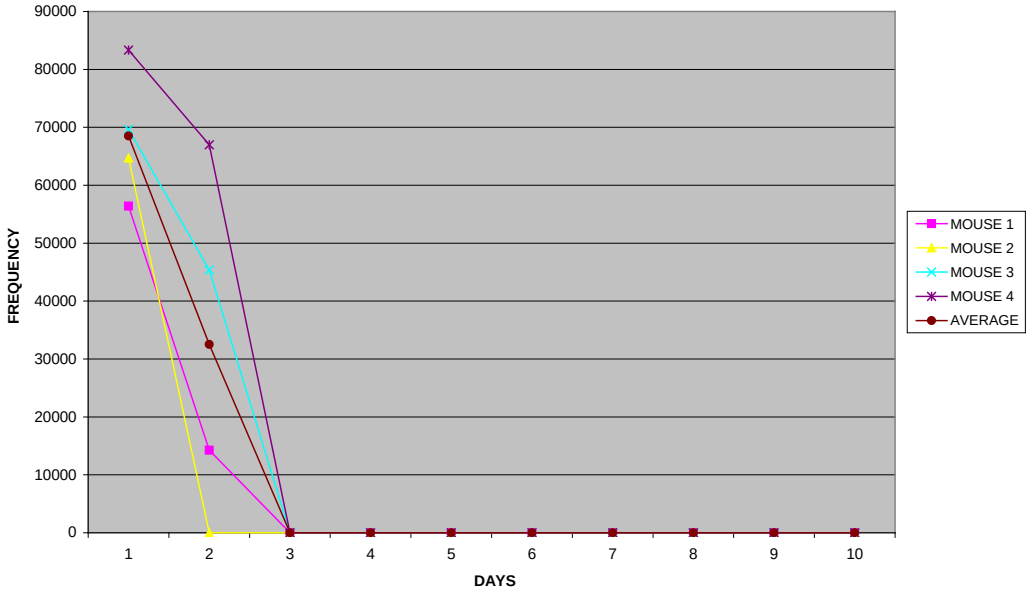

Figure 6 : Shows Parasite Response To Treatment (Chloroquine:Positive Control).

Table 1 : Average Parasite Clearance Rate (APCR) and Average Parasite Clearance Time (APCT).

HALFAN	PCR±SD	PCT±SD
HAL-AK	8653±2557	4.4±0.49
HAL-IO	6895±1010	4-2±0.40
HAL-AD	5426±3312	4.4±0.80
HAL-IK	6226±1850	4.2±0.75

IV. Discussion

The high mortality rate caused by severe malaria can be checked by adequate and effective antimalaria therapy. Malaria causes about 400-900 million cases of fever and approximately one to three million deaths annually (Breman, 20001). Meanwhile the use of chloroquine as the first line of drug in the treatment of malaria in Nigeria and other parts of the world had been stopped due to the presence of chloroquine-resistant strain in the *Plasmodium.* Resistance of *Plasmodium falciparum* has spread recently from Asia to Africa, making the drug ineffective against the most dangerous falciparum strain in many affected regions of the world (White *et al,* 2004). Unfortunately, chloroquine resistance is associated with reduced sensitivity to other drugs such as quinine, and amodiaquinine (Tinto *et al,* 2006).

The halofantrine drugs that were purchased from different locations in Ondo state and Ekiti state showed comparative efficacy in the mice infected with *Plasmodium berghei*. The drugs cleared the parasite to zero level in less than five days in all the mice treated when compared with the control. The parasite clearance clearing time (PCT) and the parasite clearance rate (PCR) showed no significant difference for the drugs ($P>0.05$) which is an indication that the drugs contain the adequate chemical composition and follow adequate stages of production.

The number of days when clearance level occurred in all the drugs differs (3-10days). This might not really show the differences in the efficacy of the drugs but rather factors like physiological state of the mice and parasite density before the administration of the drug. Other factor is depression in parasite response to treatment.

V. Conclusion and Recommendation

The result show that halofantrine drugs bought from different locations showed an effective clearance of the *Plasmodium* without significant difference and therefore it could be concluded that there was no adulterated drugs among the halofantrine drugs that were distributed and used by subjects living in Ekiti and Ondo state, south western Nigeria.

Government should adopt halofantrine drug as the first line of drug in the treatment of malaria in Nigeria and other parts of Africa. Government should also empower the drug enforcement agency in the country in search of adulterated drugs to forestall treatment failure and death from malaria parasite.

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Trade in Wild Mammalian Species for Traditional Medicine in Ogun State, Nigeria

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Abstract - There is an increasing demand for wild mammals and their parts for use in traditional medicine (TM), hence a need to document the extent of utilisation of the animals involved as a measure of the impact on biodiversity conservation.This paper examines diversity of species in trade for use in TM in Ogun State, Nigeria, the quantity of each species traded for utilisation over a period of time, and seasonal fluctuations in abundance and utilisation of species. A multi-stage stratified random sampling technique was employed. An open-ended questionnaire was administered on vendors in selected market stalls for six consecutive markets days in each of dry and rainy seasons.The study identified thirty species traded for utilisation, 13 were listed in CITES and Nigerian Decree 11(1985). Mean sales figure per dealer in a month was 61.6 ± 6.9 and 48.1 ± 5.8 carcasses in dry and rainy seasons respectively.

Keywords : *Traditional medicine, Mammals Utilisation, Wildlife trade, Ethnozoology, Ogun State.*

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Trade in Wild Mammalian Species for Traditional Medicine in Ogun State, Nigeria

Durojaye A Soewu [α], Opeyemi K Bakare [σ] & Ibukun A Ayodele [ρ]

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Keywords : Traditional medicine, Mammals Utilisation, Wildlife trade, Ethnozoology, Ogun State.

I. Introduction

Health care is one of the most important issues and major problems pertaining to the quality of life in Africa today. Hospital facilities are nowhere adequate and a considerable number of people living in the rural areas in Africa rely on traditional medicines for health care.The basis for traditional medicines and the primary ingredients used by the traditional healers are wild animal and plant species. The practice is widespread in Africa and market stalls selling plants and animal parts for medicines are common in both rural and urban markets in African towns and cities (FAO, 2007,Soewu and Adekanola 2011). Derivatives of wild plants and animals are not only widely used in traditional medicine but are also increasingly valued as raw materials in the preparation of modern medicines and herbal preparations Anon, (1999).

Wild animals in traditional medicine constitute an important exploitable natural resource that has a prominent role to play in the economy of a nation (Ayodele and Ihongbe 1995, Soewu and Ayodele 2009). Traditional medicine provide livelihood for a wide range of people, which include the traditional medical practitioners, the dealers / vendors in traditional medicinal ingredients, hunters / poachers and sometimes the intermediary sellers. Most of these people, due to their economic and social background, depend mainly on harvesting, processing and trading in wildlife as their only means of making a living (Costa-Neto, 1999; Li and Wang, 1999, Soewu 2008). However, Simmonds (1998) had surmised that as long as there is sufficient money to be made from the trade in wild animals, not only will the individual species suffer but also conservation at regional or even world level may be threatened. That the trade in wild life as trado-medicinal ingredients will continue to flourish is not in doubt, as there will always be human ailments in need of attention (Soewu 2008). The direct consequence of this would be a continued depletion of these resources in the wild as Marshall (1998) had documented that majority of wildlife traded for use in traditional medicinal preparations are collected from the wild. These wild resources are declining in population and spread, in most some cases very severely (Gaski and Johnson, 1994; Anon 1999).

Though thousands of species have been documented in indigenous health systems by ethno-botanical, anthropological and zoological researches, few researchers have examined the availability of these wild resources, the quantities in which they are used and the threats to the species and habitats in which they occur, and the nature of the trade, in wild fauna resources primarily for traditional medicine (Sodeinde and Soewu, 1999). Although there have been several quantitative studies on the bush meat trade, there is still

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paucity of information on the quantity of individual species traded for utilisation in traditional medicine and only anecdotal references are made to the nature and dynamics of this trade where available (Afolayan 1988; Sodeinde and Soewu 1999). However, in many areas demand for wild life resources for traditional medicinal preparations already exceed supply and is expected to increase substantially in years to come as human population continue to rise and as acceptance of traditional medicine and natural products increases (Marshall, 1998; Anon, 1999,Soewu and Adekanola 2011). Already reports of scarcity of species used for traditional medicine are being received with increasing frequency (Anon, 1999).

The therapeutic use of animals and animal parts to treat human ailment has been little researched, (Costa-Neto 1999, Soewu and Ayodele 2009). The recent arousal of interest in zoo therapy stems from the fact that the number of neo-tropical fauna species is declining so rapidly due to degradation of their ecosystems and in large part, poaching for therapeutic uses, that most of them are becoming extinct even before they have been studied by science (Huxtable 1992; Gaski and Johnson 1994). Kakati and Doulo (2002) reported that certain animals are already becoming rare due to indiscriminate killing and suggested that a record of indigenous knowledge related to therapeutic animal use is urgently required as a first step towards devising strategies for sustainable exploitation of these natural resources. Marshall (1998) posited that there is no indication that the level of use of medicinal wild life resources for traditional medicine will diminish. On the contrary, there is every reason to believe that the quantities of animals (and plants) required for traditional medicine will increase substantially in years to come as human population grow and acceptance of traditional medicine and natural products increases in the market (Ntiamoa-Baidu 1987, Marshall 1998, Soewu and Adekanola 2011). Moreso, the magic, superstition and dogma that surround traditional medicinal preparations are giving way to an understanding of the real basis of their curative power and consequently their social acceptance.Presently, while some researches in zootherapy are focusing on its cultural aspects, others are studying the pharmacological effects (and basis) of the substances involved, (Costa-Neto 1999).

The use for food and traditional medicine appears to exert more pressure on wild populations of animals than others in, (Bodasing 1999, Ott et al 2002). Hence a critical examination of these uses as an index of pressure on resources in the wild is a prerequisite for any conservation programme to be meaningful and effective, Chardonnet et al (2002).

Of all various uses of wild fauna and their products, use for traditional medicinal preparation stands out as the broadest and perhaps the largest route of consumptive utilisation of wild animals (Fayenuwo, 1999; Bowen-Jones and Pendy, 2000). This is a result of many factors. While the use for other purposes are selective based on traditional taboos, prejudices or formal religious dicta, use in traditional medicine transcends all barriers of institutions placed on other uses. People who would ordinarily not have utilised a particular species for other uses still find it permissible to consume or apply medicinal preparations concocted with such 'forbidden' species and some other ingredients (Soewu 2008).

It is also worthwhile to note that traditional medicine utilises a number of species that are usually not considered fit for ordinary human consumption in most parts of Nigeria. These include animals like chameleon, skink, shrew, small sized species of birds and smaller rodents and some insects to mention a few, (Fayenuwo, 1999; Banjo et al. 2001). This constitutes an additional pressure on population of animals in the wild stemming solely from the medicinal values of these animals. On the long run, this extra pressure on some species may create a significant impact on the ecosystem and the overall conservation of biodiversity.

Another factor that actively encourages the continued depletion of these resources is their market value and viability of trade in them as a source of income. Many African endangered species including our closest relatives, the great apes are moving rapidly towards extinction as a result of the illegal trade in wildlife in Central and West Africa, Goodall, (2000). Wild fauna often changes hands several times before reaching the market, allowing many individuals to profit from this trade. Hunters sell carcasses to intermediaries who supply retailers in the town (Fa 2000, Ott el al 2002; Soewu and Adekanola 2011). However, most of the trade is direct to vendors or consumers and therefore hard to quantify (Steel 1994, Pearce 1996, Bowen-Jones 1998). According to King (1994) in Cameroon, chimpanzee carcasses may fetch as much as US$20 to US$25 each and there are therefore ample incentives to hunt them, along with other large forest animals.

Traditional subsistence use of wild fauna has been changing as commercial factors have affected the socio-economies of communities that are dependent on forest for sustainance. Many of these pressures come from urbanisations and associated market economies that are creating demand for a variety of products in ever-increasing quantities. (Bowen-Jones, 1998). Although there have been several quantitative studies on the bush meat trade, there is still paucity of information on the quantity of individual species traded for utilisation in traditional medicine.

II. Methods

Study Area

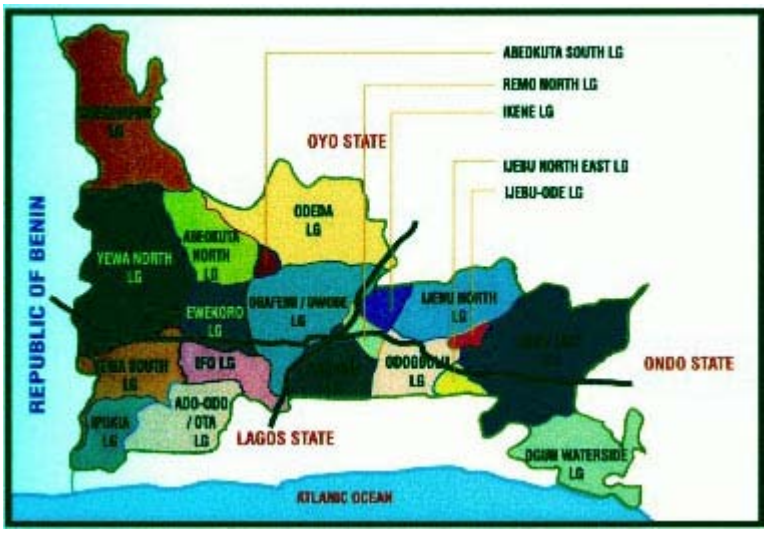

Figure 1 : Map of Ogun State Showing Local Government Areas in the Zones.

Ogun State is entirely in the tropics. Located in the Southwest zone of Nigeria with a total land area of 16,409.26 square kilometres, it is bounded on the West by the Benin Republic, on the South by Lagos State and the Atlantic Ocean, on the East by Ondo State, and on the North by Oyo and Osun States. It is situated between Latitude 6.2°N and 7.8°N and Longitude 3.0°E and 5.0°E. It has an estimated population of 3,486,683 people for the year 2005 Soewu and Ayodele (2009). Ogun State (Nigeria) is divided into four main political cum administrative zones namely Ijebu (IJB), Remo (REM), Egba (EGB) and Yewa (YEW) zones. These zones were taken as the sampling frames for the study.

Preliminary Pilot Survey

A preliminary pilot survey of the state was carried out between December 2001 and February 2002 to determine :

(i) Which two markets in each zone are the leading markets for traditional medicinal ingredients;
(ii) The number of stall/traders in identified markets that stocks and deals primarily in ingredients for traditional medicine.

This provides the basis to determine the number of traders / stalls to be involved in the main survey as a proportion of the whole number for the zone.Table 1.

Also, during this survey, the questionnaire for the main survey was subjected to trial runs to be able to establish the time needed to interview a respondent and take inventory of the stock in the stall.

Table 1 : Stalls distribution in selected markets across the zones.

Zone	Market	Number of stalls	%	No Selected
Ijebu	Oke Aje	76	64	16
	Ita Osu	43	36	9
	Sub total	119	100	25
Remo	Falawo	42	40	10
	Awolowo	63	60	15
	Sub total	105	100	25

Egba	Itoku	73	56	14
	Lafenwa	57	44	11
	Subtotal	130	100	25
Yewa	Oja garage	55	59	14
	Igbo ewe	38	41	11
	Sub total	93	100	25
TOTAL		447		100

Source : Field survey 2003.

III. Data Collection Techniques

This main study extended over a period of two years from April 2003 – March 2005. The respondents for the study are the dealers in wildlife for traditional medicinal preparations. Interviews were conducted by the authors with adequate aid from field assistants who are indigenes of the locality in view.

A stratified random sampling technique was employed in the selection of respondents. All the markets surveyed are five-day markets and the visits were made to each stall in the evening period of the market days. The market day was chosen based onhe report of the preimnary survey which established that this is the period when fresh supplies of animals are delivered to stalls and wares are fully displayed. This makes room for ease of inventory-taking and monitoring the dynamic movement of the stock. Each market and the stalls therein were given two sets of survey, one in each season of the year. In each market, the selection of stalls to be surveyed was done by the use of table of random numbers. The stalls were visited for six consecutive market days for a set of survey.

A total of one hundred dealers were interviewed, and the dynamic stock movement of their stalls taken using open-ended questionnaire to avoid yes / no answers while encouraging maximum discussion i.e. twenty-five dealers in each zone.

In each zone the dealers were selected from the two markets chosen for the survey. The number of dealers/stalls surveyed in each market was determined as the proportion contributed by the number of stalls in that market to the total number of stalls in the two markets for the zone.

On each visit to the stalls a detailed inventory of wild animal species found was taken during fixed time periods. The stock movement for each species was determined.

a) Identification of Species

All species encountered during survey were recorded with their local names in the market. To match the local names with the common English and Scientific names, due consultations and references were made to scientific publication that had previously established the names. Also the VCS i.e. Village Contact Survey method was used to identify some species. This involves showing published identification manuals and encyclopaedia with pictures and distinguishing features of animals to the dealers and some hunters for them to identify the animals with the local names. As part of the identification procedures, voucher specimens were also made available for each species. When the local name is established it is thereafter matched with the common English and the scientific names.

b) Carcass Quantification

To determine the number of carcass of each species that passed through the stall for the period, the number of that species sold out between consecutive market days were taken and summed up.

The whole animal seen at each stall on the first visit were counted and recorded separately for each species and this was taken as the initial opening stock. During subsequent visits to the stall, the remnant numbers of each species were counted and recorded. Also the number supplied to the stall after the last count was noted for the species. This allows for observation of the dynamic stock movement and the determination of the actual number sold out during the period.

Number sold out = Opening balance + Added stock – Closing balance

For some species occurring in parts, the head count approach was employed to avoid repeated counts. In this method, every head of an animal species encountered are counted as whole animals while other parts are overlooked to avoid repetition.

The main attributes of market dynamics measured during this survey are:

1. Quantity utilised by traditional medical practices as revealed by sales figures i.e. carcass number.
2. Frequency of occurrences and availability of each species
3. The average sales figure per stall / dealers for the species.

c) Seasonality and Availability

To examine any seasonality in the availability of animals on the stalls, a Latin square design was employed in deciding randomly the time of visit to each zone. The two major seasons were subdivided for convenience of study into early and late dry, early and late rain periods. The study was also designed such that markets in each zone are surveyed twice, each survey coming up at a season different from the other.

The availability of identified species for each zone was compared for the two main seasons.

d) Conservation Status of Species

To evaluate the current trade status of the species encountered during the survey, due consultations / references were made to the CITES appendices for the listing on global level. Also the Endangered Species (Control of International Trade and Traffic) Decree No 11 of 1985 was consulted to determine the present conservation status of the species in the Nigerian context.

IV. Results

a) Socio-Economic Characteristics of Respondents

Some socio-economic characteristics of the dealers in wildlife for traditional medicinal preparations were presented in Tables 2-7. Table 2 gives the gender and age distribution of respondents, table 3 shows the highest level of education attained by the respondents, while table 4 indicates their religious affiliation. Table5 gives the family sizes cum other dependants of respondents while table 6 shows whether the respondents have additional means of making a living or not. Table 7 presents the respondents' awareness of the legislative provisions / legal protection of the species they deal in as well as their awareness of the need for, and goal of conservation.

The female folk dominated the trade in trado-medicinal ingredients, having constituted over 90 percent of the dealers in all the zones of the state.

The majority of the dealers (64 percent) were aged between 40 and 60 years as at the time of the survey. While most of the dealers (53 percent) had post primary education, 12 percent of the dealers had no formal education. With respect to religious affiliation, the study shows that the majority (over 55 percent) of the dealers claimed affiliation to Islamic religion.Some dealers with either Islamic and Christianity affiliation expressed deep love for traditional religion suggesting dual affiliations in some of them. Also, the study shows that the majority (over 80 percent) of the dealers had no other means of livelihood besides the trade, and was also unaware of legislative provisions protecting wildlife species in Nigeria.

Table 2 : The Gender and Age Distribution of Respondents.

Characteristics	Dealers in traditional Medicine ingredients				
	IJB	REM	YEW	EGB	TOTAL
	Percent of respondents in each group				
Gender					
• Male	8	4	-	8	5
• Female	92	96	100	92	95
Age					
• Under 30	4	-	8	-	3
• 30-39	24	20	16	20	20
• 40-49	36	32	36	40	36
• 50-59	24	28	20	24	24
• 60-69	8	12	12	8	10
• 70 or more	4	8	8	8	7

Source : Field Survey, 2005.

Table 3 : The Highest Level of Education of Respondents.

Characteristics	Dealers in traditional medicine ingredients				
	IJB	REM	YEW	EGB	TOTAL
	Percent of respondent in each group				
• None	4	12	24	8	12
• Quoranic	-	-	-	-	-
• Primary	28	24	52	36	35
• Secondary	56	56	24	52	49
• Post Secondary	12	-	-	4	4

Source : Field Survey, 2005.

Table 4 : The Religious Affiliation of Respondents.

Characteristics	Dealers in traditional medicine ingredients				
	IJB	REM	YEW	EGB	TOTAL
	Percent of respondents in each group				
• Christianity	8	8	16	20	13
• Islam	76	68	64	64	68
• Others	16	24	20	16	19

Source : Field Survey, 2005.

Table 5 : Family and dependant sizes of Respondents.

Dealers in traditional Medicine ingredients			
Category	Male	Female	Apprentices
No. of dependants	Percent of respondents in each group		
0	12	5	4
1	19	11	17
2	26	13	33
3	22	12	29
4	15	16	11
5	3	34	4
6		4	2
7		2	
8			
9			
10			
No response	3	3	
Total	100	100	100

Source : Field survey, 2005.

Table 6 : Additional Means of Livelihood of Respondents.

	Dealers in traditional Medicine ingredients				
	IJB	REM	YEW	EGB	TOTAL
Characteristics	Percent of respondents in each group				
• Solely tradomedical trade	96	92	96	88	93
• Tradomedical & others	4	8	4	12	7

Source : Field Survey, 2005.

Table 7 a : Awareness of Legislative Provisions on Trade in Species

	Dealers in traditional medicine ingredients				
	IJB	REM	YEW	EGB	TOTAL
Characteristics	Percent of respondents in each group				
• Aware	8	4	8	8	7
• Not Aware	92	96	92	92	93

Source : Field Survey, 2005.

Table 7 b : Awareness of Conservation Needs and / or Goals.

	Dealers in traditional medicine ingredients				
Characteristics	IJB	REM	YEW	EGB	TOTAL
Have awareness	4	0	0	8	6
No awareness	96	100	100	92	94

Source : Field Survey, 2005.

b) Wildlife Species Trade Volume

Table 8 indicate the species diversity, the cumulative sales figure for each species per season as well as the total sales figure for each species encountered during the study.Thirty species were found stocked and utilised during the survey.

Table 9 gives a price index while table 10 highlights those species encountered during the survey that are listed in appendix I and II of CITES listing and schedules 1 and 2 of the Decree 11 (1985) of Nigeria.

Table 11 summarises the average number of carcasses traded per dealer in a month for dry and rainy season. Table 12 shows the average number of carcasses traded per dealer in a month across the various zones while table 13 presents theresult of Duncan multiple range tests showing significantly different means.

Table 8 : Number of Mammalian species traded over a period (20 days / season) by sampled dealers in selected 5-days markets in Ogun state, Nigeria.

		Zone	Ijebu			Remo			Yewa			Egba			All locations		
		Season	Dry	Rain	Both	Dry	Rain	Both	Dry	Rain	Both	Dry	Rain	Both	Dry	Rain	Both
English Name	**Scientific Name**	**Local Name**															
Straw-coloured fruit bat	*Eidolon helvum*	Adan	50	45	95	49	41	90	44	35	79	57	50	107	200	171	371

Savanna gerbil	*Tatera valida*	Afe	49	36	85	43	30	73	37	31	68	53	40	93	182	137	319
Roan antelope	*Hippotragus equines*	Agbagudu	10	7	17	8	4	12	6	3	9	12	9	21	36	23	59
Spotted grass mouse	*Lemniscomys striatus*	Ago	40	34	74	43	30	73	32	26	58	39	30	69	154	120	274
Whit-bellied pangolin	*Manis tricuspis*	Aika	71	58	129	68	52	68	61	47	108	76	61	137	276	166	442
Leopard	*Panthera pardus*	Amotekun	14	8	22	16	9	25	13	6	19	17	11	28	60	34	94
Shrew	*Crocidiora spp*	Asin	54	36	90	55	43	98	51	40	91	60	44	104	220	163	383
Beecrot's hyrax	*Dendrohyrax dorsalis*	Awawa	46	41	87	43	36	79	41	31	72	51	41	92	181	149	330
Multimamate rat	*Mastomys natalensis*	Eda	43	35	78	40	29	69	36	26	62	47	39	86	166	129	295
Colobus monkey	*Colobus spp*	Edun	24	22	46	23	20	43	20	15	35	26	23	49	93	80	173
African buffalo	*Syncerus caffer*	Efon	10	4	14	7	3	10	6	2	8	13	7	20	36	16	52
Serval	*Leptailurus serval*	Ekun	9	5	14	10	3	13	5	2	7	12	8	20	36	18	54
Pigmy mouse	*Mus minutoides*	Eliri	45	39	84	47	36	83	40	31	71	41	32	73	173	138	311
Nile rat	*Arvicanthis niloticus*	Emo	38	23	61	32	20	52	38	20	58	41	37	78	149	100	249
African civet	*Civettictis civetta*	Eta	25	23	48	22	20	42	20	18	38	29	23	52	96	84	180
Maxwell's duiker	*Cephalophus maxwelli*	Etu	49	41	90	43	37	80	41	34	75	51	44	95	184	156	340
Bushbuck	*Tragelaphus scriptus*	Igala	14	10	24	11	8	19	10	5	15	18	13	31	53	36	89
Patas monkey	*Erythrocebus patas*	Ijimere	41	36	77	36	30	66	31	24	55	43	39	82	151	129	280
Spotted hyaena	*Crocuta crocuta*	Ikooko	13	10	23	11	7	18	8	5	13	17	12	29	49	34	83
Geoffroy's ground	*Xerus erythropus*	Ikun	37	35	72	32	29	61	34	21	55	40	35	75	143	120	263
		Zone	Ijebu			Remo			Yewa			Egba			All locations		
		Season	Dry	Rain	Both	Dry	Rain	Both	Dry	Rain	Both	Dry	Rain	Both	Dry	Rain	Both
English Name	**Scientific Name**	**Local Name**															
squirrel																	
Gorilla	*Gorilla gorilla*	Inaki	26	22	48	22	19	41	19	16	35	31	14	45	98	71	169
Slender mongoose	*Herpestes sanguineus*	Kolokolo	23	17	40	14	10	24	11	7	18	16	11	27	64	45	109
Chimpanzee	*Pan troglodytes*	Obo	25	20	45	21	16	37	18	13	31	28	22	50	92	71	163
Tree squirrel	*Funisciurus pyrrhopus*	Okere	40	25	65	34	23	57	28	20	48	43	31	74	145	99	244
Giant rat	*Cricetomys gambianus*	Okete	87	88	175	91	83	174	87	71	158	110	92	202	375	334	709
Wild cat	*Felis silvestris*	Ologbo-oko	26	23	49	24	19	43	21	14	35	28	24	52	99	80	179
Rufous-bellied rat	*Lophuromys sikapusi*	Olose	35	23	58	30	21	51	30	24	54	41	34	75	136	102	238
Crested porcupine	*Hystrix cristata*	Oore	29	27	56	30	23	53	27	20	47	42	25	67	128	95	223
Greater cane rat	*Thryonomys swinderianus*	Oya	36	32	68	39	29	68	35	29	64	41	33	74	151	123	274
Stripped mouse	*Hybomys trivirgatus*	Eku onilakan	46	32	78	41	28	69	32	25	57	52	39	91	171	124	295
Total			***1055***	***857***	***1912***	***985***	***758***	***1691***	***882***	***661***	***1543***	***1175***	***923***	***2098***	***4097***	***3147***	***7244***

Source : Field Survey, 2005.

Thirty (30) mammalian species traded accounted for 7244 carcasses.

Cricetomys gambianus recorded the highest sales figure (n=709, 9.79%).

Table 9 : Price list of species encountered during survey.

Local Name	Season			Unit Price Range*		
	Dry	Rain	Both	Whole	Price	Part(s)	Price (NGN)
Straw-coloured fruit bat	200	171	371	x	150		
Savanna gerbil	182	137	319	x	200		
Roan antelope	36	23	59			Head	1500-3000
Spotted grass mouse	154	120	274	x	200		
Whit-bellied pangolin	276	166	442	x	1500-2500		
						Head	3000-10,000
Leopard	60	34	94			Skin(whole)	12000-20000
Shrew	220	163	383	x	100		
Beecrot's hyrax	181	149	330	x	4000	Head	600-700
Multimamate rat	166	129	295	x	150		
						Head	300-600
Colobus monkey	93	80	173			*Carcass	100-250
						Head	10000-14000
African buffalo	36	16	52			Skin(whole)	18000-22000
						Head	12000-17000
Serval	36	18	54			Skin(whole)	24000-35000
Pigmy mouse	173	138	311	x	100		
Nile rat	149	100	249	x	200		
						Head	700-1200
African civet	96	84	180			Skin(whole)	1100-2300
						Head	500-750
						Skin(whole)	1300-1500
Maxwell's duiker	184	156	340			*Carcass	400-700
						Head	3700-6500
						Skin(whole)	5500-7500
Bushbuck	53	36	89			Carcass	700
						Head	1600
Patas monkey	151	129	280			Skin(whole)	1000-2000
						Head	12000-17000
Spotted hyaena	49	34	83			Skin(whole)	15000-18000
Geoffroy's ground squirrel	143	120	263	x	700		
						Head	13000-18000
						Skin(whole)	15000-20000
Gorilla	98	71	169			Arm	4000
Slender mongoose	64	45	109			Head	3000-4500
						Head	400-800
Chimpanzee	92	71	163			Skin(whole)	1200-2300
Tree squirrel	145	99	244	x	400		
Giant rat	375	334	709	x	500		
Wild cat	99	80	179			Head	800-1400
						Skin(whole)	1300-1600
Rufous-bellied rat	136	102	238	x	150		
						Head	2300-2700
						Skin(whole with spines)	2500-3200
Crested porcupine	128	95	223				
Greater cane rat	151	123	274	x	1400-2000		
Stripped mouse	171	124	295	x	300		
Total	***4097***	***3147***	***7244***				

*Source : Field Survey, 2005.*Carcass was sold in small fragmented pieces.*

As at the time of the survey the exchange rate was NGN127 to a dollar

Table 10 : Species listed in appendix I and II of CITES and Decree 11 (1985) of Nigeria encountered during survey·

Common name		Cites listing	Decree 11 (NIG)
Colobus monkey	Colobus sp	I / II	1
Spotted hyena	Crocutta crocutta		1
Patas monkey	Erythrocebus patas	II	2
Wild cat	Felis silvestris	II	1
Gorrila	Gorrila gorilla	I	1
Slender mongoose	Herpestes sanguineus		2
Roan antelope	Hippotragus equinus		2
Serval	Leptailurus serval	II	1
Elephant	Loxodonta Africana	I	1
White bellied pangolin	Manis tricuspis	II	1
Lion	Panthera leo	II	1
Leopard	Panthera pardus	I	1
Chimpanzee	Pan troglodytes	I	1

Table 11 : Mean number of mammal carcasses traded per dealer per month in dry and rainy seasons.

Wildlife species	Mean per dealer per month by season						t-test for equality of means		
	Dry season		Rainy season		Both season		Calculated t	Significant p	Comment
Mammalian species									
Eidolon helvum	3.0 ±	0.3	2.6 ±	0.3	2.8 ±	0.2	0.99	0.33	NS
Tatera valida	2.7 ±	0.3	2.1 ±	0.3	2.4 ±	0.2	1.47	0.15	NS
Hippotragus equinus	0.5 ±	0.1	0.3 ±	0.1	0.4 ±	0.1	1.35	0.18	NS
Lemniscomys striatus	2.3 ±	0.3	1.8 ±	0.2	2.1 ±	0.2	1.39	0.17	NS
Manis tricuspis	4.1 ±	0.3	3.3 ±	0.4	3.7 ±	0.3	1.75	0.09	S (p<0.10)
Panthera pardus	0.9 ±	0.1	0.5 ±	0.1	0.7 ±	0.1	2.14	0.04	S (p<0.05)
Crocidiora spp	3.3 ±	0.4	2.4 ±	0.3	2.9 ±	0.3	1.74	0.09	S (p<0.10)
Dendrohyrax dorsalis	2.7 ±	0.3	2.2 ±	0.3	2.5 ±	0.2	1.26	0.22	NS
Mastomys natalensis	2.5 ±	0.3	1.9 ±	0.2	2.2 ±	0.2	1.34	0.19	NS
Colobus spp	1.4 ±	0.1	1.2 ±	0.1	1.3 ±	0.1	1.22	0.23	NS
Syncerus caffer	0.5 ±	0.1	0.2 ±	0.1	0.4 ±	0.1	2.01	0.05	S (p<0.10)
Leptailurus serval	0.5 ±	0.1	0.3 ±	0.1	0.4 ±	0.1	1.86	0.07	S (p<0.10)
Mus minutoides	2.6 ±	0.3	2.1 ±	0.3	2.3 ±	0.2	1.22	0.23	NS
Arvicanthis niloticus	2.2 ±	0.3	1.5 ±	0.2	1.9 ±	0.2	1.94	0.06	S (p<0.10)
Civettictis civetta	1.4 ±	0.1	1.3 ±	0.1	1.4 ±	0.1	1.09	0.28	NS
Cephalophus maxwelli	2.8 ±	0.2	2.3 ±	0.2	2.6 ±	0.2	1.28	0.21	NS
Tragelaphus scriptus	0.8 ±	0.1	0.5 ±	0.1	0.7 ±	0.1	1.58	0.12	NS
Erythrocebus patas	2.3 ±	0.2	1.9 ±	0.2	2.1 ±	0.1	1.19	0.24	NS
Crocuta crocuta	0.7 ±	0.1	0.5 ±	0.1	0.6 ±	0.1	1.41	0.17	NS
Xerus erythropus	2.1 ±	0.2	1.8 ±	0.3	2.0 ±	0.2	0.98	0.33	NS
Gorilla gorilla	1.5 ±	0.1	1.1 ±	0.1	1.3 ±	0.1	2.08	0.04	S (p<0.05)
Herpestes sanguineus	1.0 ±	0.1	0.7 ±	0.1	0.8 ±	0.1	1.74	0.09	S (p<0.10)
Pan troglodytes	1.5 ±	0.1	1.1 ±	0.1	1.3 ±	0.1	2.22	0.03	S (p<0.05)
Funisciurus pyrrhopus	2.2 ±	0.3	1.5 ±	0.2	1.8 ±	0.2	1.82	0.08	S (p<0.10)
Cricetomys gambianus	5.6 ±	0.5	5.0 ±	0.4	5.3 ±	0.3	0.98	0.33	NS
Felis silvestris	1.5 ±	0.1	1.3 ±	0.1	1.4 ±	0.1	1.39	0.17	NS
Lophuromys sikapusi	2.0 ±	0.3	1.5 ±	0.2	1.8 ±	0.2	1.41	0.17	NS
Hystrix cristata	1.9 ±	0.2	1.4 ±	0.1	1.7 ±	0.1	2.16	0.04	S (p<0.05)
Thryonomys swinderianus	2.3 ±	0.2	1.8 ±	0.2	2.1 ±	0.1	1.47	0.15	NS
Hybomys trivirgatus	2.6 ±	0.4	1.9 ±	0.2	2.2 ±	0.2	1.68	0.10	NS
	61.6 ±	6.9	48.1 ±	5.8	54.9 ±	4.6			

Table 12 : Mean number of carcasses traded per dealer by zone .

Wildlife species	Mean carcass Number per dealer per month by zone										F-test of diffrence between means			
	Ijebu		Remo		Yewa		Egba		All locations		F	Significant p		Comment
Mammalian species														
Eidolon helvum	2.9 ±	0.1	2.7 ±	0.4	2.4 ±	0.5	3.2 ±	0.6	2.8 ±	0.2	0.60	0.62		NS
Tatera valida	2.6 ±	0.4	2.2 ±	0.4	2.0 ±	0.5	2.8 ±	0.6	2.4 ±	0.2	0.52	0.67		NS
Hippotragus equinus	0.5 ±	0.2	0.4 ±	0.1	0.3 ±	0.1	0.6 ±	0.2	0.4 ±	0.1	1.22	0.32		NS
Lemniscomys striatus	2.2 ±	0.5	2.2 ±	0.4	1.7 ±	0.3	2.1 ±	0.3	2.1 ±	0.2	0.33	0.80		NS
Manis tricuspis	3.9 ±	0.4	3.6 ±	0.4	3.2 ±	0.6	4.1 ±	0.6	3.7 ±	0.3	0.52	0.67		NS
Panthera pardus	0.7 ±	0.2	0.8 ±	0.2	0.6 ±	0.2	0.8 ±	0.2	0.7 ±	0.1	0.35	0.79		NS
Crocidiora spp	2.7 ±	0.5	2.9 ±	0.4	2.7 ±	0.6	3.1 ±	0.6	2.9 ±	0.3	0.14	0.93		NS
Dendrohyrax dorsalis	2.6 ±	0.1	2.4 ±	0.4	2.2 ±	0.5	2.8 ±	0.5	2.5 ±	0.2	0.45	0.72		NS
Mastomys natalensis	2.3 ±	0.4	2.1 ±	0.3	1.9 ±	0.4	2.6 ±	0.6	2.2 ±	0.2	0.54	0.66		NS
Colobus spp	1.4 ±	0.1	1.3 ±	0.2	1.1 ±	0.2	1.5 ±	0.1	1.3 ±	0.1	1.28	0.30		NS
Syncerus caffer	0.4 ±	0.2	0.3 ±	0.1	0.2 ±	0.1	0.6 ±	0.2	0.4 ±	0.1	1.05	0.38		NS
Leptailurus serval	0.4 ±	0.1	0.4 ±	0.1	0.2 ±	0.1	0.6 ±	0.2	0.4 ±	0.1	1.15	0.34		NS
Mus minutoides	2.5 ±	0.4	2.5 ±	0.4	2.1 ±	0.5	2.2 ±	0.4	2.3 ±	0.2	0.20	0.89		NS
Arvicanthis niloticus	1.8 ±	0.3	1.6 ±	0.3	1.7 ±	0.4	2.3 ±	0.5	1.9 ±	0.2	0.71	0.55		NS
Civettictis civetta	1.4 ±	0.1	1.3 ±	0.2	1.1 ±	0.2	1.6 ±	0.2	1.4 ±	0.1	1.31	0.29		NS
Cephalophus maxwelli	2.7 ±	0.1	2.4 ±	0.3	2.3 ±	0.5	2.9 ±	0.4	2.6 ±	0.2	0.67	0.58		NS
Tragelaphus scriptus	0.7 ±	0.2	0.6 ±	0.1	0.5 ±	0.2	0.9 ±	0.2	0.7 ±	0.1	1.67	0.19		NS
Erythrocebus patas	2.3 ±	0.1	2.0 ±	0.2	1.7 ±	0.3	2.5 ±	0.4	2.1 ±	0.1	1.78	0.17		NS
Crocuta crocuta	0.7 ±	0.2	0.5 ±	0.1	0.4 ±	0.1	0.9 ±	0.2	0.6 ±	0.1	1.71	0.18		NS
Xerus erythropus	2.2 ±	0.2	1.8 ±	0.2	1.7 ±	0.5	2.3 ±	0.4	2.0 ±	0.2	0.62	0.60		NS
Gorilla gorilla	1.4 ±	0.1	1.2 ±	0.2	1.1 ±	0.2	1.4 ±	0.3	1.3 ±	0.1	0.67	0.58		NS
Herpestes sanguineus	1.2 ±	0.2	0.7 ±	0.1	0.5 ±	0.2	0.8 ±	0.2	0.8 ±	0.1	3.23	0.03		S (p<0.05)
Pan troglodytes	1.5 ±	0.1	1.1 ±	0.2	1.0 ±	0.2	1.7 ±	0.2	1.3 ±	0.1	2.77	0.06		S (p<0.10)
Funisciurus pyrrhopus	2.0 ±	0.5	1.7 ±	0.3	1.4 ±	0.3	2.2 ±	0.4	1.8 ±	0.2	0.71	0.55		NS
Cricetomys gambianus	5.3 ±	0.3	5.2 ±	0.4	4.7 ±	0.6	6.1 ±	1.0	5.3 ±	0.3	0.74	0.53		NS
Felis silvestris	1.5 ±	0.1	1.3 ±	0.1	1.2 ±	0.2	1.6 ±	0.2	1.4 ±	0.1	1.19	0.33		NS
Lophuromys sikapusi	1.7 ±	0.4	1.5 ±	0.2	1.6 ±	0.4	2.3 ±	0.5	1.8 ±	0.2	0.76	0.52		NS
Hystrix cristata	1.7 ±	0.1	1.6 ±	0.2	1.4 ±	0.3	2.0 ±	0.3	1.7 ±	0.1	1.11	0.36		NS
Thryonomys swinderianus	2.0 ±	0.2	2.0 ±	0.2	1.9 ±	0.4	2.2 ±	0.4	2.1 ±	0.1	0.17	0.92		NS
Hybomys trivirgatus	2.3 ±	0.5	2.1 ±	0.3	1.7 ±	0.4	2.7 ±	0.5	2.2 ±	0.2	1.00	0.40		NS
	57.5 ±	7.3	52.3 ±	7.3	46.5 ±	9.9	63.1 ±	11.3	54.9 ±	4.6				

Table 13 : Results of Duncan multiple range tests showing significantly different means

(a) Results for *Herpestes sanguineus*

Duncan

	N	Subset for alpha = .05	
ZONE		1	2
Yewa	10	1.8	
Remo	10	2.4	
Egba	10	2.7	2.7
Ijebu	10		4.0
Sig.		0.25	0.08

Means for groups in homogeneous subsets are displayed.

(b) Results for *Pan troglodytes*

Duncan

	N	Subset for alpha = .05	
ZONE		1	2
Yewa	9	3.4	
Remo	10	3.7	
Ijebu	9	5.0	5.0
Egba	9		5.6
Sig.		0.10	0.52

Means for groups in homogeneous subsets are displayed.

Plate 1 : Skulls of Gorilla gorilla (preserved).

Plate 2 : Gorrila gorilla arms.

V. Discussions

Utilisation of wild animals in traditional medicine cuts across all the age grades and sexes (Soewu 2008). Trado-medicinal practices also consume a vast quantity of wild mammals as revealed by the sales figure for each of the species encountered in this study (table 8). Most of these species are under pressure from over-exploitation. Regarding their conservation status, twenty-one (21) species encountered during this study (table 10) were listed in appendices 1 and 11 of CITES and the Decree 11(1985) of Nigeria as against six (6) species officially listed as endangered recorded by Kakati and Duolo (2002)

The variety of fauna species found during the study agree with the results of previous studies by several other authours (Ntiamoa-Baidu 1987; Kakati and Duolo, 1999; Costa-Neto 1999; Adeola 1992; Marshall 1998).However, the number of species encountered during this survey differ from most of the previous researches. This survey recorded 53 species while Taylor and Fox (1992) recorded 55 species in Lome Fetish Market, Togo;Kakati and Doulo (2002) recorded 23 species in a study on zoothrapeutic use by Chakhesang tribe of Nagaland in India; Costa-Neto (1999) encountered 17 species in zootherapeutic practices in Bahia, Brazil while Sodeinde and Soewu (1999) documented 45 species for southwestern Nigeria. For the bush meat markets, Fa et al (2000) reported 14 and 21 species respectively in 1991 and 1996 on Bioko Island, Equitorial Guinea, Anadu et al (1988), recorded 25 species in southwestern Nigeria while ???? (????) reported 106 species in Gabon. Although there have been several quantitative studies on the bush meat trade, there is still paucity of information on the quantity of individual species traded for utilisation in traditional medicine. On the conditions of animals supplied to their stalls, the dealers generally opined that there appears to be a general decrease in the sizes of virtually all the animals they receive over the years. It was also reported by dealers that body mass of carcasses available in the markets was on a continuous decline. Another factor that calls for concern is the fact that unlike the bushmeat trade, trade in wild animals for traditional medicine as encountered during this study readily cuts across all age grades or classes of animals: juvenile, sub-adults and adults.

a) Seasonality and Zonal fluctuations

The general trend is that more carcasses for all the species were sold during the dry season. The result of student's t test performed to compare the mean number of carcasses traded per dealer per month in dry and rainy season as presented in Table 11. Seasonal differences in number traded were found to be significant at 5% level for 4 species viz: Panthera pardus, Gorilla gorilla, Pan troglodytes, and Hystrix cristata.

The ANOVA test performed to compare the mean number of carcasses traded per dealer per month in the various zones (Table 12) revealed that there were no significant differences for all species traded across the zone except Herpestes sanguineus ($P< 0.05$) and Pan troglodytes ($P<.0.10$) The result of after-ANOVA analysis performed with Duncan Multiple Range Test for the significantly different means as presented in table 13 showed that Herpestes sanguineus was more significantly traded in Ijebu 40 ± 3.5 than all other zones put together 23 ± 2.5

The prevailing situation in the society was found to influence the trend in the demand for traditional medicines and thus, the wildlife ingredients. The respondents agreed that there are marked differences in the kind of preparations people will seek during the various period and seasons of the year. During a period of economic crises, the demand for fortune drawers and good luck charms will naturally be on the increase. In case of political crises, even if only anticipated, the demand for amulets and other preparations for protection against gun shots, cutlass and other such weapons will increase. Festive periods like Christmas and sallah celebrations are known to have involved mass movement of people from one location to another and thus, a rise in the demand for traditional medicinal preparations meant to prevent occurrence of accidents or to save users from sustaining any injury in case there is an accidents. These fluctuations in the demand for trado-medicinal preparations also influence the demand for the species involved in these preparations, thereby causing seasonal fluctuations in the demand and trade volume for such species. Season related ailments were also found to cause fluctuations in the demand for species recognised as possessing the medicinal properties to cure such ailments. For instance, common cold/catarrh and malaria which appear to have a high level of incidence during the rainy season will expectedly cause a rise in the demand for species involved in the treatment of these conditions One other factor which was found to influence seasonal changes in demand for animal species is the fear or anticipation of non-availability of such species during the forthcoming season. This may lead to panic buying by the traditional medical practitioners as well as hoarding by the dealers.

It was also established during the study that all species on the stalls visited were cropped from the wild and there were no records of any captive breeding or domestication project supplying the markets. The vendors all agreed to having procured from either larger wholesale markets or directly from hunters, and sometimes from intermediaries. They were also unanimous on receiving most of the supplies with wounds from gunshots or snares used to capture the animals.

Trade in wild animals for traditional medicine has been estimated to worth billions of dollars per year

globally. The trade for the selected markets for this study runs into excess of hundreds of millions of naira within a month (Table 9). This study however covered just a sample out of the total population of vendors in Ogun State, which in itself is one out of the thirty six states that make up the Nigerian nation.The price of species or parts was found to be influenced by the perceived medicinal value of such animal or part. Animal or its part(s) used in fortune drawers and money rituals would attract higher prices than those used for some other purposes.

The need to ensure sustainability in the exploitation of fauna resources cannot be overemphasised. This entails two basic steps: reduction in need/demand for resources in the wild for trado-medicinal practices; and improvement in the yield of these resources in the wild. Kakati and Duolo (2002) submmited that human-nature interaction must be established within its cultural dimensions for utilisation of animal resource for therapeutic purposes to be sustainable. One of the main threats to wildlife lies in the attitude of some extremist lobbying group that promotes the strict preservation of wildlife, which tends to remove all socio-economic value from wildlife.According to Chardonnet et al (2002), a complimentary approach allows conservation issues to meet with development concerns. The old-fashioned philosophy of conservation of nature and wildlife is a defensive attitude which attempts to protect nature against the consequences of development, while the modern conservation of biodiversity is a voluntary approach which intends to match the needs of people for biological resources while securing the long-term survival of the biological richness of the Earth (Chardonnet et al, 2002). While advocating effective application of punitive measures against violators of laws protecting wild fauna species, it is essential to avoid formulating policies which may be seen as trying to force dealers to abandon their trade. Modern conservation approach is obviously more appealing and promises better results.

VI. Recommendations

a) Reduction in Need / Demand

A broad based improvement in the provision of affordable health care delivery for the citizens will reduce situations that will drive the people to patronise trado-medical practices that will in turn utilise wild animals without any consideration for their sustainability.

A massive enlightenment campaign should be mounted on the ecological consequences of continued exploitation of these resources beyond their sustainable level and its attendant implications for the health status of mankind now and in the future. Wildlife conservation education should be included in the school curriculum from primary to tertiary level of education to make conservation an integral component of the live of every citizen

b) Trade Regulation

The legal machinery meant to offer protection for these animals within the country needs a comprehensive review to srike a balance between biodiversity interests and political exigencies. There is also a need to publicise the contents of national law as well as international conventions and treaties regulating trade in some of these species and, the implications of such legal provisions as the level of awareness is presently near zero among the citizenry. Adequate punitive measures should be taken in line with the law on erring members of the communities.

c) Increase in Yield

Boosting the production of desired species should be effected through several means, in-situ and ex-situ. Ex-situ methods of wildlife conservation are certainly an important method of saving species on the verge of extinction. The potentials of protected areas to conserve populations of wild animals while also serving as a source of repopulating target species cannot be over-emphasised.

Communities that play hosts to the natural habitats of the wild fauna resources should be involved as partners and beneficiaries in the management of conservation areas to make compliance with laws regulating exploitation of animals more feasible. Legitimate trade in non-protected species should be made more beneficial to theless well off rural populations and not the intermediaries or thebetter off urban dealers.

Efforts should also be intensified on captive breeding, artificial propagation and ranching of possible species. This will provide animals for other uses such as protein sources thereby reducing pressure on resources in the wild.

There is a need to carry out further studies into the quantity of wild animals utilised for traditional medicine in all other states of the country so as as to have an insight into the utilisation trend and volume at the national level.

VII. Conclusions

It is important to begin communicating wildlife conservation issues to the traditional medicine communities, i.e hunters, ingredients dealers, practitioners and end users. This allows these communities to be involved in the process to offer helpful inputs, and to plan adequately for restrictions on supply and use. If they are informed early enough, the need for increased controls might even be eliminated through voluntary cooperation and compliance. Also, the messages used to communicate conservation needs should be crafted carefully and thoroughly so as to make it comprehensible and acceptable, thereby enjoining maximum voluntary cooperation.

Prohibiting the utilisation of natural resources simply does not make sense to many people. Therefore,

the concept of wildlife conservation can be alien to them. If the need for conservation is to be accepted by people who make their livelihoods from wildlife or wildlife use for necessities such as food and medicine, then care should be taken to avoid what may be seen as ideological or culturally imperialistic approaches. It is important to accept and respect differing views of the value of wildlife, while at the same time explaining the necessities of conservation measures.

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The Effect of Some Factors on Stillbirth in Primiparous and Multiparous Holstein Cattle in Iraq

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Abstract - A total of 9691 records of calves belonged to 3076 Holstein cows and 58 sires were analyzed from 1995 to 1999 , at Nasr Dairy Cattle Station. The aim of the research is to evaluate sires geneticelly by using best linear unbiased prediction (BLUP) according to the stillbirths of their daughters after adjusting for fixed effects and to estimate heritability, phenotypic trend for the mentioned trait. Data were analayzed by using General Linear Model within SAS program to investigate the effectof some fixed factors (season and year of calving, parity sex of calf) on the stillbirths. Components of variance for the random effects in the employed mixed model were estimated by the Minimum Variance Quadratic Unbiased Estimation (MIVQUE) method. The Harvey program was also used to estimate BLUP values for sires. The overall mean of stillbirths was **11.19%** in primiparous, **8.69%** in multiparous and **9.49%** in both of them.The effect of all fixed factors was significant ($P < 0.01$) . Heritability of direct effect estimated for stillbirth rates in primiparous and multiparous and in both of them were 0.03 , 0.007 , 0.02 respectively ,whereas corresponding estimates of heritability of maternal effects were 0.04 .0.02 ,0.03 respectively .

Keywords : *Stillbirths, genetic evaluation, Herita - bility, Phenotypic trend.*

GJMR-D Classification : *FOR Code: 830302, 830301, 839803*

Strictly as per the compliance and regulations of:

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Abstract - A total of 9691 records of calves belonged to 3076 Holstein cows and 58 sires were analyzed from 1995 to 1999 , at Nasr Dairy Cattle Station. The aim of the research is to evaluate sires geneticelly by using best linear unbiased prediction (BLUP) according to the stillbirths of their daughters after adjusting for fixed effects and to estimate heritability, phenotypic trend for the mentioned trait. Data were analayzed by using General Linear Model within SAS program to investigate the effect of some fixed factors (season and year of calving, parity sex of calf) on the stillbirths. Components of variance for the random effects in the employed mixed model were estimated by the Minimum Variance Quadratic Unbiased Estimation (MIVQUE) method. The Harvey program was also used to estimate BLUP values for sires. The overall mean of stillbirths was **11.19%** in primiparous, **8.69%** in multiparous and **9.49%** in both of them.The effect of all fixed factors was significant ($P < 0.01$) . Heritability of direct effect estimated for stillbirth rates in primiparous and multiparous and in both of them were 0.03 , 0.007 , 0.02 respectively ,whereas corresponding estimates of heritability of maternal effects were 0.04 .0.02 ,0.03 respectively .

Phenotypic trend of stillbirths in primiparous was positive and non significant (**0.19%** / year) whereas negative and non-significant ($P < 0.05$) (- **0.11%**/year) in multiparous and in both of them (- **0.07%**/year). Minimum and maximum BLUP values of sires for stillbirths were **7.33** and **10.33%** respectively.

Keywords : Stillbirths, genetic evaluation, Herita - bility, Phenotypic trend

I. Introduction

Stillbirths is a trait that needs more attention especially because its rates have increased with Holstein population (Harbers *et al.,* 2003;Meyer *et al.,*2000;Berglund *et al.,*2003; Hansen *et al.,*2004). Berglund (1996) reported an increase in stillbirths in Sweden with the importation of semen from North American bulls. The cost of stillbirths to the US dairy industry has been estimated to be $ 132 million per year (Thompson *et al.,*1981). Meyer *et al.,*(2000) has revealed that each year about 7% of Holstein calves in United State die within 48 h of birth with unknown cause of death and the replacement of stillborn calves represent a substantial cost to the dairy industry at more than $125.3 million per year.

Stillbirths are defined as a calf that dies just prior to, during,or within 24 to 48 h of parturition with at least 260 days of gestation.(Philipsson *et al.,*1979; Chassange *et al.,* 1999).

Dystocia, difficulty of birth, has been implicated as the major cause of stillbirths; however, about 50% of stillborn calves were from unassisted births (Philipsson 1996).

In recent years, many studies have found stillbirths to be separate trait in primiparous (heifers) and multiparous (cows) Holstein cattle.(Meyer *et al.,*2000;Hansen, 2005; Steinbock *et al.,* 2006).

The genetic variation exists in stillbirths which consist of two parts:Direct effects (genetic variation in the calf) and maternal effects (genetic variation in the dam).The direct effects of stillbirths describe the calf's ability to survive birth.This trait is closely related to the size of the calf.The maternal effects of stillbirths describe the cow's ability to give birth to a living calf. Hence Hansen, (2005) recomonded to take in to acount the two effects when analysing stillbirths data.

Sires evaluation are almost exclusively based on field data, which are highly affected by large array of environmental factors. For this reason, Togashi *et al.,*(2004) reported the importance to adjust for environmental effects in order to accurately estimates of genetic merit of sires.

The genetic variation of stillbirths can be expressed by evaluated sires using Best Linear Unbiased Prediction (BLUP) after adjustting to environmental effects.

The aim of the present study is to evaluate sires using BLUP, estimate heritability for direct and maternal effects of stillbirths, and to determine the phenotypic trend of the previous trait in Holstein cattle in Iraq.

II. Material and Methods

Stillbirths data consist of 9691 records for the period from1990 to1999 which represent progeny of 58 Holstein sires were used in this study in Nasr Dairy Cattle Station United Company for Animal Resources Ltd. This station was established in 1987 in Al-Soueira (50 km south of Baghdad) Iraq. The herd was imported

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as 1200 pregnant Holstein heifers from the United States of America. All calves were the outcome of an artificial insemination. Records without information on season of birth, year of birth, sex of calf and parity were not included in the analyses .Records where the calf was not the result of single born were excluded. After these edits, the final data set included information on stillbirths was 9691.In this study, we defined calves as stillborn if they were recorded dead at birth or dead within 24 to 48 h after births, whereas the others were considered live-born. We assigned the value 0 for live-born and 1 for stillborn calves.

III. Statistical Analysis

General Linear Model (GLM) within SAS program was used by using three models: the first one was used to investigate the effect of season and year of calving, parity and sex of calf on the stillbirths in primiparous and multiparous cows. In addition to use the same model after excluding the data of primiparous cows to investigate the effects of the previous factors on multiparous cows.

$$Y_{ijklm} = \mu + E_i + R_j + P_k + S_l + e_{ijklm}$$

Where Y_{ijkm} is any trait considered in this study, μ is the overall mean, E_i the fixed effect of i^{th} calving season (i= 1 – 4), R_j the fixed effect of j^{th} calving year (j=1990-1999), P_k the fixed effect of k^{th} parity (k=1 – 6), S_l the fixed effect of l^{th} sex(l=1 – 2), e_{ijk} is the residual effect.

The second model used all factors in the first model but the data for parity from 2 to 6 were excluded to study the effect of the factors on stillbirths in primiparous only.

$$Y_{ijkl} = \mu + E_i + R_j + S_k + e_{ijkl}$$

Third model was used to estimate component of variance for the random effects using the Minimum Variance Quadratic Unbiased Estimation (MIVQUE) method (Rao, 1971).

$$Y_{ijklmo} = \mu + E_i + R_j + P_k + S_l + D_m + e_{ijklmo}$$

Where Dm the random effect of sires.

Heritability of direct effect (h^2a) was estimated by paternal half sibs, whereas heritability for maternal effect was estimated by submitting the following equations (Cameron, 1997):

$\sigma^2P = \sigma^2a + \sigma^2m + \sigma^2E$
$\sigma^2S = ¼\ \sigma^2a$
$\sigma^2a = 4\ \sigma^2S$
$\sigma^2D = ¼\ \sigma^2a + \sigma^2m$
$\sigma^2D = \sigma^2S + \sigma^2m$
$\sigma^2m = \sigma^2D - \sigma^2S$
$\sigma^2E = \sigma^2e - 2\ \sigma^2S$
$h^2\ m = \sigma^2m / \sigma^2P$

Where σ^2P = Phenotypic variance, σ^2a = Additive variance, σ^2m = Maternal variance, σ^2E = Variance due to permanent environment, σ^2S = Variance due to sire, σ^2D = Variance due to dam, σ^2e= Residual variance.

BLUP of sires was estimated by Harvey program (1990).Regression of phenotypic value of stillborn calves on their birth year was used to estimate stillbirth phenotypic trend (Galbraith, 2003).

IV. Results and Discussion

The overall mean of stillbirths for cows (primiparous and multiparous) was 9.49% (Table 1), the present estimation is within range obtained from many researches 4.55 – 11.8% (Agerholm *et al.*,1993; Chassagne *et al.*,1999; Meyer *et al.*,2000; Heins *et al.*,2005), whereas overall mean of multiparous was 8.69 % (Table 2), and 11.19 % for primiparous (Table 3).This finding was supported by Aurant (1972) who reported that rate of stillbirths was 50 % higher in primiparous compared with multiparous and Bar-Anan *et al.*,(1976) revealed that stillbirths percentage was 9.1% and 4.1% in primiparous and multiparous respectively.

The results of stillborn heifers, cows, and of both of them are presented in Tables 4, 5 and 6.

The effect of calving season on stillbirths for all traits was significant ($P < 0.01$). The highest estimates were in summer calving being 14.60% in heifers, 10.71% in cows and 11.36% in both, but the lowest estimates were in winter calving. These results were supported by many researches (Bar-Anan *et al.,* 1976; Lindstrom and Villa 1977; Martinez *et al.,* 1983;Erf *et al.,*1990; Meyer *et al.,* 2001).The cause of differences as a result of calving season may attribute to variation in temperatures, diseases and nutrition.

The percentage of stillbirths differ significantly by year of calving which is in agreement with some studies (Berglund, 1996; Meyer *et al.,* 2001; Hansen *et al.,* 2004).This finding was interpreted to be a reflect of differences in management among years.

Parity has a significant effect on stillbirths. This corresponds well with what has been recorded by two separate studies in Holstein submitted in the United States of America: the first one was by Martinez *et al.,* (1983) who reported that the stillbirths in the first, the second and the third calving were 10.5, 5.5 and 5.7% respectively, and the second was by Meyer *et al.,* (2000) who revealed that stillbirths were 11% in the first calving and 5.7% in the second calving.

Sex of calf had a significant effect on stillbirths percentage. Female calves had lowest estimates being 5.51% in heifers, 5.42% in cows and 7.54% in both. On the other hand the corresponding estimates of male were 12.48, 15.15 and 12.76% respectively. The results of the present study were similar to some other results obtained by Aurant, (1972), Martinez *et al.,* (1983) and

Eriksson *et al.,* (2004).The sex of calf may cause a variation in stillbirths percentage especially in cows with large body size like Holstein, which is in general, calved a large size calf and so the probability of dystocia increased and consequently the stillborns increased also, due to the high correlation between the two traits (Lindstrom and Vilva, 1977).

Estimates of heritability of stillbirths are given in Table 7.In general, all estimates were low. They reflect the importance of environment effects as an essential source in phenotypic variation of the studied traits.

The heritability of direct effect (h^2a) for stillbirths was 0.03 in heifers which is within range of 0.00 – 0.05 reported by many researches (Philipsson *et al.,* 1979; Eriksson *et al.,* 2004; Hansen, 2005; Steinbock *et al.,* 2006), whereas the h^2a in cows was 0.007 and also comes within range of 0.00 – 0.02 (Philipsson *et al.,* 1979; Eriksson *et al.,* 2004; Hansen, 2005).On the other hand h^2a of both heifers and cows was 0.02. As shown in this study, heritability of stillbirths is very low, particularly in cows (0.007); therefore, direct selection against stillbirths would relatively be ineffective. Many advantages could be provided if we selected other traits which were correlated genetically with stillbirths and had higher heritability.

The heritability estimates of maternal effects (h^2m) for stillbirths were 0.04 in heifers, 0.02 in cows and 0.03 in both of them, which were similar to estimates reported by Hansen, (2005) and Steinbock *et al.,* (2006).

Genetic evaluation of sires for stillbirths in heifers and cows using BLUP values was done and sires were ranked in descending order. The lowest and highest values are 7.33% and 10.33% respectively. This result states that there is a little genetic variation in the trait, and most variations belonged to environment effects. A similar result was obtained from Harbers *et al.,* (2000) who reported that transmitting ability of sires for stillbirths was between -3% and 3%.

Phenotypic trend of stillbirth rates in heifers for the period from 1990 to 1999 was positive and not significant (0.19% / year), negative and not significant for cows (- 0.11% / year) and both of them (- 0.07% / year) (Table 8).This finding was not supported by many researchers (Harbers *et al.,* 2000; Meyer *et al.,* 2000: Hansen, 2005) who reported positive and significant phenotypic trend in stillbirth rates.

V. Conclusion

1- Highly significant effects ($P < 0.01$) on stillbirths were found for several environmental variables including calving year, calving season, parity and calf sex. Therefore, the effects of environmental must be taken into consideration by adjusting data for these variables to provide the best estimates of genetic values and heritability.
2- Heritability estimates for stillbirths obtained for Holstein population in this study were low which were pointed to the low role of additive variation in total variation of stillbirths.
3- The phenotypic trend of stillbirths in this study was non-significant. This suggested low efficiency of animal evaluation procedures used in the herd studied.
4- Further investigation of relationship between stillbirth rates and other calving traits (dystocia, calving ease) is needed to develop a more complete understanding of biological processes resulting in the loss of calves at birth.

VI. Acknowledgements

This study would not have been possible without the support of Dr. S.S.Kalaf the manager of Nasr Dairy Cattle Station United Company for Animal Resources Ltd.The author acknowledge his cooperation in access to the farm records.

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Table 1 : Least squares means ± S.E for some factors affecting stillbirths in heifers and cows.

Factors	No. of observations	Least squares means ± S.E
Overall mean	9691	9.49 ± 0.30
Calving season		
Winter	2606	6.81 ± 0.66 c
Spring	1907	9.53 ± 0.76 ab
Summer	2457	11.36 ± 0.67 a
Autumn	2721	8.83 ± 0.64 c
Calving year		
1990	329	10.26 ± 1.68 ab
1991	332	8.19 ± 1.66 ab
1992	372	7.88 ± 1.56 ab
1993	681	7.73 ± 1.17 b
1994	871	9.16 ± 1.03 ab
1995	984	11.05 ± 0.97 a
1997	1521	7.23 ± 0.79 b
1998	1743	10.37 ± 0.73 ab
1999	1842	7.82 ± 0.72 ab

Parity		
1	3076	11.35 ± 0.56 a
2	2398	7.59 ± 0.64 b
3	1695	8.59 ± 0.76 b
4	1072	9.58 ± 0.94 b
5	794	8.44 ± 1.19 b
6	656	9.26 ± 1.10 b
Calf sex		
Male	5096	12.76 ± 0.51 a
Female	4595	5.51 ± 0.53 b

Means in the same column with no common superscripts differ significantly (P < 0.01).

Table 2 : Least squares means ± S.E for some factors affecting stillbirths in cows

Factors	No. of observations	Least squares means ± S.E
Overall mean	6615	8.69 ± 0.35
Calving season		
Winter	1679	6.29 ± 0.78 b
Spring	967	10.62 ± 0.99 a
Summer	1903	10.71 ± 0.77 a
Autumn	2066	8.19 ± 0.74 ab
Calving year		
1990	136	8.61 ± 2.49 ab
1991	176	10.82 ± 2.18 ab
1992	259	7.88 ± 1.80 ab
1993	384	8.10 ± 1.48 b
1994	549	9.80 ± 1.24 ab
1995	732	10.81 ± 1.08 a
1996	665	11.51 ± 1.12 a
1997	984	7.58 ± 0.93 b
1998	1308	10.17 ± 0.81 ab
1999	1422	7.21 ± 0.79 b
Parity		
2	2398	7.76 ± 0.64 a
3	1695	8.97 ± 0.76 a
4	1072	9.77 ± 0.94 a
5	794	8.69 ± 1.18 a
6	656	9.57 ± 1.10 a
Calf sex		
Male	3501	12.48 ± 0.48 a
Female	3114	5.42 ± 0.52 b

Means in the same column with no common superscripts differ significantly (P < 0.01).

Table 3 : Least squares means ± S.E for some factors affecting stillbirths in heifers

Factors	No. of observations	Least squares means ± S.E
Overall mean	3076	11.19 ± 0.58
Calving season		
Winter	927	8.97 ± 1.13 b
Spring	940	9.89 ± 1.12 b
Summer	554	14.60 ± 1.43 a
Autumn	655	11.92 ± 1.29 ab
Calving year		
1990	193	13.40 ± 2.34 a
1991	156	8.07 ± 2.58 b
1992	113	9.65 ± 3.02 ab
1993	297	9.10 ± 1.90 b
1994	322	11.05 ± 1.89 ab
1995	252	13.03 ± 2.11 a
1996	351	13.48 ± 1.71 a
1997	537	10.26 ± 1.39 ab
1998	435	12.78 ± 1.55 ab
1999	420	11.63 ± 1.57 ab
Calf sex		
Male	1595	15.15 ± 0.86 a
Female	1481	7.54 ± 0.89 b

Means in the same column with no common superscripts differ significantly (P < 0.01).

Table 4 : Analysis of variance for some factors affecting stillbirths in heifers and cows.

Sources of variation	D.F	Mean square
Calving season	3	8660.00 **
Calving year	9	2638.22 **
Parity	5	4007.48 **
Calf sex	1	126906.16 **
Residual	9672	888.38

*** (P < 0.01)*

Table 5 : Analysis of variance for some factors affecting stillbirths in cows.

Sources of variation	D.F	Mean square
Calving season	3	6969.80 **
Calving year	9	2288.68 **
Parity	4	984.07
Calf sex	1	81873.17 **
Residual	6597	820.41

*** (P < 0.01)*

Table 6 : Analysis of variance for some factors affecting stillbirths in heifers.

Sources of variation	D.F	Mean square
Calving season	3	3957.21 **
Calving year	9	3272.78 **
Calf sex	1	44285.17 **
Residual	3062	1033.38

*** (P < 0.01)*

Table 7 : Heritability estimates of direct effect (h^2a) and maternal effect (h^2m) of stillbirths in heifers, cows and in both.

Trait	h^2a	h^2m
Stillbirths in heifers	0.03	0.04
Stillbirths in cows	0.007	0.02
Stillbirths in heifers and cows	0.02	0.03

Table 8 : Phenotypic trends of stillbirths in heifers, cows and in both.

Trait	Phenotypic trend
Stillbirths in heifers	0.19% / year
Stillbirths in cows	- 0.11% / year
Stillbirths in heifers and cows	- 0.07% / year

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Schistosomiasis in Ogbese-Ekiti, Re-Infection After Successful Treatment with Praziquantel

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Abstract – Urinary schistosomiasis infection is one of the major public health problem facing developing countries with school age children at greater risk. Previous studies showed that Ogbese Ekiti is endemic for urinary schistosomiasis. The impact of chemotherapy was evaluated using praziquantel (40mg/kg body weight) on S.heamatobium among school pupils in Ogbese-Ekiti, Ekiti State, Nigeria. Urine samples were collected between the hours of 7.00am and 10.00am. The number of eggs in 10ml of each urine sample was calculated from the mean of two counts. At baseline, one hundred and seventy two (172) pupils were screened for eggs of the S.heamatobium out of which **75.6%** were positive with high egg intensity ranging between 40-780 eggs/10ml of urine. Out of the one hundred and seventy two screened, thirty subjects with high egg intensity (440-780 eggs/10ml of urine) were treated with praziquantel in January 2009. After 10 days post treatment, the urine samples of the thirty subjects were negative for S.heamatobium. The subjects were monitored monthly for re-infection for seven consecutive months (February – August).Re-infection was first noticed in May.

Keywords : *Schistosomiasis, praziquantel, S.heamatobium, endemicity, Macrohaematuria, Bulinus (B) globossus, cercariae.*

GJMR-A Classification : *NLMC Code: WC 810*

Strictly as per the compliance and regulations of:

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Abstract - Urinary schistosomiasis infection is one of the major public health problem facing developing countries with school age children at greater risk. Previous studies showed that Ogbese Ekiti is endemic for urinary schistosomiasis. The impact of chemotherapy was evaluated using praziquantel (40mg/kg body weight) on S.heamatobium among school pupils in Ogbese-Ekiti, Ekiti State, Nigeria. Urine samples were collected between the hours of 7.00am and 10.00am. The number of eggs in 10ml of each urine sample was calculated from the mean of two counts. At baseline, one hundred and seventy two (172) pupils were screened for eggs of the S.heamatobium out of which 75.6% were positive with high egg intensity ranging between 40-780 eggs/10ml of urine. Out of the one hundred and seventy two screened, thirty subjects with high egg intensity (440-780 eggs/10ml of urine) were treated with praziquantel in January 2009. After 10 days post treatment, the urine samples of the thirty subjects were negative for S.heamatobium. The subjects were monitored monthly for re-infection for seven consecutive months (February – August).Re-infection was first noticed in May. The rate of re-infection for the months of May, June, July and August were **11(36.7%), 13(43.3%), 17(56.7%)** and **19(63.33%)** respectively. Macrohaematuria was absent in the thirty pupils in the first five months but surfaced in the sixth and seventh months with percentage macrohaematuria of **30%** and **37.7%** respectively. Bulinus (B) globossus collected from river Ogbese is the major transmission foci shed cercariae of S.heamatobium. The study shows that mass chemotherapy, elimination of the snail intermediate host and not selective treatment will be an effective method to reduce transmission of S.heamatobium in Ogbese-Ekiti community.

Keywords : Schistosomiasis, praziquantel, S.heamatobium, endemicity, Macrohaematuria, Bulinus (B) globossus, cercariae

I. Introduction

Schistosomiasis, is the second most important parasitic disease, with over 200 million people being infected in 74 countries world wide (Remme *et al,*1993). Urinary schistosomiasis is endemic in Nigeria particularly in rural areas (Edunbgola, 1988; Adewunmi, 1991). Previous studies on urinary schistosomiasis in Nigeria has been concentrated in the Western and Northern areas (Ugbomoiko, 2000). The presence of many snail species especially the *Bulinus* species, and increased contact time with the *Schistosoma haematobium* infested freshwater habitat were thought to be responsible for the prevalence of the disease in Ogbadibo local government area, Benue State, Nigeria (Mbata *et al*, 2008). In Ekiti state, the status of urinary schitosomiasis has been well documented., although majority of the studies were based on prevalence, pathology and epidemiology (Ologunde, 2005; Ologunde, 2009).

Chemotherapy is generally recognized to be the most important, rapid and cheap method to reduce morbidity due to schistosomiasis. The treatment of schistosomiasis has been transformed with the introduction of praziquantel which is effective generally in a single dose and against all species of the parasite. Other drugs include metrozonate and oxaminiquine. In 1998 Talaat and Miller treated schistosomiasis with high success rates **(83.6%).**

McManus *et al,* 2010 in their study concluded that the inclusion of focal molluscicide, improvements in sanitation, and health education into the control scenario, may help to achieve China's target of reducing the level of schistosome infection to less than 1% by 2015. While a need for national helminth control program is confirmed in Mozambique by Augusto *et al,* 2009.

Studies on schistosomiasis in Ogbese-Ekiti carried out by our research team started in the year 2000. The prevalence at that time was 82%. The prevalence increased gradually and by 2007 it was 87.5% (Ologunde, 2009). In the year 2008, mass chemotherapy with praziquantel was carried out among the primary and secondary school pupils in the community. Although a hundred percent cure was recorded after treatment, by the year 2009, the prevalence was 75.6% among the pupils. The resurgence of the disease in 2009 despite mass chemotherapy prompted our research team to carry out this study on the rate of re-infection after successful treatment with prazinquantel. This research therefore studied the rate of *S.haematobium* re-infection seven months after successful treatment with praziquantel.

II. Methodology

a) Prevalence of Urinary Schistosomiasis

The prevalence of urinary schistosomiasis was carried out in the primary and secondary schools in Ogbese-Ekiti. Prior to collection of urine samples, all the

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school headmasters and principal were contacted for permission, cooperation and necessary briefing regarding the purpose, relevance and personal involvements of the exercise. In each of the schools, health education lectures were carried out for enlightenment about the disease. Questionnaires were administered according to the method of Edugbola *et al* (1988). Also, their urine samples were collected.

b) Collection of Urine

Urine samples were collected from 172 school children in two primary and one post primary schools in Ogbese-Ekiti between the hours of 10:00am and 11:00a.m aided by their class teachers, the samples were labeled appropriately and brought to the laboratory. Each of the sample was thoroughly mixed to ensure even distribution of contents. An aliquot of 10ml of each sample was centrifuged at 2000 rpm for 5 minutes. The supernatant (9ml) was decanted and a drop of 0.05ml of the supernatant and the number of eggs were counted. The number of eggs in 10ml of each urine sample was calculated from the mean of results of counts from four fields and recorded as number of eggs/10ml of urine. Of the 172 samples examined, 30 with high egg intensity (440-780) were selected (10 from each school) and drugs were administered to the children selected with the help of the nurses at the health centre in Ogbese-Ekiti. The drug administered was praziquantel (40mg/kg body weight) and it was taken at intervals of 4 hours after ingestion of food. Samples of urine were collected from the children for 10 days after drug administration to observe the rate of egg clearance. After total clearance i.e. successful treatment, the children were observed every month for seven months for re-infection.

III. Results

There were 172 subjects examined in the study and 130 (75.6%) were positive for at least one *S.heamatobium* egg. Egg intensity per 10ml of urine ranged from 40-780. The highest prevalence occurred among the 13-15 years age group while the lowest occurred among the 10-12 year as group. Males were more infected (79.8%) than female (70.5%) although there was no significant difference (P<0.05). The overall percentage macrohaematuria was 22%.

The results of selective chemotherapy and re-infection studies are presented in tables 3-4. The baseline average egg count was 567.3, this average baseline egg counts reduced by 54.2%, 69.9%, 99.6% and 100% on days 1,2, 5 and 10 respectively.

All the thirty subjects treated with praziquantel had zero level of *S. haematobium* on or before day 10. The results of re-infection after zero level egg count in January is presented in fig. 1-2. There was no cases of re-infection in the first three months (February, March, April). There were 11(33.7%) cases of re-infection in May, 14(43.3%) in June, 17(56.7%) in July and 14(65.3%) in August. There was no visible macrohaematuria in the first five months. In July and August, the percentage of macrohaematuria was 30% and 36.7% respectively. Female had more re-infection (71.4%) than male (56.3%). The average egg intensity/10mL of urine increased from 32.7% in May to 38.6% in June, 72.9% in July and 105.3% in August.

Results

Table 1 : Prevalence of Schistosoma haematobium Infection in Relation to Age Group among School Pupils in Ogbese-Ekiti, Ekiti State.

	TOTAL			MALE			FEMALE		
Age group	Number examined	Number infected	% infected	No of examined	No infected (prevalence	% infected	Number examined	Number infected	% infected
4-6	25	20	80.0	11	10	90.9	14	10	71.4
7-9	42	31	73.8	19	12	63.2	23	19	82.6
10-12	60	41	68.3	31	24	77.4	29	17	58.6
13-15	45	38	84.4	33	29	87.9	12	9	75.0
Total	172	130	75.6	94	75	79.8	78	55	70.5

Table 2 : Prevalence of Macrohaematuria in Relation to Sex in School Children in Ogbese-Ekiti, Ekiti State.

Sex	Number Examined	Number Infected	Macrohaematuria (%)
Male	94	75	25(25.6%)
Female	78	55	13(16.7%)
Total	**172**	**130**	**38(22.1%)**

Table 3 : Average Rate of Egg Clearance after Drug Administration.

Days	Before Drug Administration	After Drug Administration			
Average	Day 0	Day 1	Day 2	Day 5	Day 10
Egg intensity	567.3	260.00 [54.2%]	170.70 [69.9%]	2 [99.6%]	0 [100%]

The Figure in parenthesis represent percentage egg clearance

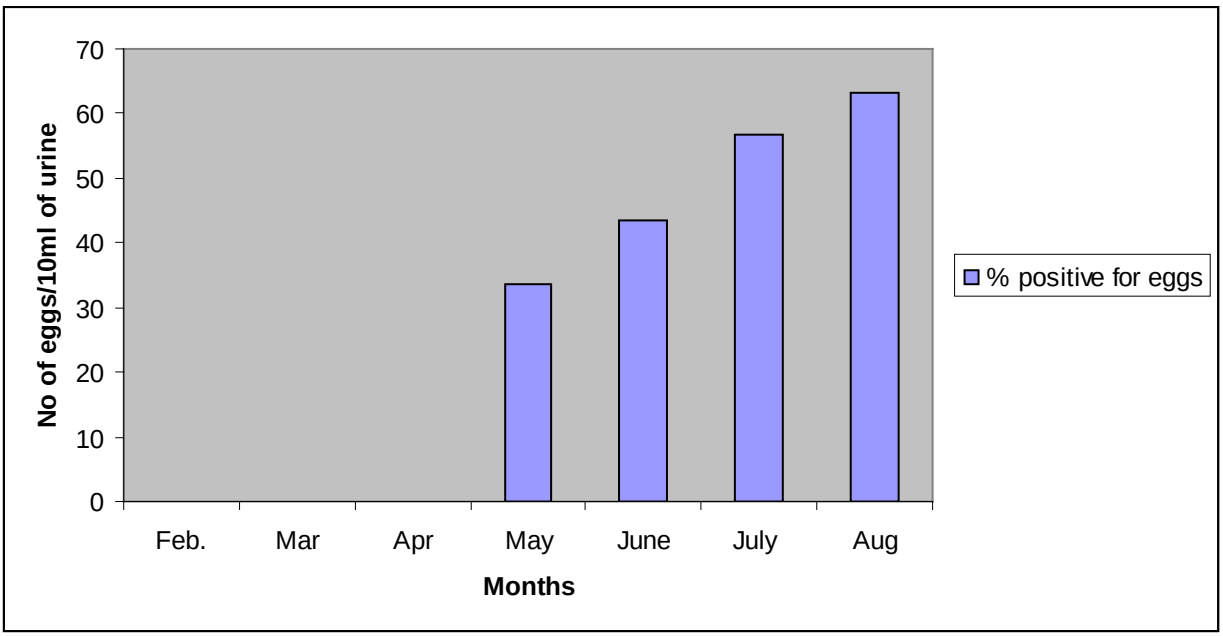

Figure 1 : Rate of Re-infection with *S.haematobium* with time.

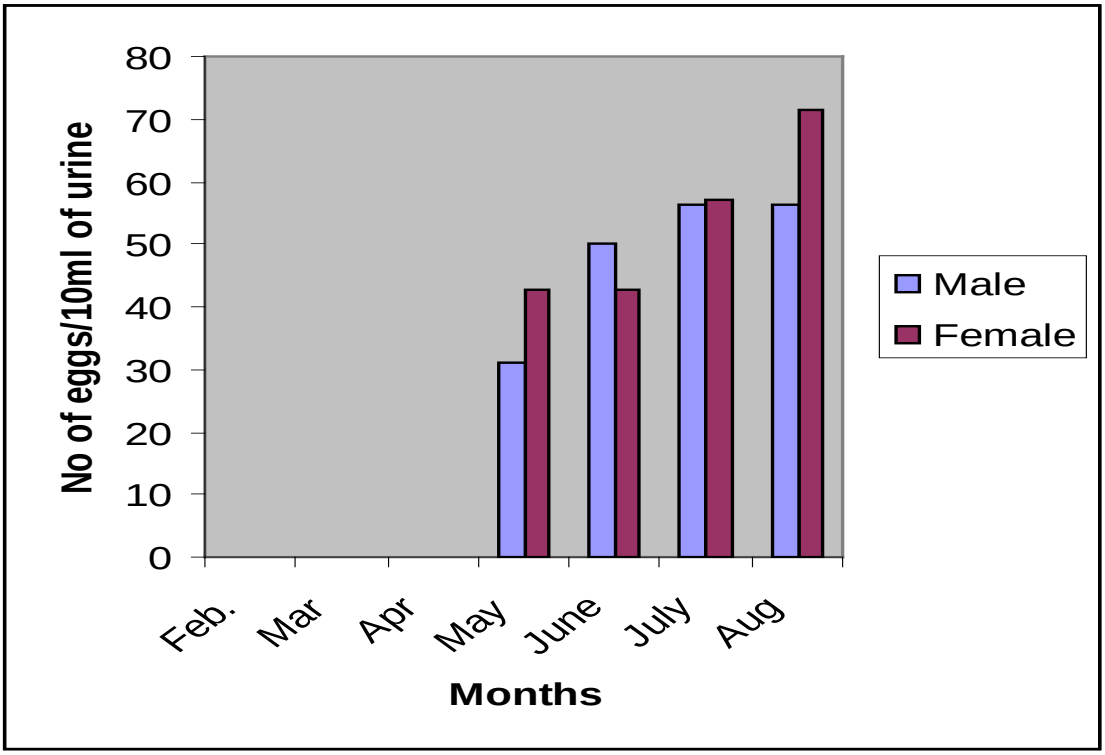

Figure 2 : Rate of Re-Infection according to Sex.

Table 4 : Rate Of Macrohaematuria With Time.

Months %	Feb.	Mar.	Apr.	May	June	July	August
Machaematobium	0	0	0	0	0	30%	36.7%

IV. Discussion

Praziquantel is rapidly metabolized and excreted predominantly via the kidney and partially via the liver and has been proved to be effective against *S. haematobium* infection at 40mg/kg body weight (Dickmand and Buhring, 1976). Talaat and Miller (1998) reported 83.6% reduction in the prevalence of *S. haematobium*, one year after treatment with praziquantel in upper Egypt. In this study, a cure rate of 100% was observed at 40mg/kg body weight within one month of treatment. There were no reported cases of re-infection within 3 months after treatment and cure in this study. This is consistent with the observation that the time limit between cercariae penetration and the appearance of the first egg in the urine is 3-4 months (Ukoli, 1990). In this study, the rate of re-infection among female is higher than that of male, although there was no significant difference ($P<0.05$). Previous studies on water contact activities in Ogbese-Ekiti confirmed that female have higher water contact activities than male (Ologunde, 2005). However, males were more infected (79.8%) than female (70.5%) before treatment but there was also no significant difference ($P<0.05$). This is in line with work of Chidi *et al* (2006). Macrohaematuria one of the indicator of urinary schistosomiasis was observed in the study. The rate was higher after cercariae reinfection than pretreated samples.

Although two brands of praziquantel bought from different locations were used in this study the results showed that adulterated praziquantel is presently not circulated in Ekiti State, Nigeria. The fact that Re-infection occurred 4 months after total cure showed that Mass Chemotherapy and not selective treatment would be an effective control measure against *S. haematobium.* Selective chemotherapy studies have different objectives; i.e. to reduce and prevent morbidity (Talaat and Miller, 1998). Several studies confirmed that incidence, re-infection and egg counts were higher in community where mass chemotherapy had not been applied (El-Enien *et al*, 1993; Webbe & Haky, 1990; Kessler *et al*, 1987; Kitron and Higashi, 1985; King *et al,* 1982). It is therefore important to carryout mass chemotherapy on all subjects adults and children in Ogbese-Ekiti community to reduce incidence, re-infection and egg intensity. Also, McManus *et al,* 2010 in

their study concluded that the inclusion of focal molluscicide, improvements in sanitation, and health education into the control scenario will reduce significantly the level of schistosome infection. It is therefore also important to control the snail intermediate host of *S. haematobium (B. globosus)* as also confirmed by the study of Ologunde (2005, 2009). Mbata *et* a*l* in 2008 showed that *schistosoma haematobium* infested freshwater habitat is responsible for the prevalence of the disease in the area studied. A national helminth control program is equally important (Augusto *et al,* 2009). Piped borne water should be provided in Ogbese-Ekiti to reduce water contact activities with river Ogbese the transmission site.

V. Conclusion and Recommendation

The study showed a high rate of re-infection and confirmed that mass chemotherapy and not selective treatment would be the most effective measure in the control of *S. haematobium* in Ogbese-Ekiti community of Ekiti-State, South-Western Nigeria.

The snail intermediate host of *S. haematobium* should be eliminated by treatment of the water with molluscicides in the month of February as confirmed by previous studies.

Health Education should also be organized to enlighten the subjects in Ogbese community about the health implication of urinary schistosomiasis.

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Direct Antisperm Antibody Examination of Infertile Men

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Abstract - The study was conducted to elucidate the correlation between direct antisperm antibody (ASA) test and some semen characters of infertile men at the Institute of Embryo Researches and Infertility Treatment at Al-Nahrain University from March 2009 to June 2010. Microscopic and macroscopic semen analysis, Direct Sperm MAR IgA test and Direct Sperm MAR IgG test were performed on semen samples. Forty-two men were included in the study, of whom, all underwent direct sperm MAR IgA test, but due to observer bias, only 36 Direct sperm MAR IgG tests were included in the study. There was a significant correlation between suspected IgA and viscosity (r=0.65, p=0.02); suspected IgA and pH (r=0.42, p=0.04); and suspected IgG and liquefaction time (r=0.44, p=0.04). A significant negative correlation was found between suspected IgG and progressive sperm motility (r=-0.44, p=0.04); suspected IgG and suspected IgA with morphologically normal sperm percentage (r=-0.65, p=0.03 and r=-0.43, p= 0.04, respectively); suspected IgG and sperm agglutination (r=- 0.54, p=0.03).

Keywords : *Direct antisperm antibody, IgA, IgG, immunological factor infertility*

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Direct Antisperm Antibody Examination of Infertile Men

Saad S. Al-Dujaily [α], Wathic K. Chakir [σ] & Sundus F. Hantoosh[σ]

Abstract - The study was conducted to elucidate the correlation between direct antisperm antibody (ASA) test and some semen characters of infertile men at the Institute of Embryo Researches and Infertility Treatment at Al-Nahrain University from March 2009 to June 2010. Microscopic and macroscopic semen analysis, Direct Sperm MAR IgA test and Direct Sperm MAR IgG test were performed on semen samples. Forty-two men were included in the study, of whom, all underwent direct sperm MAR IgA test, but due to observer bias, only 36 Direct sperm MAR IgG tests were included in the study. There was a significant correlation between suspected IgA and viscosity (r=0.65, p=0.02); suspected IgA and pH (r=0.42, p=0.04); and suspected IgG and liquefaction time (r=0.44, p=0.04). A significant negative correlation was found between suspected IgG and progressive sperm motility (r=-0.44, p=0.04); suspected IgG and suspected IgA with morphologically normal sperm percentage (r=-0.65, p=0.03 and r=-0.43, p= 0.04, respectively); suspected IgG and sperm agglutination (r=- 0.54, p=0.03).

This study, recommends the use of Direct tests instead of the indirect assays in andrology laboratories in Iraq.

Keywords : Direct antisperm antibody, IgA, IgG, immunological factor infertility.

I. Introduction

Infertility is a worldwide problem which affects approximately 15% of all couples, and is defined as, inability to conceive after twelve months of contraceptive-free unprotected intercourse (Irvine, 1998). Half of all the infertile couples remain untreated and/or unresolved. The main diagnostic groups for infertility are divided into those due to the male (25%), ovulation (25%), tubal, (20%), unexplained cause (25%) and endometriosis (5%) (Templeton, 1995).Among infertile couples, 40% are primary due to the infertility of the male partner, while in 20% of these cases, it is a combination of both male and female factors (Agarwal and Kulkarni, 2003).The common causes of male infertility are varicocele, accessory gland infection, immunological factors, congenital abnormalities, obstructive azoospermia; iatrogenic systemic and endocrine causes.

Irvine, (1998) reported that the occurrence of spontaneous antisperm antibodies (ASA) to human spermatozoa from infertile men was first described by Rumke and independently by Wilson in 1954. Antisperm antibodies (ASA) are the main cause of immunological infertility as they impair sperm function by binding to the sperm membrane (de Krester and Baker, 1999). ASA can be detected in seminal plasma, where they may be bound to the sperm surface or solubilized in the seminal plasma, or in cervical mucus, oviductal fluid or follicular fluid of women. Naturally occurring ASA in men can affect fertility by various mechanisms (Gardini et al., 1995). Some of them are mainly related to the extent of the sperm autoimmunization (e.g. sperm agglutination and impaired cervical mucus penetration); others are related to immunoglobulin (Ig)-isotype (e.g. complement-mediated sperm injury through the female genital tract); or to antigenic specificity of antisperm antibodies (e.g. interference with gametes interaction)(Gardini et al., 1995).

Antisperm antibodies can be defined as immunoglobulins of the IgG, IgA, and/or IgM isotype that are directed against various aspects of the spermatozoa (head, tail, midpiece or combination of them) (Templeton, 1995). Immunoglobulin subclasses IgA and IgG have been demonstrated in the ejaculates of men with antisperm autoimmunity. Though IgM seems to have no clinical impact, it is rarely detected alone or combined with IgA or IgG (Hijort, 1999). In Iraq, due to difficulties and high expenses in importing the kit of direct ASA test, the procedures that diagnose the antisperm antibodies in the andrology laboratories are indirect assays, using sera of men. It has been reported that anti-sperm IgG can be tested in serum but this is of little benefit as serum anti-sperm IgG neither correlates with anti-sperm Ig in semen, nor influences fertility prognosis (Esteves et al., 2007; Agostini and, Lucas, 2011). Globally, semen sample assays of men are used.

Antibodies can be measured in cervical mucus and seminal plasma as well as in serum or as an antigen-antibody complex on the surface of sperm. Direct assessment of sperm antibodies on the sperm surface is preferred to indirect measurement because capacitation of sperm might be useful in defining antibody reactivity (Esteves et al., 2007).This study is designed to find out the correlation between direct ASA test and certain semen characters of infertile men.

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II. Methods

Infertile males attending Institute of Embryo Researches and Infertility Treatment at AL-Nahrain University during the period from March 2009 to June 2010 were included in this study.

Seminal fluids were collected by masturbation after three to five days of abstinence and seminal fluid analyses were performed within one hour of sample collection. Seminal fluid analysis was performed according to WHO guide line (1999). For example, sperm concentration $\geq$ 20 million/ml, progressive sperm motility (a+b=50%), morphologically normal sperm ($\geq$ 30%), and round cells (<5 cells per high power field). All of the samples were subjected to Direct sperm MAR IgA, and sperm MAR IgG tests (Fertipro N.V. industriepark Noord 32.8730 Beemem, Belgium).

Direct Sperm MAR IgA and IgG tests are qualitative latex tests for detection of sperm antibodies of the IgA and IgG classes, respectively. For Direct Sperm MAR IgA test, the reagent used was Sperm MAR latex particles and for Direct Sperm MAR IgG test, the reagents used were Sperm MAR latex particles and Sperm MAR antiserum. For both tests, on a microslide 10 microliters of fresh semen and 10 microliters of Sperm MAR latex particles were added. The sample and the latex reagent were mixed five times with the edge of a cover glass. The cover glass was put on the mixture and the mixture was observed under a light microscope at power 40x magnification.

The result was read after 2-3 minutes. The latex particles attached to motile sperm were observed. One hundred spermatozoa were counted to determine the percentage of reactive sperms. If no attachment of beads to sperm was observed, the reading was again performed after ten minutes as described in the information booklet accompaniny with the kits (Jager et al., 1978 ; Hinting, 1988).

Samples with less than 10% reactivity were labeled as "Normal". The diagnosis of immunological infertility was "Suspected" when 10-39% of the motile spermatozoa were attached to latex particles. If 40% or more of the spermatozoa were attached, immunological infertility was "Highly Probable" as mentioned in the instructions accompanied with the kits (Jager et al., 1978). Based on these findings, the patients were divided into three groups for each, IgA and IgG, into normal, suspected, or highly probable immunological infertility.

III. Statistical Analysis

The data was expressed as mean $\pm$ standard error (SE). Further analysis was accomplished using vicariate Pearson's correlation coefficient. P-value with less than 0.05 was considered significant (Sorlie, 1995).

IV. Results

Forty-two patients were included in this study. Their age ranged between 22 to 45 years. Most of the patients had primary infertility (80.9%), whereas the remaining patients had secondary infertility. Forty-two men were subjected to Direct Sperm MAR IgA and Sperm MAR IgG tests. IgG test for thirty-six out of the total forty-two samples were included in the study; the remaining six were excluded because of bias in their results.

Table 1 shows no significant correlation between IgG and IgA with semen volume (r=0.27, p=0.22) and (r=-0.18, p=0.44) respectively. However, there was a significant negative correlation between normal IgA and semen volume (r=-0.54, p=0.03). A significant correlation was observed between suspected IgA and viscosity (r=0.65, p=0.02), and between suspected IgA and pH (r=0.42, p=0.04). There was a significant correlation between suspected IgG and liquefaction time (r=0.44, p=0.04).

Table 2 shows the correlation between both IgG and IgA with microscopic semen analysis. A significant correlation was noticed between high IgG and sperm concentration(r=0.46 p=0.04) with a significant negative correlation between suspected IgG and progressive motility (-0.44, p=0.04). There was also a significant correlation between suspected IgG and suspected IgA with morphologically normal sperm percentage (r=-0.65, p=0.03 and r =-0.43, p=0.04, respectively). The correlation coefficient for suspected IgG and sperm agglutination had a significant negative value (r=- 0.54, p=0.03).

V. Discussion

The results revealed no correlations of both IgG and IgA with semen volume, liquefaction time and pH, which are all normal in immunological infertility. However, there was a negative correlation between normal IgA and semen volume. This can be interpreted by the fact that in a sample with a low semen volume, normal IgA level is an indicator of accessory glands disorder rather than an immunological factor as the underlying cause of infertility. Several authors have stated that there is no relation between normal IgA antibodies value and abnormal semen parameters (Soffer et al., 1990). The other coefficient correlation was found between suspected IgG and liquefaction time, which means that for each semen sample taking a longer time for liquefaction, more IgG reactivity could be detected. A positive correlation of suspected IgA with viscosity and pH was also observed. This finding may be due to acute or chronic genitourinary tract infections, asymptomatic Chlamydia trachomatis infections, or an infection with human immunodeficiency virus (Naz et al., 1990; Soffer et al., 1990) .It is thought that locally

produced IgA is the most frequently implicated of humoral immune infertility (Francavilla et al., 2007).

In this study, we noticed a negative correlation between suspected IgG and sperm motility, sperm morphology and sperm agglutination .These correlations emphasized the cause of infertility. Antisperm antibodies that are located on the tail of sperm can cause the sperm to become immobilized or clumped together; and when antisperm antibodies are found on the head of sperm, they can prevent the sperm from being able to efficiently make its way through a woman's cervical mucus to the ovum (Agostini and Lucas, 2011). A significant increase in the proportion of motile sperms involved in agglutination has been reported in the presence of antisperm antibodies (Hijort, 1999).

Most of the men with antisperm antibodies had both, IgG and IgA on their spermatozoa but some had only IgG. Pure IgA reactions were not recorded and this is consistent with other studies (Bohring et al., 2001). An opsonizing effect is exerted by IgG antisperm antibodies which enhance sperm phagocytosis and lysis by peritoneal macrophages of the female and this effect is mediated by Fc-receptor for IgG (Agostini and Lucas, 2011). Cytotoxic effects due to complement activation can be produced by IgG but not IgA sperm bound antibodies (Hinting, 1988).

Aberration of the immune homeostasis may give rise to "immunological infertility". The majority of subjects with asthenozoospermia and/or oligozoospermia have a higher incidence of low complement-inhibiting activity and a reduced level of complement regulatory proteins in their seminal plasma, such that nonspecific activation of the alternative complement pathway occur, inflicting injury to sperm (Soffer et al.,1990).

In this study, six infertile patients showed significant high percentages of IgG with no agglutination in their semen. One explanation is that the autoimmune disease is at its initial stages. If those patients remained untreated for a certain period of time and were subsequently subjected to seminal fluid analysis test, notable agglutination might be noticed. However, there is variety of treatments available to help the couples struggling with ASA-mediated infertility, such as corticosteroids, intra-uterine insemination by sperm washing and *in-vitro* fertilization programs (Francavilla et al., 1997).

Direct sperm MAR IgG and Direct sperm Mar IgA tests are quick, easy, and sensitive, but require motile spermatozoa to ensure that the erythrocytes or immunobeads adhere to the sperm membrane (Jager et al., 1978). In several countries, Direct sperm Mar test kits are used to detect antisperm-IgG in semen. Positive and dubious samples are subsequently tested for antisperm IgA. IgA antibodies are more significant clinically, but very rarely occur without associated IgG; therefore, the test of antisperm IgG antibodies in semen is sufficient as the initial screening procedure (Naz et al., 1990).

Direct sperm MAR test is commercially available, cheap and quicker, and are generally considered as more suitable for routine screening of all semen analyses (Francavilla et al.,1997). Direct and indirect tests which do not detect the regional specificity of antibody link or which lack specificity for surface antigens because of concomitant exposure of internal antigens, are not suitable for delineating a diagnosis (Hinting et al., 1988).

One of the most rational ways to test for antisperm antibodies in males is by means of the direct mixed agglutination reaction (MAR) test (Bohring et al., 2001). This test is quick, easy, and sensitive, but requires motile spermatozoa to ensure that the erythrocytes or immunobeads adhere to the sperm membrane.

It is an excellent screening test if the reaction is negative or weakly positive (Bohring et al., 2001). In addition, sperm MAR is commercially available and requires no sperm processing (Esteves et al., 2007).

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Table 1 : Correlation of IgG and IgA with macroscopic seminal fluid parameters.

Kind of Ig(n)	Mean±SE In %	Volume In ml	r p	Liquefaction Time	r p	Viscosity	r p	pH	r p
IgG Normal 15	5.47±0.06	2.92±0.08	-0.22 0.31	36.0±0.63	0.28 0.33	0.20±0.03	0.27 0.40	7.76±-0.02	0.38 0.12
IgG Suspected 15	19.87±0.22	2.92±0.08	0.27 0.22	36.0±0.65	0.44* 0.04	0.2±0.04	-0.14 0.55	7.76±0.02	0.19 0.33
IgG High 6	78±0.47	2.92±0.04	------	36.0±0.33	-0.15 0.33	0.2±0.02	-0.08 0.88	7.76±0.01	0.02 0.90
IgA Normal 18	4.55±0.07	2.88±0.05	-0.54* 0.03	38.65±0.52	-0.05 0.91	0.38±0.04	-0.11 0.78	7.89±0.01	-0.03 0.88
IgA Suspected 21	16.48±0.18	2.88±0.06	-0.18 0.44	38.65±0.51	-0.08 0.66	0.38±0.04	0.65* 0.02	7.89±0.01	0.42* 0.04
IgA High 3	49.33±0.24	2.88±0.05	-----	38.65±0.53	-0.08 0.77	0.38±0.04	-0.07 0.67	7.89±0.01	0.02 0.89

Values are Mean±SE
r= Correlation coefficient
p= Probability

Table 2 : Correlation of IgG and IgA with microscopic seminal fluid parameters.

Kind of Ig Reactivity (n)	Mean ± SE	Count	r p	Progressive motility	r p	\ WBC+ germ cells	r p	Morphology	r p	Agglutination	r p	Round Cells	r p
IgG Normal 15	5.47±0.06	52.05±0.61	0.08 0.65	39.2±0.89	-0.34 0.22	1.2±0.18	0.27 0.32	35..95±0.70	-0.06 0.66	5.25±0.49	0.21 0.30	8..35±0.31	0.07 0.64
IgG Suspected 15	19.87±0.22	52.05±1.71	0.73 0.44	39.2±0.92	-0.44* 0.04	1.2±0.20	0.94 0.52	35..95±0.74	-0.65* 0.03	5.25±0.51	-0.54* 0.04	7.45±0.31	0.210 0.22
IgG High 6	78±0.47	52.05±0.76	* 0.46 0.04	39.2±0.42	0.003 0.99	1.2±0.09	0.15 0.44	35..95±0.34	-0.36 0.08	5.25±0.23	-0.10 0.57	8..35±0.31	0.10 0.66
IgA Normal 18	4.55±0.07	53.31±1.21	-0.27 0.21	41.08±0.61	-0.08 0.77	1.02±0.13	0.01 0.98	38.65±0.56	0.28 0.34	6.08±0.36	0.07 0.97	7.45±0.44	- 0.54* 0.03
IgA Suspected 21	16.47±0.18	53.31±1.21	0.20 0.88	41.08±0.61	0.09 0.90	1.02±0.13	-0.22 0.55	38.65±0.56	-0.43* 0.04	6.08±0.36	0.10 0.99	8..35±0.31	-0.13 0.88
IgA High 3	49.33±0.24	53.31±1.21	0.29 0.34	41.08±0.61	-0.02 0.87	1.02±0.13	0.15 0.67	38.65±0.56	-0.37 0.21	6.08±0.36	-0.13 0.33	7.45±0.21	-0.10 0.88

Values are Mean ± SE
r= Correlation coefficient
p= Probability

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A Study on Virulence and Signaling Mechanism Related to Biofilm Formation and Exopolysaccharide Expression in Certain *Vibrio cholerae* Environmental Isolates

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Keywords : *Vibrio cholerae environmental isolates, serogrouping, antibiogram, pathogenecity, biofilm.*

GJMR-A Classification : *NLMC Code: QW 730, QW 90, QW 55*

Strictly as per the compliance and regulations of:

A Study on Virulence and Signaling Mechanism Related to Biofilm Formation and Exopolysaccharide Expression in Certain *Vibrio cholerae* Environmental Isolates

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Abstract - *Vibrio cholerae,* causative agent of profuse watery diarrhea forms biofilm structure which is adaptive advantage for the *Vibrio cholerae.* This study was carried out to characterize certain *Vibrio cholerae* environmental strains collected from the water reservoirs surrounding Midnapore town, West Bengal, India in respect of serogroups, antibiotic susceptibility, protease activity, haemolytic activity, pathogenecity and determination of biofilm forming ability and also signaling system involved in the regulation of biofilm. Multiplex PCR assay revealed that all were from "non- O1/non O139" serogroups and *ctxA, tcpA* negative. All of them were sensitive to streptomycin, tetracycline, nalidixic acid but resistant to ampicillin, norfloxacin and chloramphenical and 70% were polymyxin B resistant. Beside this these *Vibrio cholerae* isolates were strongly hemolytic, moderate to high biofilm forming. In this study we found that these strains did not follow the biofilm controlling previously reported flagella-mediated, hapR-mediated as well as quorum sensing autoinducer(s)-mediated signal transduction pathway.

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I. Introduction

Cholera is still considered as major public health concern in developing countries as *Vibrio cholerae* has the potential to be transported internationally and has the ability to occur in explosive endemic form ignoring temporal or spatial gap (McCarthy et al., 1994). Indian subcontinent has come out as an important epicenter of cholera in most of the cholera pandemics (Kumar et al., 2009). Epidemiological data reveals that the *Vibrio cholerae* organisms belonging to O1 and O139 serogroups are etiological agent of epidemic cholera. On the other hand, *Vibrio cholerae* strains from non-O1/non-O139 serogroups are associated with sporadic cases of moderate to severe gastroenteritis and extra-intestinal infections in humans (Chen et al., 2007; Dziejman et al., 2005; Zoelsson et al., 2006, Kovacikova and Skorupski, 2002; Kaper et al., 1995). In developed countries *Vibrio cholerae* non-O1/non-O139 predominates over the enteric infections caused by *Vibrio cholerae* O1/ O139. *Vibrio cholerae* non-O1/non-O139 usually causes a less severe diarrhea than *Vibrio cholerae* O1/ O139, although certain strains, especially those that produce cholera toxin, can cause severe cholera-like disease (Datta et al., 1986). Some non O1 *Vibrio cholerae* strains despite being negative for ctxA and tcpA, the major virulence markers, were reported to cause significant mortality and morbidity in Bangladesh (Islam et al., 1988). *Vibrio cholerae* non-O1/non-O139 was again reported to be associated with cholera like disease in 1996 in Kolkata, India (Sharma et al., 1998). Earlier reports showed that epidemic strains of *Vibrio cholerae* exclusively possess ctxA and tcpA those are the most important virulence factors for cholera pathogenesis (Nair et al., 1988). But later *Vibrio cholerae* non-O1/non-O139 strains were also found to possess ctxA, tcpA (both classical and El Tor types) (Ghosh et al., 1997, Chakraborty et al., 2000).

Vibrio cholerae organisms frequently change their characteristics regarding antibiotic susceptibility producing serious problem in health management and this is due to presence of drug resistant gene which is solely responsible for the transmission and spread of the multidrug resistant properties among the pathogen (Mukhopadhyay et al., 1996). *Vibrio cholerae* O1 strains

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isolated from cholera patients in Dhaka during January 1986 were found to be sensitive to tetracycline, streptomycin, chloramphenicol, amoxicillin or nalidixic acid (Nakasone et al., 1987). Furthermore all *Vibrio cholera* El Tor biotype isolates during 1988 and 1989 in Bangladesh were sensitive to tetracycline (Siddique et al., 1989) while El Tor strains re-emerged during the 1991 epidemic in Bangladesh were tetracycline resistant (Siddique et al., 1992). In addition certain isolates from non-O1/non-O139 serogroups of *Vibrio cholerae* were found to be resistant to polymyxin B (Kiiykya et al., 1992). Polymixin B was mainly used for differentiating El Tor biotype strains from classical biotype strains as the El Tor strains were found to be resistant to polymixin B (Matson et al., 2010). Reports reveal that *Vibrio cholerae* non-O1/non-O139 strains cause pathogenesis in a complex manner and possess various virulence factors in different combinations. No single virulence factor can predict about the pathogenicity of the strain (Honda et al., 1985; Takao et al., 1985; Arita et al., 1986; Yoshimura et al., 1986). A large number of *Vibrio cholerae* non-O1/non-O139 strains are able to produce cholera like toxins, i.e., heat-stable enterotoxin, thermostable direct haemolysin, hemaagglutinin/ protease, shiga-like toxin those play significant role in the enteropathogenecity (Yamamoto et al., 1984; Ogawa et al., 1990; Kaper et al., 1994). Protease produced by *Vibrio cholerae* non-O1/non-O139 strains serves as attachment factor (s) that has important link with virulence properties as it enhances toxicity by nicking cholera toxin (Booth et al., 1984) or degrading intestinal mucin (Crowther et al., 1987). The majority of non-O1/non-O139 *Vibrio cholerae* strains are hemolytic and can lyse red blood cells (RBC) from a variety of animals by forming small pores in the cytoplasmic membrane and they show cytolytic activity against cultured cell lines (Yoshio et al., 1987; Shinoda et al., 1985; Yamamoto et al., 1990). It was previously described that highly haemolytic strains are highly enterotoxic suggesting a putative relation between haemolytic activity and enterotoxicity. Hemolysin produced by non-O1/non-O139 *Vibrio cholerae* was speculated to be an enterotoxic factor that is responsible for gastroenteritis (Yoshio et al., 1987).

Vibrio cholerae, the human pathogen is a natural inhabitant of aquatic environment. For successful survival in aquatic ecosystem in response to stresses they form association with the surface structures of crustaceans, mollusks etc. and develop the diarrheogenic disorder when human host is encountered (Yildiz et al., 2009). Non-O1/non-O139 strains are autochthonous inhabitant of the brackish water and estuarine environment in association with zooplankton and are reported to be important in evolutionary scale as they can change their serogroup through the horizontal gene transfer which is relevant mechanism for the emergence of newer variants of *Vibrio cholerae* (Rammurthy et al., 1993). Chitin, a polymer of N-acetylglucosamine and component of crustacean crab shell not only induces competence for natural transformation but also mediates the acquisition of genes conferring antibiotic resistance during the growth of a *Vibrio cholerae* strain on a crab shell fragment immersed in seawater (Meibom et al., 2005).

Biofilm is the adaptive surface growth through formation of a three dimentional multicellular-conformation. Biofilm formation is highly regulated and involves several steps. Expression of a matrix material termed exopolysaccharide (EPS) by individual cell in a biofilm is critical for the development of mature biofilm (Costerton et al., 1995; Kolter et al., 1998). This specialized structure enhances growth and survival of these organisms for their long persistence in the environmental reservoirs by providing nutrients and protection from predators and antimicrobial compounds (Donlan et al., 2002). A HapR (HA protease regulator)-dependent and a flagellum-dependent signaling pathways controlling EPS expression have been described in *Vibrio cholerae* (Zhu et al., 2002; Watnick et al., 2001). In a subset of epidemic causing *Vibrio cholerae* the LuxO-HapR regulatory loop plays leading role in EPS regulation in response to cell population density. In this subset EPS expression and biofilm formation are negatively regulated by HapR, and LuxO acts via HapR by repressing it (Zhu and Mekalanos, 2003; Vance et al., 2003). On the other hand, in another subset of *Vibrio cholerae* absence of flagella leads to excess EPS expression involving participation of sodium-driven flagellar motor and VpsR (Lauriano et al., 2004). On further investigation this subset revealed existence of quorum sensing autoinducers- and flagellum-dependent parallel but converging EPS signaling circuits independent of input of LuxO-HapR (Biswas et al., 2012, unpublished)

So, on the basis of this we conducted present investigation to characterize environmental *Vibrio cholerae* strains collected from the water reservoirs surrounding Midnapore town, in the state of West Bengal of India in respect of their serogroups, antibiotic susceptibility, protease activity including pathogenecity and also determining the potential for survival in nature through identifying biofilm forming ability of the isolated *Vibrio cholerae* strains. Besides we were also interested to find out whether HapR-mediated quorum sensing pathway or quorum sensing autoinducer(s) and flagellum-mediated parallel signaling pathways predominate in the regulation of biofilm formation in these *Vibrio cholerae* environmental isolates.

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II. Methods and Materials

a) Collection of Samples

Samples were collected during the period of January to March, 2009 from fresh water bodies

surrounding Midnapore town, southern part of state of West Bengal, India and processed by the method mentioned by Nair et al., 1988.

b) Bacterial strains and culture methods

The surface water collected from the fresh water bodies of different water reserviors was passed through sterile membrane filter (pore size 0.22 μm, Millipore) and filtered sample water was allowed to grow in alkaline peptone water (APW) containing of 1% (wt/vol) peptone and 1% (wt/vol) NaCl at pH 8.5. After 12 h incubation at 30°C the surface growth was streaked onto thiosulphate citrate bile salt (TCBS) agar plate. After screening *Vibrio cholerae* isolates by TCBS agar plate pure single colony was transferred onto Gelatin agar plate after an APW passage. Other bacterial strains used in this study were allowed to grow in Luria-Bertani (LB) broth, supplemented with specific antibiotics. LB broth without NaCl and with 10% sucrose was used for counter selection with sacB-containing plasmids. The strains of bacteria used are listed in Table 2 along with their source.

c) Multiplex PCR assay for virulence genes

Two sets of multiplex PCR assays were conducted using specific primer pairs listed in Table 1. First set of multiplex PCR assay was performed to detect serogroup using specific primer pairs for genes encoding O1 somatic antigen (rfbO1) and O139 somatic antigen (rfbO139). Second multiplex PCR was conducted to detect ctxA and tcpA (both classical and El Tor) using specific primer pairs. PCR reaction mixture and the thermal cycling conditions for PCR were carried out by the method mentioned by Kumar et al., 2009. All PCRs mentioned earlier were carried out using chromosomal DNA from these *Vibrio cholerae* environmental strains as template and isolated by the method of Sambrook et al., 2001.

d) Detection of Motility

Motility of the isolated *Vibrio cholerae* strains were tested by the method mentioned by Rasid et al., 2003 using swarm plate containing 0.3 % LB agar.

e) Detection of protease activity

Protease activity of the isolated *Vibrio cholerae* strains were detected by using a single-diffusion technique in agar gel containing skim milk as a substrate (Finkelstein et al., 1983).

f) Antibiotic susceptibility test

Antibiotic susceptibility test was done against the *Vibrio cholerae* environmental isolates by the method mentioned by Okoh and Igbinosa, 2010. Antibiotics used for this study were ampicillin (50μg/ml), streptomycin (100μg/ml), kanaycin (40μg/ml), tetracycline (50μg/ml), nalidixic acid (30μg/ml), norfloxacin (40μg/ml), chloramphenical (20μg/ml) and polymyxin B (300 IU).

g) Detection of haemolysis

Haemolytic ability of the bacterial isolates was examined on Blood agar media using sheep blood. *Vibrio cholerae* non O1 strains inoculated in Brain Heart Infusion Broth (BHB) were spread on to sheep blood (5% vol/vol) agar and incubated at 37◦ C for 24 hours. Blood agar plates were examined for haemolysis around the colonies (Singh et al., 1996).

h) Toxicity to adult mice

Crude toxin was prepared from the each of the bacterial isolates by the method mentioned by Kiiyukia et al., 1992. Toxicity to adult mice was determined through the intra-peritoneal injection of 0.5-ml of crude toxin into 4-week-old male mice. Four animals were used per strain. Epidemic strain MO10 lac- O139 Bengal was used as a positive control. The number of dead mice was recorded after 48 h.

i) Biofilm Formation Assay

Biofilm assay was performed by the method originally mentioned by Watnick et al., 2001with further modifications made by Lauriano et al., 2004. The strains were grown in LB broth overnight and after that normalised to identical densities using OD600. Then 5 μl of culture was inoculated into 500 μl of LB broth in 10-ml sterile borosilicate test tubes and kept statically at 30◦C for 22 hours. The tubes were rinsed thoroughly with distilled water to remove all non-adherent cells. Further the tubes were incubated for 30 minutes after addition of 600 μl of 0.1% (w/v) crystal violet and were rinsed again with distilled water. Lastly 1 ml of dimethyl sulfoxide was added, the test tubes were vortexed and kept standing for 10 minutes, and the OD570 was measured.

Table1 : Oligonucleotide primer sequence.

Target gene or encoding region	Primer sequence (5'-3')
O1*rfb1* P-A	GTTTCACTGAACAGATGGG
O1*rfb1* P-B	GGTCATCTGTAAGTACAAC
O139*rfb1*P-A	AGCCTCTTTATTACGGGTGG
O139 *rfb1*P-B	GTCAAACCCGATCGTAAAGG
CtxA P-A	CTCAGACGGGATTTGTTAGGCACG
CtxA P-B	TCTATCTCTGTAGCCCCTATTACG
tcpA, cl P-A	CACGATAAGAAAACCGGTCAAGAG
tcpA, cl P-B	ACCAAATGCAACGCCGAATGGAGC
tcpA, ET P-A	GAAGAAGTTTGTAAAAGAAGAACAC
tcpA, ET P-B	GAAAGGACCTTCTTTCACGTTG
HapR P-A	GCTCTAGAGCGACCTCTTGCTCAGAAATC
HapR P-B	GCGGATCCGCGTTTTTCGATTGATGCGTC
HapR P-C	GCGGATCCCAAGTCTCCGTTGCAACAGTG
HapR P-D	GCGCGTCGACGCTGGCCATGTTATCGACATC
FlaA P-A	GCTCTAGACTACTGCAATAACGAGATTGC
FlaA P-B	GCGGATCCGTCACAGCATCAGTAACCTGC
FlaA P-C	GCGGATCCCATCCAAACCACGAGATTGCG
FlaA P-D	GCGGTCGACATGACCATTAACGTAAATAC
CqsA P-A	GCTCTAGAATGCATTTAACGAAAATA
CqsA P-B	GCGGATCCAACAGGAGATGAACGAAATAC

CqsA P-C	GCGGATCCTGTTGTTCTTCCAGTAATGAC
CqsA P-D	GCGGTCGACATGAACAAGCCTCAACTTCC
LuxS P-A	GCTCTAGAATGCCATTATTAGACAGTTT
LuxS P-B	GCGGATCCATCTTTGTTTGGCATAGTAA
LuxS P-C	GCGGATCCTGCGGCACTGCGGCGATGCA
LuxS P-D	GCGGTCGACTTAGTGAACCTTCAGCTCAT
VpsLPr-A	GCGAATTCATATTGTTCTGTTTTTCCTTTC
VpsLPr-B	GCGGATCCCGAGTATTCTGCTTTTTTCCTTCATC GC

for 10 minutes, and the OD_{570} was measured.

j) Plasmid construction

All plasmid constructs and strains used in this study are displayed in Table 2. All PCRs were performed using MO10 lac- chromosomal DNA as template and specific primer pairs which were designed depending on the specific gene sequence from complete *Vibrio cholerae* genome sequence (Kwok et al., 1990; Heidelberg et al., 2000). SM10 λpir was used for the propagation of the pir-dependent plasmid and conjugation in *Vibrio cholerae*. The mutations were performed in the choromosome of the bacterial isolates via homologous recombination method (Lauriano et al., 2004).

ΔhapR, ΔflaA, ΔcqsA and *ΔluxS,* plasmid constructs had been constructed by the method described by Lauriano et al., 2004 with further modification in our laboratory. Around 160-500 bp fragment 5' of the gene deletion was generated through PCR using specific primer A and B presented in Table1. This PCR generated fragment of gene deletion after digestion with XbaI and BamHI was ligated into similarly digested suicide vector pKEK229, pir dependent derivative of CVD442 (Correa et al., 2000) to produce plasmid constructs containing deletion. Similarly around 160-500 bp fragment 3' of the gene deletion was PCR generated using the corresponding primer C and D listed in Table 1. The PCR generated 3' fragment of the gene deletion digested with BamHI and SalI was ligated into similarly digested pKEK229 containing 5' of the gene deletion mentioned earlier.

The promoter-lacZ fusion containing transcriptional reporter plasmid of vpsL was prepared by PCR amplification of the respective promoter using primer pairs Promoter A and B for corresponding gene (Table 1). PCR generated fragment was digested with EcoRI and BamHI, and ligated into the corresponding sites of plasmid vector pRS551 (Simons et al., 1987).

k) β - Galactosidase assays

Vibrio cholerae environmental strains were transformed with plasmid pSH117, the transcriptional reporter construct (Table 1). Bacterial cells grown in LB broth were harvested at OD600 of ~ 0.2 to 0.4, permeabilized with chloroform and sodium dodecyl sulfate and assayed for β-galactosidase activity following the method mentioned by Miller 1992.

III. Result and Discussuion

The Gram-negative bacterium *Vibrio cholerae* is inhabitant of aquatic ecosystems as well as a human pathogen. *Vibrio cholerae* causes the profuse watery diarrhea called cholera by colonizing the small intestine and subsequently producing a potent enterotoxin, cholera toxin (CT) (Norris et al., 1974; Rabbani et al., 1990; WHO 1980). Cholera is endemic in southern Asia, parts of Africa and Latin America, where seasonal outbreaks occur widely and sanitation is rudimentary (Kaper et al., 1995). In the areas of endemic infection it appears in regular seasonal pattern and also as explosive outbreaks (Glass et al., 1982; Kaper et al., 1995), indicating a possible role of environmental factors in triggering the epidemic process. Majority of *Vibrio cholerae* strains in the environmental reservoir have been observed to belong to the non-O1/non-O139 serogroups. Although the *Vibrio cholerae* non-O1/non-O139 isolates usually gives rise to the sporadic diarrheal disorders and limited outbreaks certain non-O1/non-O139 strains are also reported to be involved in cholera-like epidemics (McCormack et al., 1969). In this context we developed an interest to study the endemic environmental isolates in relation to their virulence properties including serogroups. In addition we made an attempt to understand their persistence ability in the environmental reservoir and also the signaling system confirming the environmental persistence in these organisms. With this aim we collected water samples from different fresh water bodies recognized as water reservoirs and used for the domestic purposes in the surroundings of Midnapore town, West Bengal, India during the period of January to March, 2009 and finally isolated twelve *Vibrio cholerae* strains (Table 2).

Table 2 : Plasmid and strains used in this study.

Strains	Description	Source
Vibrio cholerae strains		
SB100 to SB 117	Environmental isolates	Collected from fresh water bodies surrounding Midnapore town, West Bengal, India
***Escherichia coli* strains**		
SM10λ *pir*	*Thi thr leu tonA lacY supE* RP4-2-Tc:: Mu λ *pirR6K* Km^r	Laboratory collection
pKEK229	R6K *ori sacB mob* Amp^r	Laboratory collection
pSH101	*Δ hapR* in pKEK229	This study
pSH102	*Δ flaA* in pKEK229	This study
pSH103	*Δ cqsA* in pKEK229	This study
pSH104	*Δ luxS* in pKEK229	This study
pSH117	*vpsL-lacZ* in pRS551	This study

a) Serogrouping of the Vibrio cholerae isolates

We performed multiplex PCR assay with the primer pairs specific for serogroups O1 and O139 (Table 1). All the *Vibrio cholerae* isolates were found to be from non-O1/non-O139 serogroups (Table 3a). In another multiplex PCR we found that these non-O1/non-O139 strains were negative for ctxA and tcpA those are major virulence markers (Table 3a). Previous report also suggested that *Vibrio cholerae* strains isolated from environment are mostly non-O1/non-O139 than O1 and O139 in riverine and estuarine areas and are also CT (cholera toxin) and TCP (toxin co regulated pilus) negative (Karaolis et al., 1998). However report shows that some members of *Vibrio cholerae* non-O1/non-O139 predominate over O1 and O139 strains in producing gastroenteritis and also produce cholera-like toxin (Janada et al., 1988).

b) Antibiotic susceptibility among Vibrio cholerae environmental isolates

Multi-drug-resistant property is found to get continuously increased among the different pathogenic *Vibrio cholerae* strains from time to time and in different geographic locations producing serious treatment crisis and this is due to presence of self-transmissible transposon-like element (SXT element) encoding resistance to sulfamethoxazole, trimethoprim and streptomycin in *Vibrio cholerae* O139 and O1 (Waldor et al., 1996). Depending upon the several case reports on the increased changes in the pattern of drug resistance we performed antibiotic susceptibility test against different antibiotics. Our study showed that all the isolated *Vibrio cholerae* strains (100%) were sensitive to streptomycin (100µg/ml), tetracycline (50µg/ml), nalidixic acid (30µg/ml) but 100 % were resistant to ampicillin (50µg/ml), norfloxacin (40µg/ml) and chloramphenical (20µg/ml) and 70% were resistant to polymyxin B (300IU), but few strains were found to be sensitive (58%) as well as resistant (41.7%) to kanamycin (40µg/ml) (Table 3a). These results revealed that multiple antibiotic resistant properties were present among these bacterial strains. Previous studies had shown that *Vibrio cholerae* is resistant to streptomycin, tetracycline and sensitive to ampicillin but our findings in the present study differed from those reports. We also observed that most of our *Vibrio cholerae* isolates belonging to non-O1/non-O139 serogroups were polymixin B resistant. Polymyxin B is an antimicrobial peptide which is mainly used to differentiate the classical and El Tor biotypes of *Vibrio cholerae* as El Tor biotypes are so long identified as polymyxin B resistant (Chatterjee et al., 2003). However Kiiyekya et al., 1992 reported that some non-O1/non-O139 strains were also becoming resistant to polymyxin B. We predict that due to changing environment in aquatic reservoir they have evolved either acyltransferase like enzyme which confers resistance to antimicrobial peptide drug polymixin B or may have evolved some other strategy for lipid acylation for their survival. Beside this antimicrobial peptide resistance was reported to have a close association with virulence and also to confer greater fitness within the host for their survival (Jyl et al., 2010 Poyart et al., 2003; Somerville et al., 1999). Our observation of newer pattern of antibiotic susceptibility in these non-O1/non-O139 environmental strains supports the concept of substantial mobility in genetic elements encoding antibiotic resistance in these *Vibrio cholerae* strains.

c) Pathogenecity

Pathogenesis of *Vibrio cholerae* non-O1/non-O139 is very complex involving combination of several virulence factors and suggestion is there that a single factor can not be responsible for the enteropathogenecity (Honda et al., 1985; Takao et al., 1985; Arita et al., 1986; Yoshimura et al., 1986). We examined protease activity, which is also considered as virulence factor in *Vibrio cholerae* in these isolated non-O1/non-O139 environmental strains those were already identified to be negative for ctxA and tcpA. We observed that all of them were able to hydrolyze protein (Table-3b). According to previous report protease activity has an important role in adhesion to surface including virulence expression in *Vibrio cholerae* (Booth et al., 1984).

Earlier report also suggested that *Vibrio cholerae* hemolysin/cytolysin acts as a virulence factor for contributing to the pathogenesis in case of C. elegans infection via a CT- and TCP-independent process (Vaitkevicius et al., 2006) and non-O1/non-O139 strains are found to produce El Tor like hemolysin. Non-O1/non-O139 strains were reported to be involved in the fluid accumulation in rabbit ileal loop and infant mice as well as in adult rabbit (Yoshio et al., 1987). So, depending on these reports we studied the haemolytic activity among the isolated non-O1/non-O139 *Vibrio cholerae* environmental strains and surprisingly we found that all isolates caused complete haemolysis of erythrocytes of sheep blood (Table 3b). We further examined the toxicity of crude toxin prepared from the isolated non-O1/non-O139 environmental strains to adult mice and 75% of the mice died after 48 hours (Table 3c). In present investigation we identified that crude toxin was lethal to adult mice and we predict that death of each mice was caused probably due to presence of enterogenic haemolysin in these non-O1/non-O139 envoironmental strains. Yamanoi et al. (1980) demonstrated that mice were killed by intraperitoneal injection of live cells of *Vibrio cholerae* non-O1/non-O139 isolates. Another report by Yoshio et al. (1987) also described non-O1/non-O139 haemolysin as enterogenic.

d) Biofilm formation and related signaling systems

Vibrio cholerae organisms from the non-O1/non-O139 serogroups are frequently found in

aquatic environment. In aquatic ecosystems they exist both as free-living organism and also in association with zooplanktons (Rivera et al., 2001). During their life cycle both in aquatic environment and eukaryotic host they face a number of stresses i.e., chlorine water, antibiotics, bactericidal agents etc. and to combat stresses they have evolved an adaptive feature, known to be formation of biofilm on biotic and abiotic surfaces. Biofilm is a three-dimentional conformation of surface-attached bacterial cells that confers the bacterial cells in it fitness for survival by overcoming the adversities and thus ensures their long persistence in nature. The stability of biofilm structure is critically determined by expression of exopolysaccharide (EPS) (Yildiz et al., 1999; Wai et al., 1998). In this study we were interested to understand the biofilm forming ability of the newly isolated non-O1/non-O139 environmental strains. We observed that all of these *Vibrio cholerae* non-O1/non-O139 strains were able to produce moderate to high biofim (Fig.1). Then to draw correlation between biofilm formation and EPS expression in these isolates we studied the expression of *vpsL*, the first gene from the *vpsL-Q* operon in the vps biosynthetic gene cluster as a marker for EPS expression using promoter *vpsL-lacZ* fusion transcriptional reporter construct. In each isolate expression of *vpsL* was well correlated with the biofilm formation (Fig. 2). This strongly suggests that the biofilm formation in the present isolates was dependent on expression of exopolysaccharide. On the basis of these observations we suggest that these non-O1/non-O139 environmental strains of *Vibrio cholerae* have the capability to persist in the environment for long period through the formation of biofilm. Different environmental factors determine the expression of virulence associated genes and also other appropriate sets of genes required for their growth in each niche. Earlier reports have suggested the existence of a hapR-dependent quorum sensing pathway and a flagellum-dependent signal transduction cascade, respectively to control the exopolysaccharide (EPS) expression, biofilm formation and expression of virulence genes like cholera toxin (CT) and toxin coregulated pilus (TCP) in two different subsets of the epidemic strains of *Vibrio cholerae* (Zhu et al., 2002; Watnick et al., 2001; Lauriano et al., 2004).

On the basis of this we were eager to develop an understanding regarding the above mentioned signaling cascade that is functional in the present environmental isolates. In our study we observed that deletion in *hapR* and *flaA* as well as quorum sensing autoinducers deficiency did not cause any transition in colony morphology, EPS expression and biofilm formation in these environmental isolates. Hence our observations suggest that these non-O1/non-O139 *Vibrio cholerae* isolates do not follow the biofilm controlling HapR-mediated, flagella-mediated as well as quorum sensing autoinducer(s)-mediated signal transduction pathways those have been observed previously to take leading role in epidemic isolates.

Table 3 a : Serogrouping & antibiotic susceptibility of *Vibrio cholerae* environmental isolates.

Samples	O1 *ctxA & tcpA*	O139 *ctxA & tcpA*	Antibiogram
SB100	–	–	Amp^r,Chl^r, $Nalidixic^s$, $Strep^s$,$Norflox^r$, Polymixin B^S, Tetracycline ^{s}, Kan ^{s}
SB101	–	–	Amp^r,Chl^r, $Nalidixic^s$,$Strep^s$,$Norflox^r$, Polymixin B^r, Tetracycline ^{s}, Kan ^{r}
SB102	–	–	Amp^r,Chl^r, $Nalidixic^s$,$Strep^s$,$Norflox^r$, Polymixin B^S, Tetracycline ^{s}, Kan ^{s}
SB103	–	–	Amp^r,Chl^r, $Nalidixic^s$,$Strep^s$,$Norflox^r$, Polymixin B^r, Tetracycline ^{s}, Kan ^{s}
SB104	–	–	Amp^r,Chl^r, $Nalidixic^s$,$Strep^s$,$Norflox^r$, Polymixin B^S, $Tetracycline^s$, Kan ^{r}
SB105	–	–	Amp^r,Chl^r, $Nalidixic^s$,$Strep^s$,$Norflox^r$, Polymixin B^r, $Tetracycline^s$, Kan ^{s}
SB106	–	–	Amp^r,Chl^r, $Nalidixic^s$,$Strep^s$,$Norflox^r$, Polymixin B^S, Tetracycline ^{s}, Kan ^{s}
SB107	–	–	Amp^r,Chl^r, $Nalidixic^s$,$Strep^s$,$Norflox^r$, Polymixin B^r, Tetracycline ^{s}, Kan ^{s}
SB108	–	–	Amp^r,Chl^r, $Nalidixic^s$,$Strep^s$,$Norflox^r$, Polymixin B^S, Tetracycline ^{s}, Kan ^{r}
SB109	–	–	Amp^r,Chl^r, $Nalidixic^s$,$Strep^s$,$Norflox^r$, Polymixin B^r, $Tetracycline^s$, Kan ^{r}
SB110	–	–	Amp^r,Chl^r, $Nalidixic^s$,$Strep^s$,$Norflox^r$, Polymixin B^r, $Tetracycline^s$, Kan ^{s}
SB111	–	–	Amp^r,Chl^r, $Nalidixic^s$,$Strep^s$,$Norflox^r$, Polymixin B^r, $Tetracycline^s$, Kan ^{r}
SB112	–	–	Amp^r,Chl^r, $Nalidixic^s$,$Strep^s$,$Norflox^r$, Polymixin B^r, Tetracycline ^{s}, Kan ^{r}
SB113	–	–	Amp^r,Chl^r, $Nalidixic^s$,$Strep^s$,$Norflox^r$, Polymixin B^r, $Tetracycline^s$, Kan ^{r}
SB114	–	–	Amp^r,Chl^r, $Nalidixic^s$,$Strep^s$,$Norflox^r$, Polymixin B^r, Tetracycline ^{s}, Kan ^{r}
SB115	–	–	Amp^r,Chl^r, $Nalidixic^s$,$Strep^s$,$Norflox^r$, Polymixin B^r, $Tetracycline^s$, Kan ^{r}
SB116	–	–	Amp^r,Chl^r, $Nalidixic^s$,$Strep^s$,$Norflox^r$, Polymixin B^r, Tetracycline ^{s}, Kan ^{r}
SB117	–	–	Amp^r,Chl^r, $Nalidixic^s$,$Strep^s$,$Norflox^r$, Polymixin B^r, Tetracycline ^{s}, Kan ^{r}

Table 3 b : Pathogenicity of *Vibrio cholerae* environmental isolates.

Samples	Protease activity	Haemol ysin production
SB100	+	+
SB101	+	+
SB102	+	+
SB103	+	+
SB104	+	+
SB105	+	+
SB106	+	+
SB107	+	+
SB108	+	+
SB109	+	+
SB110	+	+
SB111	+	+
SB112	+	+
SB113	+	+

Table 3 c : Toxicity of extracellular products of *Vibrio cholerae* non-O1/ non-O139 environmental strains.

Samples	No. of dead adult mice after injection of intraperitoneal injection (n = 4)
SB100	3
SB101	4
SB102	3
SB103	3
SB104	3
SB105	2
SB106	4
SB107	3
SB108	4
SB109	3
SB110	2
SB111	3
SB112	3
SB113	4
SB114	3
SB115	2
SB116	3
SB117	2

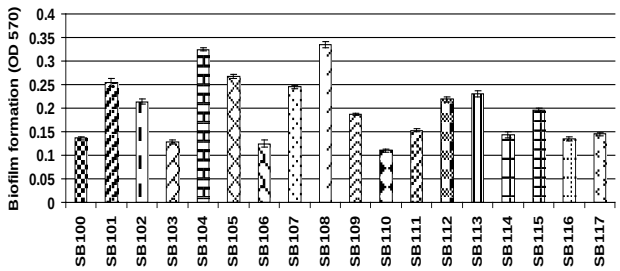

Fig.1. Biofilm formation by different *Vibrio cholerae* environmrental isolates at 30◦C for 24-hr Biofilm formation ability by the different *Vibrio cholerae* environmrental isolates were measured by biofilm formation assay. Biofilm formation by (A) SB100, SB101,SB102,SB103,SB104,SB105,SB106,SB107,SB108,SB109,SB110,SB111,SB112,SB113,SB114,SB115,SB116 and SB117 are presented. OD570 values indicate quantity of biofilm produced. Error bars are standard deviations.

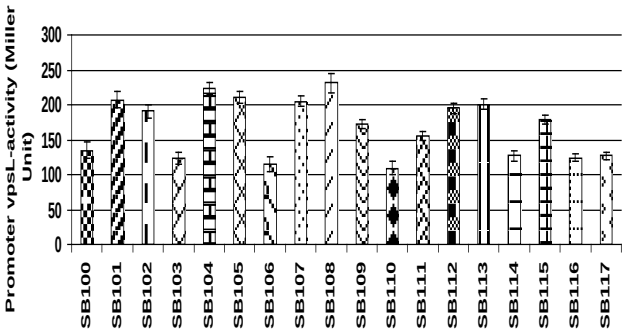

Fig.2 Comparison of EPS expression among different non-O1/non-O139 *Vibrio cholerae* environmental isolates. Transcription level of *vps*L was

examined as marker for *EPS* expression by β-galactosidase assay using *vpsL* promoter-lacZ fusion transcriptional reporter construct.

IV. Conclusion

The results of present investigation on isolated environmental *Vibrio cholerae* organisms those belonged to the non-O1/non-O139 serogruops and negative for both ctxA and tcpA have appeared to be significant as these organisms demonstrated certain new pattern in terms of antibiotic susceptibility. Moreover these organisms were found to be positive in protease activity, haemolysin production, potent in enteropathogenecity and biofilm forming ability. They also differed in cellular signaling mechanism controlling exopolysaccharide expression and biofilm structure as per prior description (Zhu et al., 2002; Watnick et al., 2001; Lauriano et al., 2004). We infer that the environmental clones of non-O1/non-O139 *Vibrio cholerae* those have demonstrated certain alternative behaviors than previous descriptions but have come out with potentially lethal virulence properties reflects further the significance of endemic reservoirs for future epidemics.

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Neuroprotective Effect of Hydro Alcoholic Extract of Annonasquamosa Linn Against 6- OHDA-Lesion Model of Parkinson's Disease in Male Sprague-Dawlely Rats: A Behavioral, Biochemical and Histological Study

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Abstract - Parkinson's disease (PD) is a chronic neurodegenerative disorder characterized by the progressive loss of dopaminergic neurons of substantianigra pars compacta in the ventral midbrain leads to the reduction of dopamine being released into the striatum. These processes are then responsible for the clinical features of PD including bradykinesia, resting tremor, rigidity, and difficulty in initiating movements.

Keywords : *Parkinson disease; 6-OHDA; Dopamine; Monoamine oxidase enzyme; Acetyl Cholinesterase; Annonasquamosa Linn.*

GJMR-C Classification : *NLMC Code: QU 34*

Strictly as per the compliance and regulations of:

Neuroprotective Effect of Hydro Alcoholic Extract of Annonasquamosa Linn Against 6-OHDA-Lesion Model of Parkinson's Disease in Male Sprague-Dawley Rats: A Behavioral, Biochemical and Histological Study

D.Sivaraman [α] , B.Kartika [σ] & Dr.P.Muralidharan [σ]

Abstract - **Parkinson's disease (PD) is a chronic neurodegenerative disorder characterized by the progressive loss of dopaminergic neurons of substantianigra pars compacta in the ventral midbrain leads to the reduction of dopamine being released into the striatum. These processes are then responsible for the clinical features of PD including bradykinesia, resting tremor, rigidity, and difficulty in initiating movements.**

a) Aim

Aim of the present study is to investigate the neuroprotective effect of hydro alcoholic extract of Annonasquamosa Linn (HAEA) in male Wistar Sprague-Dawley rats subjected to unilateral 6-OHDA lesion model.

b) Materials and Methods

6-OHDA is the most commonly employed agent for the induction of experimental Parkinsonism in rodents. The locomotion, muscular strength and behavioral response were significantly reduced in 6-OHDA lesioned group. Gait analysis and beam walk test were assessed to evaluate gait abnormalities, motor-coordination and postural balance. Pole test and catalepsy test was performed to evaluate the measure for bradykinesia. 6-OHDA injected rats were subjected to Rotarod, Open field spontaneous activity, Force swim test and Grid test to evaluate the motor coordination, locomotion, muscular strength, and catching reflex.

The results showed that HAEA significantly ameliorated high degree of bradykinesia and also showed improvement in gait and balance dose dependently.

HAEA significantly restored the motor deficits and behavioral changes caused by 6-OHDA. HAEA at both the dose level of 200mg/kg and 400mg/kg significantly ($p<0.01$) restores the dopamine level in the basal ganglia and antagonizing the excitatory effect of cholinergic neurons by increasing brain dopamine and Acetyl Cholinesterase (AchE) level. It was further observed that HAEA showed significant decreases in Monoamine oxidase enzyme (MAO) and Glutamate level when compare to 6-OHDA lesioned group. HAEA showed potent antioxidant activity by increasing the brain anti oxidant enzyme level, thus reestablishing the correct the balance between dopamine and acetylcholine and also to antagonize the damage due to oxidative stress.

c) Conclusion

Our results suggested that the HAEA have promising neuroprotective activity against 6-OHDA lesioned experimental Parkinsonism. Ameliorating effect of HAEA may be due to its potential antioxidant activity and might provide an opportunity to management neurological abnormalities in Parkinson's disease conditions.

Keywords : Parkinson disease; 6-OHDA; Dopamine; Monoamine oxidase enzyme; Acetyl Cholinesterase; Annonasquamosa Linn.

I. Introduction

The primary deficit in Parkinson's disease (PD) is a loss of the dopaminergic neurons in the substantianigra pars compacta which provides dopaminergic innervations to the striatum i.e. caudate and putamen. The present understanding of the pathophysiology of PD traced to neurochemical investigations which demonstrates that striatal dopamine content is reduced to 80%, which leads to the loss of neurons from substantianigra [1].

In Parkinson's disease, destruction of cells in the substantianigraresults in the degeneration of neurons responsible for secreting dopamine in the neostriatum. Thus the normal modulating inhibitory influence of dopamine on cholinergic neurons in the neostriatum is significantly diminished, resulting in overproduction or a relative overactivity of acetylcholine by the stimulatory neurons. This triggers a chain of

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abnormal signaling, resulting in loss of the control of muscle movements resulting in the Parkinsonism degeneration of the control of muscle movement [2].

The prevalence of Parkinson's disease in industrialized countries is estimated at 0.3% of the general population and about 1% of the population older than age 60 years [3,4] .People of all ethnic origins can be affected, and men are slightly more prone to the disorder [5,6].

In India about two thousand medicinal plants are found. Annonasquamosa Linn belonging to family Annonaceae, is a tree, cultivated throughout India [7]. Plant possesses antidiabetic [8] and anti-mycrobial property [9].The purpose of the study was to evaluate for the anti-parkinsonism activity of leaves of the plant. Anonasquamosa Linn leaves are found to have Cardiotonic and several alkaloids, quinoline, squamone and bullatacinone, terpene derivatives, flavanoids, polyphenols, dopamine, squamoline and a novel diazepine, squamolone as major active constituents and have Strong anti-oxidant properties.

Hence the present study was designed to study the neuroprotective effect of hydro alcoholic extract of Annonasquamosa Linn (HAEA) in male Wistar Sprague-Dawley rats subjected to unilateral 6-OHDA lesion model.

II. Materials and Methods

a) *Plant Material*

The leaves of Annonasquamosa Linn .were collected from Anna university campus in chennai in the month of August 2010 and identified by Dr.Sasikala Ethirajulu, Asst.Director, Pharmacognosy department, Siddha central research institute, Arumbakkam, chennai and a voucher specimen was deposited at C.L.Baid Metha College of Pharmacy for future reference.

b) *Preparation of Extract of HAEA.*

Fresh leaves were collected, shade dried and grinded to get a coarse powder with an electric blender and about 1000g of the powder was subjected to hot solvent extract using water and ethanol (4:10)in soxlet extractor at 40-50°C. The filtrate collected after extraction was evaporated to dryness at 45°C in hot air oven till liquid to semisolid mass was obtained. It was then stored in airtight containers in a refrigerator below 100 °C, the resulted extract yield was 17.61%, the appearance of extract was gummy resin in nature with dark brown color.

c) *Phytochemical screening*

Phytochemical screening of the HAEA extract was performed using the reagents and chemicals as follows.

- Alkaloids with Mayer's, Hager's and Dragendorffs reagent [10]
- Flavonoids with the use of sodium acetate, ferric chloride and amyl alcohol
- Phenolic compounds and tannins with lead acetate and gelatin
- Carbohydrate with Molish's, Fehling's and Benedict's reagent [11]
- Proteins and amino acids with Millon's, Biuret Xanthoprotein test
- Saponins test using the hemolysis method
- Sterols with 5% potassium hydroxide
- Steroids with Libermann Burchard's test [12]
- Saponins with foam test
- Terpenes with thionyl chloride
- Glycosides with ferric chloride, acetic acid and concentrated sulphuric acid
- Gum tested using Molish's reagent and Ruthenium red
- Coumarin by 10% sodium hydroxide and Quinones by concentrated sulphuric acid.

These were identified by characteristic color changes using standard procedures [13]

The screening results were as follows:

carbohydrate, flavonoid, flavones, tannins, protein, phenol and glycoside

Alkaloids +; Carbohydrates +; Proteins and amino acids +; Steroids -; Sterols -; Phenols +, Flavonoids +; Flavones+ Gums and mucilage +; Glycosides +; Saponins -; Terpenes -, and Tannins +ve. where + and - indicates the presence and absence of compounds.

d) *Acute toxicity study [14]*

This was performed for the extracts to ascertain safe dose by the acute oral toxic class method by the Organization of Economic Cooperation and Development (OECD). A single administration of starting dose of 2000 mg/kg body weight/po of the HAEA was administered to three female and male rats, and the rats were observed for three days to evaluate considerable changes in body weight and other signs of toxicity.

Repeating the experiment with the same dose level of HAEA for more seven days, we observed the body weight change and toxicity sign for totally fourteen days.

e) *Experimental Animals*

Colony inbreed strains of Male Sprague-Dawley rats of 250-300 g body weight were used for pharmacological studies, four different groups with 6 animals in each, were housed at controlled temperature (25±2°C) and light dark cycle (12/12 hr); on a standard rodent diet(Hindustan lever pvt ltd., banglore) and water ad libitum. Animals were handled carefully and acclimatized to laboratory conditions at least 1 week prior to experimentation. The experimental protocol was approved by Institutional animal ethics committee (IAEC) of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals).

IAEC Reference number: IAEC/XXX/02/CLBMCP/2010 dated 22.09.2010

f) Induction of Parkinson's disease in rodents [15].

Rats were weighed and anaesthetized with Xylaxine (5mg/kg) and Ketamine (100 mg/kg). Hair depletory was applied to head and scrubbed to remove hair. 1unit adrenaline was injected by IP route using insulin syringe to prevent the bleeding. Each rat was placed in a stereotaxic instrument (David Kopf Instruments, Tujunga CA, USA). A midsagital dorsal skull incision was made through the skin and the skin was retracted. The soft tissues overlaying on the skull were then removed. The landmark of the skull, bregma and lambda were identified and the skull was oriented such that both points were positioned at the same horizontal level. After cleaning the underlying fascia, a small hole was made using a driller with sharp end, by rotary movements of the needle through the skull over coordinates corresponding to the site of interest. The coordinates were estimated from the rat brain atlas (Franklin and Paxinosie AP – 5.0, ML + 1.5, DV – 8.0). ICV (Intra cerebro ventricular injections were given using micro syringe (Hamilton Company, Nevada, USA) connected by PE-10 polyethylene tubing to a 29 gauge internal cannula (Plastics One). Projected 8mm below the guide cannula.

The internal cannula was inserted into the guide cannula and a volume of 4 μl was delivered over a period of 4 min and the needle left in place for an additional 5 min before retraction (8 µg 6-hydroxydopamine in 4 µl saline containing 0.02% ascorbic acid in the left substantianigra). An air bubble was created into the polyethylene tube to avoid the mixing of drug solutions with disttiled water. The movements of air bubble inside the polyethylene tubing confirmed the drug administration. The internal cannula was held in position for another 1 min to prevent backflow and complete dispersal of the drugs or peptides.

After surgery, the animals were then allowed to recover for a week under antimicrobial cover of cefotaxime (50 mg/kg/day, s.c.) once a day for three days. After recovery from anesthesia, wound sites were inspected and cleaned on regular basis. Following surgical intervention animals were treated with extract for 1 week by Oral route. Rats with any neurological and motor deficits were excluded from the study. The animals were housed individually in home cages during recovery period. To avoid infection, Soframycin antibiotic ointment (Aventis Pharma Ltd., Pune) was applied on the wound. During this period, rats were habituated to the experimental protocol to minimize non-selective stress.

g) Experimental procedure

Male Sprague dawley rats of body weight 250-300g were randomized into 4 groups. Each group have 6 animals (n= 6 per group). The groups and treatment are designated as follows.

GROUP I : Animals (Control) treated with (0.9 % saline, p.o)
GROUP II : Animals (Negative control) with 6-OHDA (8 μg 6-hydroxydopamine)(I.C.V) and treated with saline (p.o)
GROUP III : Animals with 6-OHDA (I.C.V) lesion and treated with 200mg/kg of HAEA (p.o)
GROUP IV : Animals with 6-OHDA (I.C.V) lesion and treated with 400mg/kg of HAEA (p.o)

h) In-vivo behavior Examination

i. *Rota rod test [16].*

Rota rod unit consists of a rotating spindle (diameter 7.3cm)d individual compartments for each rat with varying rotational speeds. Initially animals were trained for four training sessions on consecutive days (each constituted by a maximum of 10 trails) to achieve the maximal performance. The animals were exposed on a rotating rod at 10r.p.m at 5 min intervals with the cutoff period of 180 seconds. Average retention time spent on rod was calculated.

ii. *Open field spontaneous activity [17].*

Animals were placed in the corner of an open field (33×33×30cm) chamber with floor grated into squares under weak light. Number of square crossed (in 5 mins) was counted manually in triplicates as forepaw criterion (horizontal activity). Number of observations including grooming and rearing (vertical activity) was also measured.

iii. *Pole test [18].*

Animal was placed head upward on the top of a vertical rough surfaced pole (diameter 1cm and height 55cm). Each rat was habituated to the apparatus on the day prior to testing, then allowed to descend five times. The total time taken by the animal to turn completely downward and climb down to the floor was recorded with the maximum duration of 120 seconds. Even if the rat descended part away and fell the rest of the way, the behavior was scored until it reached to the floor. When the rat was not able to turn downward and instead dropped from the pole, TLA was taken as 120 seconds because of maximal severity.

iv. *Catalepsy test [19].*

Catalepsy was measured by placing the animal on a flat horizontal surface with both hind limbs on square wooden block of 3cm in height and the latency in seconds required to move the limbs from the block to the ground was measured.

v. *Beam walking test [20].*

Motor coordination and balance were assessed by measuring the ability of the rat to transverse a narrow beam to reach dark goal box in beam walking test. The beams consisted of stationary wooden narrow flat beam (L 100cm × W1cm) placed at a height of 100cm from

the floor. The animals were trained in beam for ten trials with one minute interval. During experiment animals were monitored and rewarded with food pellets in the goal box. Time taken to transverse the beam from start box to goal box and number of stepping errors were measured.

vi. *Grid test [21].*

The grid apparatus consisted of a horizontal mesh (total size 12×2cm, openings 0.5×2cm) mounted 20cm above a hard surface, thus discouraging falling, but not leading to the injury in case of falling. The apparatus consists of a 3 inch wall that made up of any opaque sturdy material. Rat were lifted by their tail and slowly placed in the centre of the horizontal grid and supported until they grabbed the grid with both their fore and hind paws. The grid was then inverted so that the rat was hanging upside down for 30seconds and the maximum hanging time was measured.

vii. *Gait analysis [22].*

To measure the gait animals were trained to walk through a narrow alley leading into their home cage. Once trained paper was placed along the alley floor and each animal fore limbs and hind limbs were brushed with non toxic paint. Animals were kept at the beginning of the alley. As they walked through that alley into their home cage, they left their paw prints on the paper. By measuring the distance between paw prints stride length was determined.

viii. *Force Swim-test [23].*

Force swim test was carried out in water tubs (40cm length×25cmwidth×16cm height). Water was kept at the depth of 12cm and the temperature was maintained at 27±2 ◦C. The animals were wiped dry immediately after the experiment using a dry towel and returned to cages kept at 27±2 ◦C.

i) Estimation of Neurotransmitters and Metabolic Enzymes

i. *Acetylcholine esterase Estimation [24].*
ii. *Estimation of Monoamine Oxidase B [25].*
iii. *Estimation of Dopamine [26].*
iv. *Estimation o f Glutamate [27].*
v. *Estimation of Total Proteins*

Total protein was estimated in brain using the method described by (Lowry et al., [28].

j) Estimation of Anti Oxidant Enzymes

i. Assay of Superoxide dismutase (SOD) [29].
ii. *Estimation of Catalase (CAT) [30].*
iii. *Estimation of Lipid Peroxidation [31].*
iv. *Estimation of Glutathione Peroxidase (GPx) [32].*
v. *Estimation of Glutathione Reductase (GR) [33].*
vi. *Estimation of ascorbic acid [34].*
vii. *Methods for Histopathological sectioning staining*

The rats from each group were anesthesized by intra peritoneal injection of Ketamine and xylazine. The brain was carefully removed without any injury after opening the skull. The collected saline was washed with ice cold normal saline and fixed in 10% formal saline (10 ml of formaldehyde in 90 ml of physiological saline). Paraffin embedded sections were taken 100 μm thickness and processed in alcohol –xylene series and stained with Haematoxyli- Eosin dye. The sections were examined microscopically for histopathological changes.

k) Statistical Analysis

The statistical analysis was carried by one way ANOVA followed by Dunnet's "t" test. (P values < 0.001) was considered statistically significant, it was calculated using graph pad prism 5.

III. Results

a) Effect of HAEA on Rotarod test (Motor co-ordination)

The motor co-ordination in the control group was significantly ($p<0.001$) high when compared with the 6-OHDA treated animals. Which was found by noting the time taken by the animals to stay on the rotating rod? One-way ANOVA indicated that the decrease in motor coordination was improved with HAEA. Treatment at dose level of 200mg/kg and 400mg/kg showed the significant ($p<0.001$) increase in the motor co-ordination when compared with 6-OHDA injected group. Results are shown in Table 1.

b) Effect of HAEA on Open field spontaneous activity (Locomotor)

The significant ($p<0.001$) decrease in the number of square crossed, grooming and rearing (Locomotor) in the 6-OHDA injected animals were observed. With the treatment of 200mg/kg of HAEA, animals showed the significant ($p<0.01$) increase in the no. of sq crossed and grooming ($p<0.001$), but less significant ($p<0.05$) increase in rearing. With the treatment group 400mg/kg dose of HAEA, animals showed significant ($p<0.01$) increase no. of square crossed, grooming and rearing when compared with 6-OHDA treated animals. Results are shown in Table 2.

c) Effect of HAEA on Pole test (Bradykinesia)

The significant ($p<0.001$) increase in the time taken by the animal to turn downward and time taken to reach floor was observed in 6-OHDA treated animals. With the treatment of 200mg/kg and 400mg/kg dose of HAEA, animals showed the significant ($p<0.01$ and $p<0.001$) decreased time to turn downward and also showed less significant ($p<0.05$) decrease in time taken to reach the floor. Whereas, on treatment with 400mg/kg dose of HAEA, animals showed significant ($p<0.001$) decreased time to reach the floor when compared with 6-OHDA treated group. Results are shown in Table-8 and Histogram 3:- 3.1 and 3.2

d) Effect of HAEA on Catalepsy

In the control group catalepsy was not observed. When 6-OHDA injected group compared with control group, it showed a significant ($p<0.001$) rise in cataleptic rigor. Group treated with 200mg/kg and 400mg/kg dose of the HAEA showed significant ($p<0.01$ and $p<0.001$) decrease in cataleptic rigor when compared with 6-OHDA treated group. Results are shown in Table 4.

e) Effect of HAEA on Beam walking (Motor co-ordination and Balance)

There is a significant ($p<0.001$) increase in the number of foot errors and time taken to travel was observed in 6-OHDA treated animals. With the treatment of 200mg/kg and 400mg/kg dose of HAEA, animals showed the significant ($p<0.01$ and $p<0.001$) decrease in number of foot errors and time taken to travel when compared with the 6-OHDA treated group. Results are shown in Table 5.

f) Effect of HAEA on Grid test (Muscular strength and catching reflex)

6-OHDA injected group compared with control group, showed a significant ($p<0.001$) decrease in hanging time. Group treated with 200mg/kg and 400mg/kg dose of HAEA showed significant ($p<0.01$ and $p<0.001$) improvement in hanging time when compared with 6-OHDA treated group. Results are shown in Table 6.

g) Effect of HAEA on Gait

The stride length and fore paw stance width in 6-OHDA treated animals was significantly ($p<0.001$) decreased when compared with the control group while hind paw stance width in 6-OHDA treated animals was significantly ($p<0.001$) increased when compared with the control group. With the treatment of 200mg/kg and 400mg/kg dose of HAEA, animals showed significant ($p<0.01$ and $p<0.001$) increase in stride length and fore paw stance width and significant ($p<0.05$ and $p<0.01$) decrease in hind paw stance width. When compared with the 6-OHDA treated group. Results are shown in Table 7.

h) Effect of HAEA on Forced swim test (motor impairment)

6-OHDA injected group on comparison with control group, showed a significant ($p<0.001$) decrease in swim time. Group treated with 200mg/kg and 400mg/kg dose of HAEA showed significant ($p<0.001$) increase in swim time when compared with 6-OHDA treated group. Results are shown in Table 8.

i) Effect of HAEA on Acetylcholine esterase activity

The Acetylcholine esterase activity was found to be significantly ($p<0.001$) decreased in 6-OHDA treated animals when compared with control group. Treatment with HAEA 200mg/kg and 400mg/kg significantly ($p<0.01$ and $P<0.01$) increased activity of acetylcholine esterase whem compared to 6-OHDA treated group. Results are shown in Table 9.

j) Effect of HAEA on Monoamino oxidase B

The monoamino oxidase B levels in 6-OHDA treated group was found to be significantly ($p<0.01$) high than the control group. HAEA 200mg/kg and 400mg/kg treatment significantly ($p<0.01$) decreased the Monoamino oxidase-B in 6-OHDA lesion animals. Results are shown in Table 10.

k) Effect of HAEA on Dopamine

When compared to the control group, 6-OHDA treated group showed significant ($p<0.001$) decrease in the dopamine level in striatum. 200mg/kg and 400mg/kg administration showed significant ($p<0.01$ and $p<0.001$) increase in dopamine content when compared to 6-OHDA lesion animals. Results are shown in Table 11.

l) Effect of HAEA on Glutamate

The Glutamate content in 6-OHDA injected group was significantly ($p<0.001$) higher than control group. On treatment with 200mg/kg and 400mg/kg showed significant ($p<0.05$ and $p<0.001$) decrease in glutamate level. Results are shown in Table 12.

m) Effect of HAEA on Total protein

Group treated with 6-OHDA had shown significant ($P < 0.001$) lower brain total protein level when compared with control group animals Brain total protein level of those animals treated with HAEA 200mg/kg and 400mg/kg significantly ($P<0.01$ and $p<0.001$) increased when compared with the 6-OHDA lesion animals. Results are shown in Table 13.

n) Effect of HAEA on Superoxide Dismutase (SOD) Activity

A significant ($p<0.001$) decrease in the brain tissue Superoxide dismutase (SOD) was observed in 6-OHDA lesion animals when compared to control animals. Treatment with HAEA at doses of 200mg/kg and 400mg/kg showed significant ($p<0.05$ and $p<0.01$) increase in 6-OHDA lesion animals. Results are shown in Table 14.

o) Effect of HAEA on Catalase (CAT) Activity

A significant ($p<0.001$) decrease in the brain CAT was observed in 6-OHDA lesion animals when compared to control animals. Treatment with HAEA at doses of 200mg and 400mg/kg showed significant ($p<0.01$) when compared to 6-OHDA induced 6-OHDA lesion animals Results are shown in Table 15.

p) Effect of HAEA on Lipid Peroxidation (LPO)

The content of Lipid peroxidation in the brain tissue was significantly increased ($P<0.001$) in 6-OHDA injected animals when compared with control group. Treatment with HAEA at doses of 200mg/kg and 400mg/kg p.o showed significant ($p<0.01$and $p<0.001$)

decrease in LPO in 6-OHDA lesion animals. Results are shown in Table 16.

q) Effect of HAEA on Glutathione Peroxidase (GPx)

The activity of Glutathione Peroxidase (GPx) in brain was significantly decreased ($p<0.001$) in 6-OHDA administered animals when compared with control group. HAEA at200mg/kg and 400mg/kg doses showed significant ($p<0.01$ and $p<0.001$) increase when compared to 6-OHDA lesion animals. Results are shown in Table 17.

r) Effect of HAEA on Glutathione reductase (GR)

The activity of Glutathione reductase (GR) in brain was significantly decreased ($p<0.001$) in 6-OHDA administered animals as compared to control group. HAEA at 200mg/kg and 400mg/kg doses showed significant ($p<0.01$ and $p<0.001$) increase in GR when compared with 6-OHDA lesion animals. Results are shown in Table 18.

s) Effect of HAEA on ascorbic acid (vitamin c)

When compared with control animals the amount of ascorbic acid was significantly ($P<0.001$) reduced for the 6-OHDA lesion groups. HAEA at doses of 200mg/kg and 400mg/kg showed significant ($p<0.01$ and $P<0.001$) increase in ascorbic acid amount when compared with 6-OHDA lesion animals. Results are shown in Table 19.

t) Effect of HAEA on Neuronal Degeneration

There was a increase in neuronal degeneration and decrease in the number of neuronal cells in brain striatal region in 6-OHDA lesion group (negative control) group when compared with the control group .The group treated with 200mg/kg and 400 mg/kg HAEA showed the significant decrease in brain cell edema and improves the neuronal configuration when compared with negative control group as shown in Fig no: 1.

Table 1 : Effect of HAEA on Rotarod test.

Groups	Control I	Negative Control (6-OHDA) II	HAEA(200mg/kg) + 6-OHDA III	HAEAS(200mg/kg) + 6-OHDA IV
Retention time (Sec)	108.8 ± 2.839	$33.28 \pm 1.797^{a***}$	$61.00 \pm 2.739^{b***}$	$84.25 \pm 2.689^{b***}$

Comparisons were made between aControl Vs Negative control; bNegative control Vs Treatment groups
Values are expressed as mean ± SEM of 6 animals.
Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

Table 2 : Effect of HAEA on Open field spontaneous activity.

Groups	Control I	Negative Control (6-OHDA) II	HAEAS(200mg/kg) + 6-OHDA III	HAEAS(200mg/kg) + 6-OHDA IV
No. of Sq. crossed	22.44 ± 1.078	$8.770 \pm 0.253^{a***}$	$12.47 \pm 0.3373^{b**}$	$16.62 \pm 0.4845^{b***}$
Rearing	9.650 ± 0.432	$4.963 \pm 0.413^{a***}$	$7.268 \pm 0.176^{b*}$	$13.621 \pm 1.006^{b***}$
Grooming	6.853 ± 0.434	$2.728 \pm 0.229^{a***}$	$5.138 \pm 0.237^{b***}$	$5.203 \pm 0.137^{b***}$

Comparisons were made between aControl Vs Negative control; bNegative control Vs Treatment groups
Values are expressed as mean ± SEM of 6 animals.
Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

Table 3 : Effect of HAEA on Pole Test.

Groups	Control I	Negative Control (6-OHDA) II	HAEAS(200mg/kg) + 6-OHDA III	HAEAS(200mg/kg) + 6-OHDA IV
Time taken to turn downward	4.655 ± 0.206	9.548 ± 0.240^{a}***	8.553 ± 0.166^{b}**	8.343 ± 0.148^{b}***
Time taken to reach the floor	3.513±0.192	11.46 ± 0.354^{a}***	9.853 ± 0.357^{b}*	7.463 ± 0.463^{b}***

Comparisons were made between aControl Vs Negative control; bNegative control Vs Treatment groups
Values are expressed as mean ± SEM of 6 animals.
Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

Table 4 : Effect of HAEA on Catalepsy.

Groups	Control I	Negative Control (6-OHDA) II	HAEAS(200mg/kg) + 6-OHDA III	HAEAS(200mg/kg) + 6-OHDA IV
Latency period (Sec)	0.0 ± 0.0	9.163 ± 0.561^{a}***	7.493 ± 0.214^{b}**	6.490 ± 0.209^{b}***

Comparisons were made between aControl Vs Negative control; bNegative control Vs Treatment groups
Values are expressed as mean ± SEM of 6 animals.
Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

Table 5 : Effect of HAEA on Beam walking.

Groups	Control I	Negative Control (6-OHDA) II	HAEAS(200mg/kg) + 6-OHDA III	HAEAS(200mg/kg) + 6-OHDA IV
Time taken to travel (Sec)	10.25 ± 0.812	25.65 ± 0.484^{a}***	21.47 ± 0.914^{b}**	12.25 ± 0.952^{b}***
No. of foot errors	0.0 0.0	6.688 ± 0.186^{a}***	5.228 ± 0.337^{b}**	3.863 ± 0.252^{b}***

Comparisons were made between aControl Vs Negative control; bNegative control Vs Treatment groups
Values are expressed as mean ± SEM of 6 animals.
Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

Table 6 : Effect of HAEA on Grid test.

Groups	Control I	Negative Control (6-OHDA) II	HAEAS(200mg/kg) + 6-OHDA III	HAEAS(200mg/kg) + 6-OHDA IV
Hanging time(Sec)	2.61 ± 0.331	$6.713 \pm 0.179^{a***}$	$9.510 \pm 0.374^{b**}$	$14.26 \pm 0.723^{b***}$

Comparisons were made between aControl Vs Negative control; bNegative control Vs Treatment groups
Values are expressed as mean ± SEM of 6 animals.
Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

Table 7 : Effect of HAEA on Gait.

Groups	Control I	Negative Control (6-OHDA) II	HAEAS(200mg/kg) + 6-OHDA III	HAEAS(200mg/kg) + 6-OHDA IV
Stride length(cm)	6.290± 0.267	$1.490 \pm 0.113^{a***}$	$3.00 \pm 6.296^{b**}$	$5.510 \pm 0.196^{b***}$
Forepaw stance width (cm)	1.990 ±0.053	$1.490 \pm 0.113^{a**}$	$1.918 \pm 0.050^{b**}$	$1.938 \pm 0.051^{b**}$
Hindpaw stance width (cm)	2.863 ±0.045	$3.343 \pm 0.092^{a***}$	$3.088 \pm 0.004^{b*}$	$3.03 \pm 0.011^{b**}$

Comparisons were made between aControl Vs Negative control; bNegative control Vs Treatment groups
Values are expressed as mean ± SEM of 6 animals.
Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

Table 8 : Effect of HAEA on Forced Swim Test.

Groups	Control I	Negative Control (6-OHDA) II	HAEAS(200mg/kg) + 6-OHDA III	HAEAS(200mg/kg) + 6-OHDA IV
Time (Sec)	84.17 ±1.138	$62.33 \pm 0.494^{a***}$	$66.50 \pm 0.428^{b***}$	$70.00 \pm 0.365^{b***}$

Comparisons were made between aControl Vs Negative control; bNegative control Vs Treatment groups
Values are expressed as mean ± SEM of 6 animals.
Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

Table 9 : Effect of HAEA on Acetylcholine esterase activity.

Groups	Control I	Negative Control (6-OHDA) II	HAEAS(200mg/kg) + 6-OHDA III	HAEAS(200mg/kg + 6-OHDA IV
nmoles acetyl thiocholinehydrolysed/min/ mg protein	2813 ±122.6	$1698 \pm 49.90^{a***}$	$2293 \pm 955.8^{b**}$	$2415 \pm 72.40^{b**}$

Comparisons were made between aControl Vs Negative control; bNegative control Vs Treatment groups
Values are expressed as mean ± SEM of 6 animals.
Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

Table 10 : Effect of HAEA on Monoamino Oxidase- B.

Groups	Control I	Negative Control (6-OHDA) II	HAEAS(200mg/kg) + 6-OHDA III	HAEAS(200mg/kg) + 6-OHDA IV
nmoles/min/ mg	5845 ± 245.5	7238 ± 119.4[a]**	5995 ± 288.7[b]**	5845 ± 277.2[b]**

Comparisons were made between aControl Vs Negative control; bNegative control Vs Treatment groups
Values are expressed as mean ± SEM of 6 animals.
Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

Table 11 : Effect of HAEA on Dopamine.

Groups	Control I	Negative Control (6-OHDA) II	HAEAS(200mg/kg) + 6-OHDA III	HAEAS(200mg/kg) + 6-OHDA IV
dopamine (ng/mg tissue)	664.3 ± 9.772	391.2 ± 15.30[a]***	441.9 ± 18.27[b]**	570.3 ± 14.47[b]***

Comparisons were made between aControl Vs Negative control; bNegative control Vs Treatment groups
Values are expressed as mean ± SEM of 6 animals.
Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

Table 12 : Effect of HAEA on Glutamate.

Groups	Control I	Negative Control (6-OHDA) II	HAEAS(200mg/kg) + 6-OHDA III	HAEAS(200mg/kg) + 6-OHDA IV
nmoles/gm	72588 ±394.8	87605 ± 301.5[a]***	83510 ± 1535[b]*	82713 ± 304.2[b]**

Comparisons were made between aControl Vs Negative control; bNegative control Vs Treatment groups
Values are expressed as mean ± SEM of 6 animals.
Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

Table 13 : Effect of HAEA on Total Protein.

Groups	Control I	Negative Control (6-OHDA) II	HAEAS(200mg/kg) + 6-OHDA III	HAEAS(200mg/kg) + 6-OHDA IV
mg/gm of tissue	1.868 ± 0.109	0.8775 ±0.0442[a]***	1.463 ± 0.743[b]**	1.543 ±0.106[b]***

Comparisons were made between aControl Vs Negative control; bNegative control Vs Treatment groups
Values are expressed as mean ± SEM of 6 animals.
Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

Table 14 : Effect of HAEA on Superoxide Dismutase (SOD) Level.

Groups	Control I	Negative Control (6-OHDA) II	HAEAS(200mg/kg) + 6-OHDA III	HAEAS(200mg/kg) + 6-OHDA IV
unit/mg protein	10.81± 0.5070	6.668± 0.2244[a]***	7.890± 0.09656[b]*	8.740± 0.2074[b]**

Comparisons were made between aControl Vs Negative control; bNegative control Vs Treatment groups
Values are expressed as mean ± SEM of 6 animals.
Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

Table 15 : Effect of HAEA on Catalase (CAT) Level.

Groups	Control I	Negative Control (6-OHDA) II	HAEAS(200mg/kg) + 6-OHDA III	HAEAS(200mg/kg) + 6-OHDA IV
n moles/H2O2/ deconsumed /min/mg protein	2813±122.6	1698±49.90[a]**	2293±155.8[b]**	2415±72.40[b]**

Comparisons were made between aControl Vs Negative control; bNegative control Vs Treatment groups
Values are expressed as mean ± SEM of 6 animals.
Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

Table 16 : Effect of HAEA on Lipid peroxidation (LPO).

Groups	Control I	Negative Control (6-OHDA) II	HAEAS(200mg/kg) + 6-OHDA III	HAEAS(200mg/kg) + 6-OHDA IV
nmoles of TABRS/ mg protein	2.343± 0.07192	3.935± 0.1028[a]***	3.310± 0.1646[b]**	2.915±0.7735[b]***

Comparisons were made between aControl Vs Negative control; bNegative control Vs Treatment groups
Values are expressed as mean ± SEM of 6 animals.
Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

Table 17 : Effect of HAEA on Glutathione Peroxidase (GPx).

Groups	Control I	Negative Control (6-OHDA) II	HAEAS(200mg/kg) + 6-OHDA III	HAEAS(200mg/kg) + 6-OHDA IV
units/min/mg protein	32.84±0.3824	22.12±0.6462[a]**	25.16±0.6525[b]**	29.34±0.3424[b]***

Comparisons were made between aControl Vs Negative control; bNegative control Vs Treatment groups
Values are expressed as mean ± SEM of 6 animals.
Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

Table 18 : Effect of HAEA on Glutathione reductase.

Groups	Control I	Negative Control (6-OHDA) II	HAEAS(200mg/kg) + 6-OHDA III	HAEAS(200mg/kg) + 6-OHDA IV
nmoles of NADPH oxidised	33.12±0.4908	24.49±0.3512[a]***	27.24±0.4923[b]**	28.98±0.6632[b]***

Comparisons were made between aControl Vs Negative control; bNegative control Vs Treatment groups
Values are expressed as mean ± SEM of 6 animals.
Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

Table 19 : Effect of HAEA on Ascorbic acid.

Groups	Control I	Negative Control (6-OHDA) II	HAEAS(200mg/kg) + 6-OHDA III	HAEAS(200mg/kg) + 6-OHDA IV
ng/mg protein	890.8±10.14	450.8±10,53[a]**	534.5±26.99[b]*	661.0±23.82 [b] ***

Comparisons were made between aControl Vs Negative control; bNegative control Vs Treatment groups
Values are expressed as mean ± SEM of 6 animals.
Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

Histopathology of Nigro striatal region in rat brain

Figure 1a : Group - I

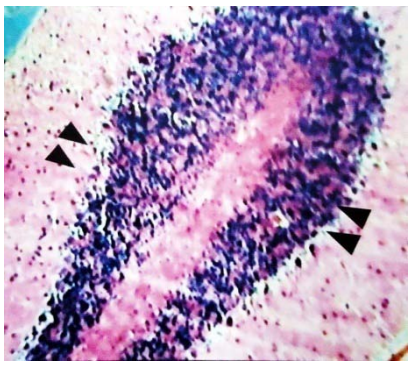


Figure 1b : Group - II

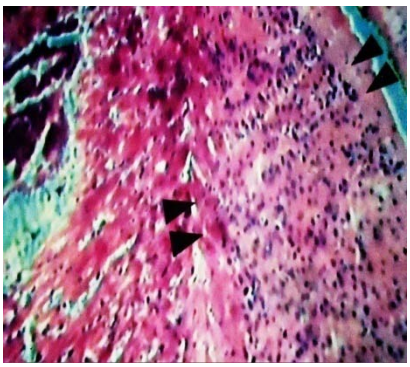


Figure 1c : Group - III

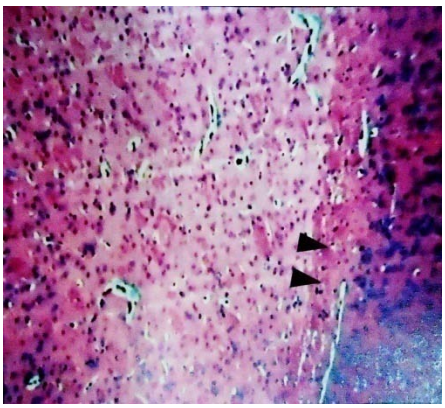
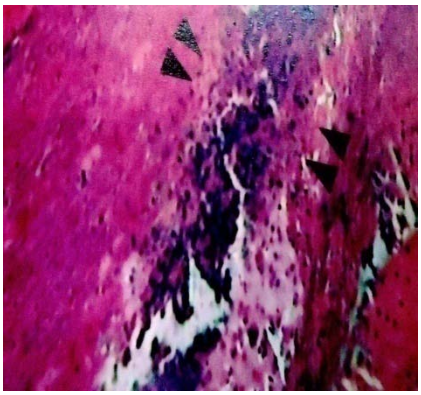


Figure 1d : Group - IV



IV. Discussion

From long time plants have been used as source of drugs for the treatment of Parkinsonism in developed as well as developing countries. Many species have been reported to have anti-parkinsonism effect. Working on the same line, we have undertaken a study on Annonasquamosa for its Anti-parkinsonism property along with its anti oxidant potential.

Acute oral toxicity studies of HAEAS were performed by using OECD 423 guidelines. Studies did not exhibit any lethality or any profound toxic reactions at a dose of 2000mg/kg/p.o. According to the (OECD) 423 guidelines for acute oral toxicity study LD50 dose of 2000mg/kg/p.o of HAEA was found to be safe.

6-OHDA is the most commonly employed agent for the induction of experimental Parkinsonism animal models. It is a selective catecholaminergic neurotoxin as it is thought that it is taken up by the dopamine transporter (DAT), that selectively destroys catecholaminergic neurons and it is typically injected unilaterally, since bilateral injections cause high mortality Furthermore, intra cerebral injection of 6-OHDA into the rat nigrostriatal pathway has been shown to permanently degenerate virtually all dopaminergic neurons in the substantia nigra pars compacta leading to stable motor deficits over time [35]. It is reported that the underlying mechanism of 6-OHDA on dopaminergic neurons is believed to be through a number of mechanism that includes its uptake through a dopamine transporter and leading to mitochondrial dysfunction by inhibiting complexes I and IV of the mitochondrial respiratory chain and increases free radicals formation. As a final effector of Dopamine neural cell death. It seems to induce a caspase 3-dependent apoptotic mechanism [36].6-OHDA is also attributed to the formation of various oxidants and impairs antioxidant enzyme levels which leads to the increase in oxidative stress by the production of ROS 6-OHDA can auto-oxidize to semiquinone and superoxide radical which is considered to be key pathogenesis of Parkinsonism [37]. Currently many evidences suggest the role of antioxidants as a neuroprotective compound by preventing 6-OHDA -induced dopamine depletion in rat.

The loss of dopaminergic neurons due to 6-OHDA administration leads to behavioral changes which are associated with an onset of motor deficits such as Bradykinesia, muscular rigidity, resting tremor and gait

abnormalities with poor postural balance are characterized symptoms of PD [38]. Our study found that when 6-OHDA injected rats were subjected to Rotarod, Open field spontaneous activity, Force swim test and Grid test they reveals poor motor coordination, locomotion, muscular strength, and catching reflex. In 6-OHDA lesion animal's motor impairment was prevented and the latency period was increased by HAEAS. The locomotion, muscular strength and behavioral response were significantly reduced in 6-OHDA lesion group where HEA significantly restored the motor deficits dose dependently. Gait analysis and Beam walk test were assessed to evaluate gait abnormalities, motor-coordination and postural balance. Nigrostraital damage gives the measure for gait abnormalities and also balance. This study revealed that 6-OHDA lesion group had poor gait and balance, where the treatment with HAEAS significantly improved gait and balance dose dependently. Pole test and catalepsy test were assessed to evaluate the measure for bradykinesia.Our results showed a high degree of bradykinesia in 6-OHDA lesion group and treatment with HAEA significantly ameliorated high degree of bradykinesia.

Dopamine, a neurotransmitter plays a key role in parkinson's disease. Degeneration of dopaminergic neurons from substantianigra results in the progressive loss of neostriatal nerve endings which are associated with the body movements and motor control [39]. Our study results showed a marked decrease in the dopamine level and neuronal cell death in brain of 6-OHDA lesioned group, when treated with HAEA it showed significant restoration of dopamine level and also prevention of neuronal cell death in brain.

In neostriatum, dopamine makes the synapses with cholinergic neurons through its inhibitory influence on its activity. In PD the loss of dopaminergic neurons leads to its diminished inhibitory activity on cholinergic neurons and overproduction of acetylcholine. Acetylcholine is generally degraded within the synapse by acetylcholinesterase (AChE).For decades AChE has been recognized for decades as a marker for cholinergic pathways in brain located on both pre and postsynaptic membranes. Overall AChE activities are also affected in the brains of patients with PD [40].Our study results showed that the 6-OHDA induced group showed the decrease in AChE activity and when treated with HAEAS it showed significant amelioration.

Monoamine oxidases (MAO) are belonging to the class of enzymes involved in the oxidative deamination of biogenic amines such as the neurotransmitters dopamine, norepinephrine and serotonin. The MAO- B form is predominant in the brain. In pathogenesis of PD MAO-B is well known for its property to generate free radicals. Increased expression of MAO-B leads to the deficiency of the neurotransmitter dopamine. MAO-B concentration and activity have been reported to be increased in several regions of the central nervous system in association with Parkinson's disease [41]. In the present study, it was observed that there was an increase in activity of MAO in 6-OHDA lesioned group and when it was treated with HAEAS, significant inhibition of MAO-B enzyme activity in brain. It may be due to alkaloids and flavonoids constituents in present in HAEAS.As it was reported that MAO-B can be inhibited by some plant-derived alkaloids, anthraquinones, flavanoids and phenols [42].

Glutamate is an excitatory amino acids are the primary neurotransmitters that mediate synaptic excitation which plays a dual role as neurotransmitter and in excess it plays as neurotoxic in CNS. It has been reported as its role in the pathogenesis of the neuronal degeneration in PD ativation of NMDA receptor gated ion channels by excess glutamate leads to Ca2+ influx and facilitates the formation of ROS such as Nitric oxide [43]. In the present study, it was observed that there was an increased level of glutamate in 6-OHDA lesion group and when it was pretreated with HAEAS, significant reduction in glutamate level in striatum.

Oxidative stress is nothing but increased oxidation due to increased amount of potential oxidants and decreases in the antioxidant levels. Small amounts of the neurotoxin are formed from the metabolism of dopamine itself by auto oxidation or enzymatic catabolism via MAO deamination leads to production of HVA and DOPAC, hydrogen peroxide can be converted to highly toxic hydroxyl radicals via iron-mediated Fenton reactions causes' production of ROS, higher levels of free radical production and free radical damage. It was reported that changes in oxidative damage or in antioxidant status in nervous tissue are found in the pathogenesis of Parkinson's disease [44].

Certain studies points to oxidative stress as a component of the pathological mechanisms accompanying nigral cell death and increased lipid peroxidation (LPO), impaired glutathione metabolism, and enhanced superoxide activity in PD.6-OHDA in rat brain generate ROS as it can auto-oxidize to semiquinone and superoxide radical (the most common free radical in the body) and leads to the neurotoxicity. Superoxide Dismutase (SOD) is an enzyme that repairs cells and reduces the damage done to them by superoxide [45].In the present study, it was observed that there was a decreased activity of SOD in 6-OHDA lesion group and when it was treated with HAEAS, significant restoration of SOD level in striatum. The detoxification of free radicals prevents cell damage which generally prevents the progress of LPO, TBARS is a reliable marker of LPO. In our study we found the elevated level of LPO in 6-OHDA lesion group, and significant reduction in LPO on pre-treatment with HAEAS was observed. The oxidative stress also causes damage to proteins, which leads to impaired protein

synthesis [46] when compared to 6-OHDA lesion group, pre-treatment with HAEA restored the protein level in brain.

Glutathione is a tripeptide, which plays a major defense system against oxidative stress in the brain. Glutathione peroxidase (GPx) is an enzyme which plays an important role in removing excess of free radicals and hydroperoxidases and Glutathione reductase (GR) is an enzyme which provides pool for GSH that protects the membrane from toxification. Dysfunction of this system leads to the oxidative damage in PD [47]. We found the significant decrease in the GPx and GR levels in 6-OHDA lesion group and found the significant restoration on pretreatment with HAEA.

The catalase (CAT), which was found at a very low level of activity in the brain, detoxifies hydrogen peroxide to water and prevents ROS. Our studies found the significant increase in CAT levels in 6-OHDA lesion group and found the significant restoration on pretreatment with HAEA.

Due to the oxidative damage by ROS generation in neurons and tissues of brain, level of Ascorbic acid level get reduced in neurons and tissues in brain [48]. Our results found to have a significant increase in the activity of ascorbic acid which was restored on pretreatment with HAEAS when compared to 6_OHDA lesion group.

Our histopathological study revealed striatal neuronal damage and decrease in neuronal cell number in 6-OHDA lesion group, but on pretreatment with HAEA has significantly protected striatal neuronal damage against 6-OHDA toxicity and showed increase in neuronal cell number.

V. Conclusion

In conclusion the results obtained from the present study, indicates that HAEAS protective effect may be due to its activity against free radicals by its antioxidant effect, inhibiting activity of MAO-B and elevation in the dopamine level in the brain. Further studies are required to establish the anti-Parkinsonism activity of Annonasquamosa in terms of molecular mechanism(s) involved in the activity.

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Effects of Tai Chi on the Metabolism of Breath and Energy of the Senior Citizen

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Abstract - The purpose of this article is to find out the different of index of metabolism of breath and energy between the senior citizen who has often trained 24 style Tai Chi for a long time and the new learner when they just finished 24 style Tai Chi, and the results tell us that the index of testing of the senior citizen who has often trained 24 style Tai Chi is much better than the new learner, so the results indicate Tai Chi can improve the function of metabolism of breath and energy of the senior citizen which as a science proof of the senior citizen who trains the Tai Chi.

Keywords : *Tai Chi; energy metabolism ; breath frequency.*

GJMR Classification : *NLMC Code: QU 120, QU 125, WR 102*

Strictly as per the compliance and regulations of:

Effects of Tai Chi on the Metabolism of Breath and Energy of the Senior Citizen

Liu Zongxiang [α] & Wen Jiao [σ]

Abstract - The purpose of this article is to find out the different of index of metabolism of breath and energy between the senior citizen who has often trained 24 style Tai Chi for a long time and the new learner when they just finished 24 style Tai Chi, and the results tell us that the index of testing of the senior citizen who has often trained 24 style Tai Chi is much better than the new learner, so the results indicate Tai Chi can improve the function of metabolism of breath and energy of the senior citizen which as a science proof of the senior citizen who trains the Tai Chi.

Keywords : Tai Chi; energy metabolism ; breath frequency.

I. Introduction

Tai Chi is a main major division of Chinese martial art, which has the characteristics of slow, soft and ethereal. The movement required to be such as spinning, to go as a cat, round-trip must be folded, advance and retreat will transform into each other, and there are rules of dynamic and quiescent, equality and connect style by style. Styles should be round, no bump, quiet, no distracting thoughts, not intended to push and so on. Tai Chi can improve the ability of, breathe and sport systems, also has some medical prevention and treatment of insomnia and neurasthenia, improving anxiety and tension. [1] The senior citizen belongs to the special community, whose cardiovascular system is very fragile, and the abilities of breath and sport are begin to decline. This article compares the data of senior citizen who training Tai Chi for a long time and the beginners, which can provide a scientific basis for Tai Chi exercise science.

II. Object and Method

a) Object

Ten Tai Chi trainer from Tai Chi Association of Inner Mongolia Autonomous Region as the experimental group, whose Tai Chi average training time is 8 years. Ten Tai Chi fancier as the control group, whose Tai Chi average training time is less than 3 months.

Table 1 : Basic Information of Research Object.

groups	age/year	height/cm	weight/kg	training time/year
The experimental group	69.56 ± 7.85	171±6.8	67.4 12.4±	8.5±2.7
the control group	72.16 ± 8.12	166±4.6	67.2±6.8	

b) Methods

i. *Literature summary method*

Refer to reports, journal and outstanding master's and doctor degree paper as the material of this article.

ii. *Interview method*

After interviewing coaches of Tai Chi and exports of sports Physiology, we take the advices and instructions as the base of this article.

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iii. *Experimentation*

We used Germany CORTEX-3B sports heart-lung function testing machine to remote test the gas metabolism of our subjects during Tai Chi, input the data to computer, dealing with SPSS software.

Experimentation process :

1. Subjects are tested in laboratory where the temperature is 23℃, obtaining data at the same time. We divide 24 style Tai Chi into four phases based on the tips of Tai Chi: Phase 1: Commencing form~Play Pipa; Phase 2: Repulse Monkey~Single Whip; Phase 3: Wave Hands Like Clouds~Strike Opponent's Ears with Both Fists; Phase 4: Turn and Kick with Left Heel~Closing Form. The whole Tai Chi takes 5min45s. Testing index: quiet state before exercise; exercise; after exercise. [2]
2. Heart-lung function test: 3min before exercise is the quiet state; process of playing Tai Chi is the experiment state; 5min after playing Tai Chi is the recovery state. The annals start from the quiet state to the recovery state.

III. Results and Analysis

a) Analysis of the changes of energy metabolism of senior citizen during Tai Chi exercise.

Table 2 : changes of energy metabolism of quiet state, in exercise, and after exercise.

state	The experimental group	The control group
quiet state	1.25±0.18	1.09±0.33
Phase 1	3.26±1.52	2.02±1.28
Phase 2	3.78±1.57*	3.10±2.44
Phase 3	3.71±2.55*	3.43±2.01
Phase 4	1.79±0.40	1.20±0.49
1min after exercise	1.57±0.31	1.08±0.39
2min after exercise	1.37±0.20	1.17±0.27
3min after exercise	1.42±0.38	1.13±0.37
4min after exercise	1.37±0.37	1.15±0.38
5min after exercise	1.47±0.22	1.12±0.38

(marked with * when P<0.05 compared with control group)

Data from Table 2, Metabolic Equivalent of experimental group is 1.25±0.18 MET, and the control group is 1.09±0.33 MET, there is no significant differences. From the Commencing form, Metabolic Equivalent trends to raise, in the Phase 3 Strike Opponent's Ears with Both Fists, Metabolic Equivalent compared to be lower. There is significant difference of Metabolic Equivalent between Phase 2 and Phase 3. 5min after exercise recover to quiet state, two groups' Metabolic Equivalent are 1.47±0.22 MET and 1.12±0.38 MET. From Table 2 we can see the energy expenditure of experimental group is higher than control group, so the load of experimental group when they playing Tai Chi is much higher than control, the quality of Tai Chi motions of control group is lower than the experimental group. If you insist on playing Tai Chi for a long time can achieve the purpose of losing fat. [3]

Table 3 : Changes of respiratory quotient of quiet state, in exercise, and after exercise.

state	The experimental group	The control group
quiet state	0.89±0.11	0.87±0.05
Phase 1	0.85±0.03	0.8±0.06
Phase 2	0.97±0.07	0.86±0.03
Phase 3	1.03±0.07	0.87±0.03
Phase 4	1.01±0.19	0.96±0.22
1min after exercise	0.96±0.09	0.92±0.12
2min after exercise	0.94±0.08*	0.93±0.08
3min after exercise	0.93±0.07*	0.87±0.02
4min after exercise	0.93±0.09	0.83±0.01
5min after exercise	0.92±0.06	0.84±0.02

(marked with * when P<0.05 compared with control group)

The respiratory quotient of every phase raises like waves during the whole exercise, and higher in Phase 4 and before recovery (Table 3).The level of oxygen consumption recovers to quiet state 5min after exercise. The average of oxygen consumption is above 0.8 during the whole exercise, the result suggests the main energy supply system is sugar and fat.

Compared with two groups, there is no significant difference during quiet state and exercise, but the respiratory quotient of the experimental group is higher than the control group during recovery, the results suggest that the level of energy supply is the same during quiet state and exercise, but the experimental group's is much lower than the control group's during recover, there is significant difference when 2min and 3min after exercise. The results suggest that the oxygen consumption of experimental group is lower than the control group, which has faster recovery. Based on the oxygen consumption ratio, people who want to consume more energy and lose fat needs to train Tai Chi again and again for a long time. [2]

b) Effects of Tai Chi on the heart function of the senior citizen.

i. *Effects of Tai Chi on the heart rate*

Table 4 : Changes of the heart rate of quiet state, in exercise, and after exercise.

state	The experimental group	The control group
quiet state	81.27±4.18	84.06±4.93
Phase 1	96.45±5.12	103.61±5.78
Phase 2	104.54±5.62	112.73±5.90
Phase 3	131.46±3.98	134.55±4.76
Phase 4	150.09±4.21	159.63±3.86
1min after exercise	133.21±5.76	150.54±4.56
2min after exercise	120.78±6.49	143.54±3.74
3min after exercise	110.67±6.09	132.09±3.72
4min after exercise	90.84±5.74*	120.73±4.67
5min after exercise	83.43±4.53*	103.12±4.84

The heart rate significant increases during the whole Tai Chi, and which keeps high level, the heart rate shown a clear upward trend, the max of the heart rate shows in the Phase 4(Table 4), so Tai Chi is an exercise which heart rate and intension increase gradually.

The heart rate of experimental group is lower than the control group during the whole phases, and shows significant difference in recovery time, that suggests it can improve the function of heart if you insist on training Tai Chi. The research discovered the heart rate keeps 90~130 BPM during Tai Chi exercise, which belongs to low to medium intensity exercise, so Tai Chi is a safe, gentle, soft sport. [4]

ii. *Effects of Tai Chi on the breath frequency*

Table 5 : Changes of the breath frequency of quiet state, in exercise, and after exercise.

state	The experimental group	The control group
quiet state	17.91±3.66	18.69±6.28
Phase 1	26.61±6.00	19.52±2.59
Phase 2	27.48±5.64	24.29±5.46
Phase 3	28.03±7.75	23.01±3.14
Phase 4	24.52±4.04	22.9±3.74
1min after exercise	23.66±4.47	21.26±3.38
2min after exercise	20.78±2.76	20.61±3
3min after exercise	19.37±3.37	20.04±3.92
4min after exercise	18.98±3.15	19.98±3.37
5min after exercise	18.08±2.69	19.78±2.23

The breath frequency of the experimental group is 17.91±3.66 times/min and which the control group is 18.69±6.28 times/min in quiet state, the breath frequency begin to raise from the Commencing form, the frequency increases go with time, and relates to the load of exercise. The max breath frequency shows in the Phase 3, recovery to the quiet level 5min after exercise. And there is no significant difference between two groups.

iii. *Effects of Tai Chi on the Relative Oxygen Uptake*

Table 6 : Changes of the Relative Oxygen Uptake of quiet state, in exercise, and after exercise.

state	The experimental group	The control group
quiet state	4.4±0.60	3.74±1.16
Phase 1	15.06±5.48	10.24±4.49
Phase 2	18.25±5.78*	13.95±8.46
Phase 3	21.96±8.84*	11.6±7.00
Phase 4	14.52±0.90*	9.35±1.6
1min after exercise	5.48±1.03	3.87±1.3
2min after exercise	5.06±1.05	4.07±1
3min after exercise	4.74±1.14	3.91±1.38
4min after exercise	5±1.39	4.14±1.32
5min after exercise	5.18±0.73	3.89±1.4

The relative oxygen uptake of the experimental group is 4.4±0.60 and which the control group is 3.74±1.16 in quiet state, and raises when begin to exercise, the relative oxygen uptake of the experimental group is much higher than the control group, which range is bigger and faster, descendent when Strike Opponent's Ears with Both Fists(Table 6). There is significant difference from Phase 2 the Phase 4.

The relative oxygen uptake of the experimental group is much higher than the control group when training Tai Chi, suggesting that Tai Chi can improve the oxygen transport system, the function of heart pump blood and the ability of muscular tissue uptakes oxygen, consequently to meet the needs of oxygen of body.[5]

iv. *Effects of Tai Chi on the time of expiration*

Table 7 : Changes of the time of expiration of quiet state, in exercise, and after exercise.

state	The experimental group	The control group
quiet state	2.17±0.59	2.92±2.01
Phase 1	1.11±0.24	1.33±0.15
Phase 2	1.18±0.36	1.64±0.5
Phase 3	1.39±0.58	1.99±0.31
Phase 4	1.68±0.27	1.94±0.33
1min after exercise	1.56±0.28	2.01±0.42
2min after exercise	1.76±0.18	1.95±0.31
3min after exercise	1.76±0.17	2.09±0.41
4min after exercise	1.7±0.19	1.81±0.31
5min after exercise	1.69±0.23	1.84±0.19

There is no significant difference of the time of expiration between two groups during the whole testing. The time of expiration became shorter from Commencing form, begin to stabilize from Play Pipa, and raises clearly after exercise (Table 7).

Table 8 : Changes of the time of breathe in of quiet state, in exercise, and after exercise.

state	The experimental group	The control group
quiet state	1.4±0.28	2.3±2.18
Phase 1	1.08±0.33	1.08±0.13
Phase 2	1.03±0.27	1.18±0.2
Phase 3	1.1±0.29	1.27±0.14
Phase 4	1.12±0.17	1.35±0.26
1min after exercise	1.07±0.17	1.37±0.29
2min after exercise	1.19±0.21	1.37±0.27
3min after exercise	1.13±0.21	1.39±0.32
4min after exercise	1.2±0.19	1.14±0.18
5min after exercise	1.14±0.22	1.19±0.21

Based on the data of Table 8, the time of breathe in became shorter when begin to train Tai Chi, but there is no significant difference, the time of breathe in kept a stable state during the whole 4 Phases and which increased clearly during recovery time, there in no significant difference between the experimental group and the control group. So someone who trained Tai Chi for a long time can meet the needs of oxygen of body in a shorter time, which suggests the ability of muscular tissue uptakes and utilize oxygen of experimental group is better than the control group.[6]

v. *Effects of Tai Chi on the Tidal Volume*

Table 9 : Changes of the time of Tidal Volume of quiet state, in exercise, and after exercise.

state	The experimental group	The control group
quiet state	0.99±0.91	0.57±0.11
Phase 1	1.15±0.1	0.87±0.15
Phase 2	1.06±0.27	0.85±0.14
Phase 3	1.07±0.28	0.84±0.22
Phase 4	0.74±0.1	0.61±0.13
1min after exercise	0.66±0.1	0.57±0.08
2min after exercise	0.7±0.08	0.6±0.05
3min after exercise	0.68±0.11	0.53±0.03
4min after exercise	0.62±0.04	0.53±0.07
5min after exercise	0.61±0.04	0.54±0.07

The Tidal Volume of two groups increase when begin to train, and the experimental group's data is higher than the control group during the whole test (Table 9). Which suggests that the depth of breath of the experimental group is higher than the control group, under the conclusion of there is no significant difference in the breath frequency, and enhanced the ability of breathing reserve and the lung ventilation. [7] And the energy and oxygen consumption of the breathe muscle are decreased correspondingly, so the senior citizen who trained Tai Chi for a long time will use different style

IV. Conclusions

1. Insisting on training Tai Chi is good for losing body fat because the effect of consuming the body energy of Tai Chi is obvious.
2. Appropriate quantity and intension of Tai Chi can improve the cardiovascular function and vital capacity, promote the function of heart-lung and energy metabolize.
3. Tai Chi can strengthen senior citizen's physique, promotes the health.

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Regulatory Privatisation, Social Welfare Services and its Alternatives

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Abstract - The strategy of price liberalisation and privatisation had been implemented in Sudan over the last decade, and has had a positive result on government deficit. The investment law approved recently has good statements and rules on the above strategy in particular to pharmacy regulations. Under the pressure of the new privatisation policy, the government introduced radical changes in the pharmacy regulations. To improve the effectiveness of the public pharmacy, resources should be switched towards areas of need, reducing inequalities and promoting better health conditions. Medicines are financed either through cost sharing or full private. The role of the private services is significant. A review of reform of financing medicines in Sudan is given in this article. Also, it highlights the current drug supply system in the public sector, which is currently responsibility of the Central Medical Supplies Public Corporation (CMS).

Keywords : *Sudan, Healthcare, Medicines, Regulatory authorities, Pharmacy Management.*

GJMR-I Classification : *NLMC Code: W 322, W 323*

Strictly as per the compliance and regulations of:

Regulatory Privatisation, Social Welfare Services and its Alternatives

Gamal K. M. Ali [α] & Abdeen M. Omer [σ]

Abstract - The strategy of price liberalisation and privatisation had been implemented in Sudan over the last decade, and has had a positive result on government deficit. The investment law approved recently has good statements and rules on the above strategy in particular to pharmacy regulations. Under the pressure of the new privatisation policy, the government introduced radical changes in the pharmacy regulations. To improve the effectiveness of the public pharmacy, resources should be switched towards areas of need, reducing inequalities and promoting better health conditions. Medicines are financed either through cost sharing or full private. The role of the private services is significant. A review of reform of financing medicines in Sudan is given in this article. Also, it highlights the current drug supply system in the public sector, which is currently responsibility of the Central Medical Supplies Public Corporation (CMS). In Sudan, the researchers did not identify any rigorous evaluations or quantitative studies about the impact of drug regulations on the quality of medicines and how to protect public health against counterfeit or low quality medicines, although it is practically possible. However, the regulations must be continually evaluated to ensure the public health is protected against by marketing high quality medicines rather than commercial interests, and the drug companies are held accountable for their conducts.

Keywords : Sudan, Healthcare, Medicines, Regulatory authorities, Pharmacy Management.

I. Introduction

The World Health Organisation (WHO, 2009) has defined drug regulation as a process, which encompasses various activities, aimed at promoting and protecting public health by ensuring the safety, efficiency and quality of drugs, and appropriateness accuracy of information (WHO, 2009). Medicines regulation is a key instrument employed by many governments to modify the behaviour of drug systems. The regulation of pharmaceuticals relates to control of manufacturing standards, the quality, the efficacy and safety of drugs, labelling and information requirements, distribution procedures and consumer prices (Sibanda, 2004). To assure quality of medicines, in most countries registration is required prior to the introduction of a drug preparation into the market. The manufacturing, registration and sale of drugs have been the subject of restricts regulations and administrative procedures worldwide for decades (Lofgren, and Boer, 2004). Nobody would seriously argue drugs should be proven to be 100% safe. No set of regulations could achieve that goal, because it is impossibility and all drugs carry some risk (Lexchin, 1990).

Stringent drug regulation was introduced across many countries in the 1960s following the thalidomide disaster, and had since been embraced by the industry as a commercial essential seal of safety and quality (Lofgren, and Boer, 2004). In spite of the measures, many countries, especially developing ones face a broader range of problems. In several developing countries drug quality is a source of concern. There is a general feeling there is a high incidence of drug preparations, which are not of acceptable quality (Shakoor, Taylor, and Behrens, 1997). For example, about 70% of counterfeit medicines were reported by developing countries (Helling-Borda, 1995). Reports from Asia, Africa, and South America indicate 10% to 50 % of consider using prescribed drugs in certain countries may be counterfeit (Rudolf, and Bernstein, 2004). For instance, in Nigeria fake medicines may be more than 60 -70% of the drugs in circulation (Osibo, 1998), and 109 children died in 1990 after being administered fake Paracetamol (Alubo, 1994). In Gambia the drug registration and control system resulted in the elimination of 'drug peddlers' and certain 'obsolete and harmful' drugs, as well as a large decrease in the percentage of brand and combination drugs (Jallow, 1991). The percentage of drugs failed quality control testing was found to be zero in Colombia, but 92% in the private sector of Chad (WHO/DAP, 1996). Hence, it is very difficulty to obtain an accurate data. The proportion of drugs in the USA marketplace are counterfeit is believed to be small - less than 1 percent (Rudolf, and Bernstein, 2004). (Andalo, 2004) reported two cases of counterfeit medicines found their way into legitimate medicine supply chain in the UK in 2004.

Poor quality drug preparations may lead to adverse clinical results both in terms of low efficacy and in the development of drug resistance (Shakoor, Taylor, and Behrens, 1997). Regulations are the basic devices employed by most governments to protect the public health against substandard, counterfeit, low quality medicines, and to control prices. Thus, thorough knowledge of whether these regulations produce the intended effects or generate unexpected adverse

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consequences is therefore critical. The World Health Organisation (WHO) undertook a number of initiatives to improve medicines quality in its member states and promote global mechanisms for regulating the quality of pharmaceutical products in the international markets. But,there are not any WHO guidelines on how to evaluate the impact of these regulations. There are numerous reports concerning drug regulations (Ratanwijitrasin, Soumerai, and Weerasuriya, 2001), but the published work on the impact of these regulations on the quality of medicines moving in the international commerce has been scarce. Findings from most published studies lack comparable quantitative information that would allow for objective judging whether and by how much progress on the various outcomes have been made by the implementation of the pharmaceutical regulations. To ignore evaluations and to implement drug regulation based on logic and theory, is to expose society to untried measures in the same way patients were exposed to untested medicines (Ratanwijitrasin, Soumerai, and Weerasuriya, 2001).

The present policy of the national health–care system in Sudan is based on ensuring the welfare of the Sudanese inhabitants through increasing national production and upgrading the productivity of individuals. A health development strategy has been formulated in a way that realises the relevancy of health objectives to the main goals of the national development plans. The strategy of Sudan at the national level aims at developing the Primary Health Care (PHC) services in the rural areas as well as urban areas. In Sudan 2567 physicians provide the public health services (554 specialists, 107 medical registrars, 1544 medical officers, 156 dentists, and 206 pharmacists (Gamal and Omer, 2008)). Methods of preventing and controlling health problems are the following :

- Promotion of food supply and proper nutrition.
- An adequate supply of safe water and basic sanitation.
- Maternal and child health-care.
- Immunisation against major infectious diseases.
- Preventing and control of locally endemic diseases, and
- Provision of essential drugs.

This will be achieved through a health system consisting of three levels (state, provincial and localities), including the referral system, secondary and tertiary levels.

Pharmacy management should be coordinated and integrated with other various aspects of health. The following are recommended:

- Community must be the focus of benefits accruing from restructures, legislature to protect community interest on the basis of equity and distribution, handover the assets to the community should be examined; and communities shall encourage the transfer the management of health schemes to a professional entity.
- The private sector should be used to mobilise, and strengthen the technical and financial resources, from within and without the country to implement the services, with particular emphasis on utilisation of local resources.
- The government should provide the necessary financial resources to guide the process of community management of pharmacy supplies. The government to divert from provision of services and be a facilitator through setting up standards, specifications and rules to help harmonise the private sector and establish a legal independent body by an act of parliament to monitor and control the providers. Government to assist the poor communities who cannot afford service cost, and alleviate social-economic negative aspects of privatisation.
- The sector actors should create awareness to the community of the roles of the private sector and government in the provision of health and pharmacy services.
- Support agencies assist with the financial and technical support, the training facilities, coordination, development and dissemination of health projects, and then evaluation of projects.

II. Health and Pharmacy Systems

The health system in Sudan is characterised by heavily reliance on charging users at the point of access (private expenditure on health is 79.1 percent (WHO, 2004)), with less use of prepayment system such as health insurance. The way the health system is funded, organised, managed and regulated affects health workers' supply, retention, and the performance. Primary Health Care was adopted as a main strategy for health-care provision in Sudan and new strategies were introduced during the last decade, include:

- Health area system.
- Polio eradication in 1988.
- Integrated management of children illness (IMCI) initiative.
- Rollback malaria strategy.
- Basic developmental need approach in 1997.
- Safe motherhood, making pregnancy safer initiative, eradication of harmful traditional practices and emergency obstetrics' care programmes.

The strategy of price liberalisation and privatisation had been implemented in Sudan over the last decade, and has had a positive result on government deficit. The investment law approved

recently has good statements and rules on the above strategy in particular to health and pharmacy areas. The privatisation and price liberalisation in healthy fields has to re-structure (but not fully). Availability and adequate pharmacy supplies to the major sectors. The result is that, the present situation of pharmacy services is far better than ten years ago.

The government of Sudan has a great experience in privatisation of the public institutions i.e., Sudanese free zones and markets, Sudan telecommunications (Sudatel) and Sudan airlines. These experiences provide good lessons about the efficiency and effectiveness of the privatisation policy. Through privatisation, government is not evading its responsibility of providing health-care to the inhabitants, but merely shifting its role from being a provider to a regulator and standard setter. The drug financing was privatised early in 1992. Currently, the Federal Ministry of Health (FMOH) has privatised certain non-medical services in hospitals such as catering services, security and cleanings.

The overall goal of the CMS ownership privatisation is to improve access to essential medicines and other medical supplies in order to improve health status of the inhabitants particularly in far states (e.g., Western and Southern States).

Establishment of alternative ownership for the CMS can be achieved by selling the majority of shares to the private sector. This will achieve the following objectives:

- High access to essential medicines of good quality and affordable prices to the states' population and governments.
- Efficiency and effectiveness in drug distribution system to avoid the serious pitfalls and incidences that reported during the last ten years in the CMS.
- Equity by reaching all remote areas currently deprived from the formal drug distribution channels.
- Improvement of the quality and quantity of delivery of medicines to the public health facilities.

The above objectives are expected to:

- Increase geographical and economic access to essential medicines in all states (i.e., in both rural and urban areas) to reach at least 80% of the population (currently less than 50% of population have access to essential medicines).
- The tax collection from the new business becomes more efficient and will increase after privatisation. The tax revenues could be used to finance other health-care activities.
- If the government reserves some shares (not more than 50%) in the new business, then its shares' profit could be used to finance free medicines project in hospitals outpatients' clinic, and other exempted medicines e.g., renal dialysis and haemophilic patients treatment.

III. Privatisation of Public Pharmaceutical Supplies

The term privatisation has generally been defined as any process aims to shift functions and responsibilities (totally or partially) from the government to the private. In broader meaning, it refers to restrict government's role and to put forward some methods or policies in order to strengthen free market economy (Aktan, 1995). Privatisation can be an ideology (for those who oppose government and seek to reduce its size, role, and costs, or for those who wish to encourage diversity, decentralisation, and choice) or a tool of government (for those who see the private sector as more efficient, flexible, and innovative than the public sector) ((Kamerman et al., 1989), and (Gormley, 1991)). (Scarpaci, 1991) contends that "the invisible hand of the market is more efficient and responsive to the consumer needs and the public administrative budgets consume large portion of tax monies that could otherwise be used for service delivery". The emphasis is on improving the efficiency of all public enterprises, whether retained or divested.

a) Privatisation may take many forms including:

- The elimination of a public function and its assignment to the private sector for financial support as well as delivery (police, and fire departments, schools, etc.). Opponents characterise this as "load-shedding" (Bendick, 1989).
- Deregulation is the elimination of government responsibility for setting standards and rules concerning goods or services (Gormley, 1996 and 1997).
- Assets sales are the selling of a public asset (city buildings, sports stadiums) to private firms.
- Vouchers are the government provided or financed cards or slips of paper that permit private individuals to purchase goods or services from a private provider (food stamps) or circumscribed list of providers (Kettl, 1995).
- Franchising is the establishment of models by the public sector that is funded by government agencies, but implemented by approved private providers.
- Contracting is the government financing of services, choice of service provider, and specification of various aspects of the services laid out in contracts with the private-sector organisation that produces or delivers the services.
- User fees are the public facilities such as hospitals maximise their income or finance some goods from private sources, either through drug sales or other services. This kind of privatisation is applied in

Sudan since early 1990s, as the health financing mechanism (especially for medicines).

In Sudan, the government has decided to distance itself from direct involvement in business, and thus to divest most of its interests whether in loss or profit making public enterprises. The public reform programme was set firmly in the context of the broader reforms, which were introduced in 1992. It had become clear the previous policies had delivered very disappointed results. This reform based on the transfer of activities vested with the government institutions to the private sector. It signalled the government intention to reduce its presence in the economy, to reduce the level and scope of public spending and to allow market forces to govern economic activities. Privatisation also forms part of the government strategy of strengthening the role of the private in the development to achieve the vision of the 25 years strategy in which the private sector will be the engine for economic growth. The privatisation started in 1992 by liberalization of local currency, foreign exchange transactions, internal and external trade, prices and health services (e.g., user fee as a mechanism of drug financing and other services). This reform had led to greater reliance on individual initiative and corporate accountability rather than on government as a decision-maker in business matters.

The privatisation policy goal is to improve the performance of the public sector companies. So, they can contribute to the growth and the development of the economy by broadens ownerships, participation in management, and stimulation domestic and foreign private investment.

The following are the primary objectives, which have been defined in the government's policy statement on public sector reform:

- Improve the operational efficiency of enterprises that are currently in the public sector by exposing business and services to the greatest competition for the benefit of the consumer and the national economy.
- Reduce the burden of public enterprises on the government's budget by spreading the shares' ownership as widely as possible among the population.
- Expand the role of the private sector in the economy (permitting the government to concentrate on the public resources) on its role as provider of basic public services, including health, education, social infrastructure, and to compact the side effects of the privatisation.
- Encourage wider participation of the people in the ownership and management of business.

In pursuing the primary objectives the privatisation policy aims to transform the performance of most significant enterprises in the public sector and ensure liquidation of all viable and non-viable public enterprises as soon as possible through commercialisation, restructuring and divesture.

Public sector reform efforts are thus aimed at reducing government dominance and promoting a larger role for the private sector, while improving government's use of resources. Movement towards those goals in some countries is supported by components of a structural adjustment loan, which helped initiate the programme and establish the legislative and institutional base.

Opponents argue, the original objectives of state ownership were to ensure the corporate sector of the economy was in national hands rather than being controlled by either foreign investors or the minorities that enjoyed business dominance upon independence. A further objective was to use investment in state firms to accelerate development in a situation, in which private sector was reluctant to take risks.

IV. Medicines Legislation Framework in Sudan

The availability of medicines in Sudan is controlled on the basis of safety, quality and efficacy. Thus, the government effects control in accordance with the Pharmacy, Poisons, Cosmetics and Medical Devices Act 2001 and its instruments. The Federal or State Departments of Pharmacy (DOP) and directives issued orders. The primary objective of both Federal and States' Departments of Pharmacy is to safeguard public health by ensuring all medicines and pharmaceuticals on the Sudan market meet appropriate standards of safety, quality and efficacy. The safeguarding of public health is achieved largely through the system of medicines' registration and licensing of pharmacy premises.

The first Pharmacy and Poisons Act was enacted in 1939. This Act had been amended three times since then. In 2001 amendments, cosmetics and medical devices were also brought under its purview. Thus, the name was changed to Pharmacy, Poisons, Cosmetics and Medical Devices Act (hereafter the Act). The Act regulates the compounding, sale, distribution, supply, dispensing of medicines and provides different levels of control for different categories e.g., medicines, poisons, cosmetics, chemicals for medical use and medical devices.

The Act makes provision for the publication of regulations and guidelines by the Federal Pharmacy and Poisons Board (FPPB), the pharmaceutical regulatory authority and its executive arm - the Federal General Directorate of Pharmacy (FGDOP). The FGDOP regulates mainly four aspects of medicines use: safety, quality, efficacy and price. Traditionally, governments in many countries, particularly developed nations have attempted to ensure the efficiency, safety, rational prescribing, and dispensing of drugs through pre-

marketing registration, licensing and other regulatory requirements (Ratanwijitrasin, Soumerai, and Weerasuriya, 2001). When applying to register the medicine manufacturers and importers are required to furnish the FGDOP with a dossier of information including among others, the indication of the medicine, its efficacy, side effects, contraindication, warnings on usage by high risk groups, price, storage and disposal (MOH, 2001).

The role of FGDOP includes among others :

1. Regulation and control of the importation, exportation, manufacture, advertisement, distribution, sale and the use of medicines, cosmetics, medical devices and chemicals;
2. Approval and registration of new medicines - the Act requires FGDOP should register every medicine before be sold or marketed. Companies are required to submit applications for the registration of medicines for the evaluation and approval;
3. Undertake appropriate investigations into the production premises and raw materials for drugs and establish relevant quality assurance systems including certification of the production sites and regulated products;
4. Undertake inspection of drugs' whole and retail sellers owned by both public or private sectors;
5. Compile standard specifications and regulations and guidelines for the production, importation, exportation, sale and distribution of drugs, cosmetics, etc.
6. Control of quality of medicines: This will be done by regular inspection and post-marketing surveillance;
7. Licensing of pharmacy premises (i.e., pharmaceutical plants, wholesalers and retail pharmacies);
8. Maintain national drug analysis laboratories for the pre- and post- marketing analysis of medicines;
9. Coordination with states departments of pharmacy to ensure the enforcement of the Act and its rules and directives.

V. Public Sector Medicines Supply System

In Sub-Saharan Africa countries (Sudan is not an exceptional) discussions about medicine distribution system reform have concentrated on ways to improve sustainability and quality of access to essential medicines. These discussions also include debate on the impact of privatisation of public drug supply organisations on effectiveness, efficiency, quality and cost of medicines in the public health facilities, as well as on the respective role of the public and private sectors (Leighton, 1996).

Until the mid 1980s, governments in Africa assumed responsibility for providing drugs to the inhabitants in some countries such as Mali and Guinea. The private distribution of all drugs including aspirin was illegal (Vogel et al., 1989). In many countries e.g., in Sudan there were two parallel government distribution systems. The public health network of hospitals and health centres were gratuitously distributed drugs. In the public sector pharmacies, the drugs were sold to the public at subsidised prices.

During the 1990s, Sudan initiated a number of initiatives to establish drug-financing mechanisms as part of the health reform process and decentralised decision-making at a state level. In 1992 when a law was passed, medicines were not anymore free-of-charge (i.e., privatised) in public health system. The aim of the government is to increase equitable access to essential medicines, especially at states' level. As a result the Central Medical Stores, which was responsible for medicines supply system of the public health facilities, became an autonomous drug supply agency, and renamed as the Central Medical Supplies Public Corporation (CMS) and operated on cash-and-carry basis. It was capitalised and an executive board was installed. Since that time, it implied the states and federal hospitals have to buy their own medicines, other medical supplies. They organised their own transport means and distribution to their primary health-care facilities and hospitals. In addition, all hospitals became financially autonomous entities and had to organise their own medicines procurement system.

The public drug supply system has not been working throughout Sub-Saharan Africa including Sudan. There are serious shortages or no medicines at all, particularly in rural areas. A study in Cameroon found the rural health centres received only 65% of the stock designated for them, and 30% of the medicines arrived at the centres did not reach the clients. The loss rate after arrival in hospitals was estimated at 40% (Stephens, 1982). In Sudan, Graff and Evarard (2003) who visited the country on a WHO mission reported, "Although the cash-and-carry system took off well, but lack of sufficient foreign exchange hampered the CMS procurement activities and resulted in low stock levels of all medicines and even stock out of life-saving products. Hospitals had to purchase the medicines from elsewhere and often had to buy from private sector. Overall hospitals' budgets were tied to allocate drug budget and sales income was not sufficient to cover the purchase of needed medicines supplies. This resulted the medicines were not available most of the times. The in- or outpatients with their prescriptions were directed to the private pharmacies. In 2003, Khartoum Teaching Hospital-the biggest hospital in Sudan (not far than 5 km away from the CMS) had medicine stock of only LS 83,000 (US$ 31). This would not fill one prescription for an anaemic patient as a result of renal failure. This is a common practice that patients or their relatives are given prescriptions to buy any pharmaceutical supplies that are needed including drugs and other disposables

from private sector pharmacies.

Many ministries of health, services' providers and researchers have identified many characteristics that lead to poor performance in Africa public drug supply systems. These characteristics include:

(1) Absence of competition :

Competition is the best way to ensure the goods and services desired by the consumer are provided at the lowest economic cost. Given the customers (i.e., public health facilities) freedom of choice enables market forces to provide sustained pressures on companies to increase efficiency. Privatised companies generally operate in a competitive market environment.

(2) Insufficient funding :

For example in Sudan with exception of Khartoum, Gezira and Gedaref states, all the states have no enough funds to establish efficient drug supply system. In spite of being profit-making organisation, the CMS failed to avail such funds during the past 14 years.

(3) Inefficient use of available resources :

The CMS since it was established in early 1990s working as a profit-making organisation. Due to the absence of privatisation the CMS engaged in an instalment of repackaging joint venture pharmaceutical factory in 1999 and recently announced its commitment to build a pharmaceutical city with not less than US$ 20 million, despite the lack of life-saving medicines in the public health facilities. Such amount could be sufficient to establish a reliable supply system for all states of Sudan. The lack of prioritisation is a typical symptom and sign of most public organisations.

(4) Poor management :

There are a number of constraints inherent in operating government drug supply service.
These constraints comprise :

a) Civil servants are hired, rather than persons with business experience and skills. Managers confront different challenges in public setting. They are not easily hired or fired. The lack of accountability results from the lack of shareholders, who would be free to remove incompetent administrators.

b) Even if the services can recruit outside of civil service, the wages are often too low to attract experienced managers. In addition, the managers do not share in dividends or other monetary activities as do private managers and incentives for doing well are often attenuated in a bureaucracy.

c) There are cultural and structural conditions that promote corruptions including enormous pressure of wages earners to support an extended family and a strong incentive to more than their fixed government wage, traditional gift giving practice and a proprietary view of public offices (Van der Geest, 1982).

VI. Privatisation of the Cms's Ownership

The public sector drug supply institutions have not succeeded (CMS is not exceptional) so far in organising a reliable and regular essential drug supply for the public health facilities (Huss, 1996). One of the most criticisms of the public drug supply system generally in Africa and particularly in Sudan, is how badly they are internally managed. There are those who agree the greater amount of real pharmaceutical resources could be made available to the public health-care system and the access to essential medicines could be significantly increased, if managerial efficiency of the system improved (Akin, 1987). Given the limitation of the public sector - due to constraints inherent in operating a government drug supply organisation even after autonomous experience - and the stabilised role of the private sector organisations such as private pharmaceutical sectors organisations (rapid increase in importing companies, manufacturers and pharmacies). Telecommunications, e.g., Sudatel is one of the obvious solutions of choice for the government pharmaceutical policy would be to privatise the ownership of the CMS to the extent possible.

VII. Advantages of Private Agencies

There are many arguments in favour of privatisation of public institutions. Advocates of this method claim privatisation have the following advantages (Savas, 1987; Hartley, 1986; De Hoog, 1984; Moore, 1987; and Ascher, 1987).

- Privatisation is efficient and effective because it fosters and initiates competition. The competition among firms drives the cost down. Empirical studies clearly prove the cost of the services provided by the government is much higher than when the services are provided by private contractors. For example CMS's declared mark-up on cost (35%) amounted to 2.3 times the private mark-up (15%). In addition, private sector pays taxes, customs and other governmental fees (CMS exempted).
- Privatisation also provides better management than the public management. Because decision making under privatisation is directly related to the costs and benefits. In other words, the privatisation fosters good management because the cost of the service is usually obscured.
- Privatisation would help to limit the size of government at least in terms of the number of employees. On the other hand, it is a fact that overstaffing is common in publicly owned enterprises.

- Privatisation can help to reduce dependence on a government monopoly, which causes inefficiencies and ineffectiveness in services.
- Private sector is more flexible in terms of responding to the needs of citizens. Greater flexibility in the use of personnel and equipment would be achieved for short-term projects, part-time work, etc. Bureaucratic formalities are very common when government delivers the service. Less tolerance and strict hierarchy in bureaucracy are the reasons of the inflexibility in publicly provided services.

VIII. Medicines Supply System

The Act, for the first time in Sudan has given the responsibility of veterinary medicines to separate committees. The Ministry of Animal Resources took the law "in hand", and started the registration of veterinary medicines and the licensing of the veterinary medicines premises. The conflict in the shared authorities between the Ministry of Health and the chairman of the FPPB lead to the freezing of the Board since October 2002. The FGDOP continues in the process of medicines registration, inspection of the pharmaceutical premises and the licensing as before establishment of FPPB.

The Act also obliges the states' governments to take all steps necessary to ensure compliance with marketing of registered medicines in licensed premises. But, the weaknesses of the regulatory infrastructure and lack of political commitment at state levels, the leakage of low quality, unregistered medicines to those states are highly suspected. This left the door widely opened for informal marketing of medicines particularly in far states. The states regulatory authorities should take the advantage of the legal authority granted by the Sudan constitution and the Pharmacy, Poisons, Cosmetics and the Medical Devices Act 2001 to enforce the regulations and increase the frequency of the inspection visits to drug companies and retail pharmacies.

Experience has shown the poor regulation of medicines can lead to the prevalence of substandard, counterfeit, harmful and ineffective medicines on the national markets and the international commerce. The Sudanese pharmaceutical legal framework was described as one of the strictest pharmaceutical system in the region. One of the great loopholes in this system was found to be the increased number of non-registered medicines-governmental sources such as the Central Medical Supplies Public Organisation (CMSPO) and not-for-profit non-governmental Organisations (NGOs). Respondents were hopeful the double standard of rules enforcement would be lifted after the new national unity government take over, arguing the current situation in which public organisations (such as the CMSPO) sell non-registered medicines to the private pharmacies could enhance trading of counterfeit medicines and create unfair competition environment.

One of the respondent reported, "It is disturbing, in spite of the existence of appropriate legislation, illegal distribution of medicines by the CMSPO. The CMSPO continues to flourish, giving the impression the government is insensitive to harmful effect on the people of medicines distribution unlawfully, and some are of doubtful quality". During the past three years the CMSPO started to sell unregistered medicines to the private pharmacies. The CMSPO practice (he added) will undermine the inspection and medicines control activities and ultimately jeopardise the health of the people taking medication.

Not surprisingly all respondents strongly agreed the increased number of sources of non-registered medicines will lead to entrance of low quality medicines. This result is inline with the WHO recommendation, which encourages the regulatory authorities and state members` government to register all medicines before the marketing. The medicines imported by public sector organisations are not excluded (Bryman, 2004).

The FGDOP should define the norms, standards and specifications necessary for ensuring the safety, efficacy and quality of medicinal products. The availability, accuracy and clarity of drug information can affect the drug use decisions. The FGDOP does not have a well-developed system for pre-approval of medicines labels, promotional, and advertising materials. The terms and conditions under, which licenses to import, manufacture and distribute will be suspended, revoked or cancelled. This should be stringently applied to public, private and not-for-profit NGOs drug supplies organisations.

The predominant view, shared between the medicines' importers is the current pharmacy legislation to some extent satisfactory and managed to prohibit the marketing of low quality medicines. The recent post-marketing study carried by the National Drug Quality Control Laboratories, suggested the power of the current regulation is overestimated (WHO, 1991). The finding of this article indicates the application procedures of the current measures to ensure the quality of medicines should be revisited. The technical complexity of regulations, political, commercial and social implications, makes necessary a degree of mutual trust between concerned stakeholders (i.e., suppliers, doctors, pharmacists, consumer representatives and government agencies).

IX. Discussions

The study reveals the need for further research to find out how efficient the regulatory authorities at both federal and state levels are. The research also needed to discover whether or not counterfeit medicines are sold on the Sudanese market.

From the data obtained in this article some inferences could be made:

1. The brad outlines remain intact, but preventing drug smuggling across national boarders (Sudan shares frontiers with 9 countries) is hard to police.
2. The enforcement of the Act and its regulation governing the manufacture, importation, sale, distribution and exportation of medicines are not adequate enough to control the illegal importation and sale of medicines in Sudan.
3. The splitting of the drug regulatory authority between two ministries and the marketing of unregistered medicines by public drug suppliers (namely the CMSPO, and RDFs), and NGOs undermine the quality of medicines and ultimately jeopardise the health of the people taking medication.

In the light of the findings the following recommendations could be useful at various levels:

1. There is an urgent need for government to implement the provisions of existing Act.
2. The government should adequately equip and fund the National drug Analysis laboratories to start active post-marketing surveillance.
3. A more spirited effort need to be made by FGDOP and the States' Departments of Pharmacy to ensure all the medicines on the pharmacies' shelves are registered and come from legal sources.
4. The states' departments of pharmacies are not in existence should be re-established and invigorated. They should be adequately funded to be able to acquire the necessary facilities for their operations.
5. The CMSPO should stop importation, manufacture and distribution of unregistered medicines. It should also cease selling the tenders' product to the private pharmacies. The latter practice undermines the inspection outcomes, because it makes inspectors task too difficult (i.e., cannot identify the source of medicine whether it is CMSPO or not).

X. Rational of the Cms Privatisation

Even in the absence of broader adjustment context, however, it has long been clear the CMS reform is needed and indeed is not to avoidable. Patients, administrators (at both hospitals and ministries of health), doctors and other health-care professionals, the regulatory authority and others are being fully aware the performance of the CMS is so poor and ill people are really suffered even after the privatisation of medicines financing in 1992. Although it is profit-making organisation, neither the Ministry of Finance nor FMOH is getting proper or even any returns from the CMS. The Ministry of Finance after more than 14 years still have to inject annual money to cover the cost of certain budget lines such as free medicines projects. In addition, the following are main three justifications, which summarise the inefficiency of the CMS as a public organisation:

- There is a widespread dissatisfaction with the situation of pharmaceuticals in public facilities. For instance, 79% of the population pay for their medicines out of pockets (WHO, 2004). The access to essential medicines in Sudan is still less than 50% (Quick, 1997).
- Yet, the cost has been immense and it is continuing. There is no satisfactory estimate of the total capital invested in the CMS. Rather than receiving a sustained flow of dividends from its investments, the Ministry of Finance still financing the free medicines and certain diseases drugs. For example, in Khartoum State RDF, with small capital (US$ 2 million) - compared to the CMS big employed capital (more than US$ 20 Million) approximately 10 times that of the Revolving Drug Funds (RDF)-support Ministry of Health activities with two billion every year. In contrast, the CMS pays nothing to health services since it was established in 1992. Instead, the strong stream of dividends and tax revenues, which should support public spending on other health activities, is lost. Hence, it is the poor who suffer as a result.
- Violation of pharmaceutical regulation at the expenses of the public health by creating a big loophole in the pharmaceutical legal framework, which will inevitably leads to marketing of counterfeit medicines. This practice also suppresses the private sector (the government encourages it heavily to grow) by making inappropriate barriers to the private sector provision of drugs.

This is not to say the CMS has no future: there are substantial investment opportunities. Many can be turned around under new ownership and will succeed. It has been the experience of state enterprises worldwide that, in both socialist economies and in mixed economies, it is exceedingly difficult to remain competitive:

- If run by a board of public servants with multiple objectives and without real accountability to shareholders.
- The constraints from government on investment and other business decisions.
- If cut off by virtue of ownership from the latest technologies, marketing and management trends.

The basic points are :

- Public sector boards and civil servants are not in touch with markets and commercial trends.
- Government-run companies have conflicting objectives that do not stress commercial accountability and thus jeopardise survival and commercial success.

Reform is a matter of practical necessity rather than ideology. For example, the government of Cuba

has still committed to socialist policies, and has recently chosen for pragmatic reasons, to privatise its telephone company. The final pragmatic reason impelling the government towards swift public sector reform is the resources are being misused.

XI. Strategies to Overcome the Cms Privatisation Obstacles

It is not surprising some obstacles and resistance from the CMS member of staff will confront this reform. The following strategies will help to overcome such resistance and obstacles:

- Consensus should be built by negotiation with relevant ministries, public and private sectors, and interest groups so that all "buy into" the process and negotiated the goals.
- Promotion research and development, demonstration and dissemination of information for the current situation. WHO mission 2003 Report will be of great value and expected outcomes with more focus on the patients after adoption of user fee policy.

XII. The Role of the FMOH

Private enterprise functions most efficiently if market forces are allowed to operate independently and completely unfettered. Nonetheless, some FMOH involvement is necessary to ensure the availability of proper use of good quality and affordable pharmaceuticals. So FMOH will continue its current responsibility for importing, licensing, inspecting and regulating the distribution system without any discrimination between different organisations including the new established business, facilitating the development of adherence to the national drug list in the public health facilities, encourage purchasing of registered medicines from the least cost reliable sources, quality control of medicines and maintenance of quality through out the system, and enforcement of price control system. The FMOH could also be involved in informing private distributors and the public about the appropriate use of medicines.

At the public health facilities, however, freedom-of-choice arguments that would justify a laissez-fair approach to private sector importing do not apply. There is the overriding merit-good aspects of medicines need, the related requisites of availability, cost-efficiency, and quality control. Some pharmaceuticals are more cost-effective than others. Therefore, the enforcement of a government-mandated essential drug list lowers the real resource cost of a given quantity of pharmaceuticals necessary for alleviation of common diseases. Standard treatment guidelines alleviate unsuitable medicating practices particularly over-medication, and reduce costs to consumers.

XIII. Conclusions

The CMS reform is stronger today than it was in the early 1990s when the reforms were started. There are many highly committed and able individuals throughout the public sector in the absence of the single-minded pursuit of commercial success. Also, in the long-term interest of employment growth and the public at large, narrower concerns have prevailed. Managements and boards are less able and less willing to impose accountability for results on themselves and their employees. Stock-out of life saving items is common, and sanctions for non-performance are often absent altogether. To overcome those common symptoms of all public owned enterprise, and achieve the strategic objectives of the FMOH by increasing the access of population to the essential medicines. The privatisation of the CMS's ownership is the best solution of choice. By resurrecting competition, which could be achieved mainly through privatisation of the CMS ownership, many of the mentioned pitfalls can be avoided. The new business should be responsible (of course without any kind of monopoly) for drug supply and distribution to the public health facilities on competition basis. The initial capital of the drug stocks for the different health facilities should be given by this new business by signing a clear agreement with interested states' ministries of health.

XIV. Recommendations

By resurrecting competition, which could be achieved mainly through privatisation of the CMS ownership, many of the mentioned pitfalls can be to avoided. The new business should be responsible (of course without any kind of monopoly) for drug supply and distribution to the public health facilities on competition basis. The initial capital of the drug stocks for the different health facilities should be given by this new business by signing a clear agreement with interested states' ministries of health.

The government may retain a special (or "golden") share ranging from 30% to 50% to protect a newly privatised business from unwelcome take-over on national security grounds, or as temporary measure, to provide an opportunity for management to adjust to the private sector. The special share requires certain provisions in the articles of incorporation of a company may not be changed without the specific consent of special shareholder. The presence of a special share is useful tool but is not intended to be a government straitjacket on the management. The management and not the government are generally responsible for ensuring the special share's provisions are observed (Omer, 1994; and Gibbon, 1996). In order to develop a free market in shares, special shares should be time limited as far as possible. The purpose of privatisation is to remove the government from ownership of the CMS.

In some cases, especially where there are major uncertainties about the probable market of the business, for example, United Kingdom and other governments have sold their ownership interest gradually in several times over a period of years (Gibbon, 1996; Bryman, 2004; MOH, 2003; and Andalo, 2004).

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88

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Design and Simulation of Leg-Exoskeleton Suit for Rehabilitation

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Abstract - In everyday activities, the injuries to lower limb are considered to rehabilitate in urgent priority. The rehabilitation is to restore the patient's physical, sensory, and mental capabilities due to injury, illness, or disease in normal condition. The typical habilitation for lower limb is separated in three types , which ar e active , passiv e and active -assi stive mode. Active mode is one important aspect of rehabilitat ion for increasing or maintaining joint function providing appropriate resistance to the muscles to increase endurance and strength . For passive mode , patients cannot activ ely participate in rehabilitation and no effort is required from them. Patient's lower limb will be driven by exoskeleton suit. This rehabilitation motion is influenced by bone surfaces within the joint, joint capsule, ligaments, tendons, and muscles acting on the joint. In this paper, the new hardware design is introduced and the possible motions of each joint are simulated. The relative positions of each joint are determined by Denavit- Hartenberg method. In simulation the revolute, speed and acceleration of each joint in left side are studied to construct the real hardware.

Keywords : *Lower limb joint, rehabilitation, exoskeleton suit.*

GJMR-B Classification : *NLMC Code: WB 29, WB 320, WM 308*

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I. Introduction

Injuries to lower limb joints, especially the knee and ankle are most of all musculoskeletal disorders. Because of the importance of the lower limb in everyday activities such as walking, running, the injury to these joints is urgently considered in practice. An exoskeleton suit is a powered mobile machine supplied at least part of the activation-energy for joint movement. The suit may be designed to assist and protect human to aid the survival in other dangerous environments. One of the main purposes uses an exoskeleton suit to enable a soldier to carry heavy weight during running or climbing including armor or weapon. Most of exoskeleton suit is designed by using hydraulic system. Another application could be therapy habilitation, nursing in particular. An exoskeleton suit could reduce therapy process for patient to be trained by one therapist.

Takehito et al. [1] described force-feedback mechanism of the rehabilitation system for upper limbs with active and passive mode. Steven K. Charles et al. [2] purposed a test bed to study the potential of using robots to assist in and quantify the neuro-rehabilitation of motor function. Their work focused on wrist rehabilitation, which provides three rotation degrees of freedom. Ming-Shaung Ju et al. [3] designed a robot system to assist for rehabilitation of patients with neuromuscular disorders by performing various movements. Their controller was stable in limited range of movement and force. Ahathe Koller-Hodac et al. [4] purposed a new way to assist patella mobilization during knee rehabilitation programs. They use the robotic device to perform exercises on regular basic during therapy process. S.Slavic et al. [5] developed the concept of mobile gait rehabilitation system and presented the simulation results to demonstrate the translation degrees of freedom between support platform and exoskeleton device. Robort Riener et al. [6] purposed new human-centered robotic for rehabilitation of gait in patients with movement disorders. Pengju Sui et al. [7] presented device for the ankle rehabilitation and types of motion and analyzed kinematics and workspace. Tobias Nef and Robert Riener [8] explained the principle of exoskeleton for shoulder movement providing motion of the center of joint. The current research of exoskeleton suit from Pin Wang and K.H. Low [9] developed a natural and tunable rehabilitation gait system to assist the gait rehabilitation with body weight support. Mohamed Bouri et al. [10] developed the device introducing a stationary rehabilitation system. Their work presented the flexibility and diversity of use for diagnosis. Qingling Li et al. [11] presented wearable rehabilitation robot for hemiplegic patients.

II. Leg-Exoskeleton Suit System Design

The system of leg-exoskeleton suit is properly designed to hold with human leg by belts integrated with DC motors with encoder and force sensors, microcontroller and drive system. At shaft of DC motor at each joint of the leg-exoskeleton suit, encoders are integrated to measure the rotational angle of hip, knee and ankle joints. In design, the leg-exoskeleton suit has six degree of freedom. Each leg has three DOFs for hip, knee and ankle as shown in figure 1. The weight of the leg exoskeleton suit is very important. The structure is not only light but also ensured for its durability. Thus, aluminum alloy is selected to be the structure of

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the leg exoskeleton suit. The array of force sensors are attached under belts at foot area, near under knee joint and under hip joint to measure force exerted by the human that use as input in active mode. The hardware of leg exoskeleton suit is driven by six motors mounted with encoders. The motor drive system, encoders and sensors are exchange information with microcontroller.

Then microcontroller will send the information to PCstation to display the information of leg-exoskeleton suit and sensors. The PC-station can receive the command from user with GUI application.

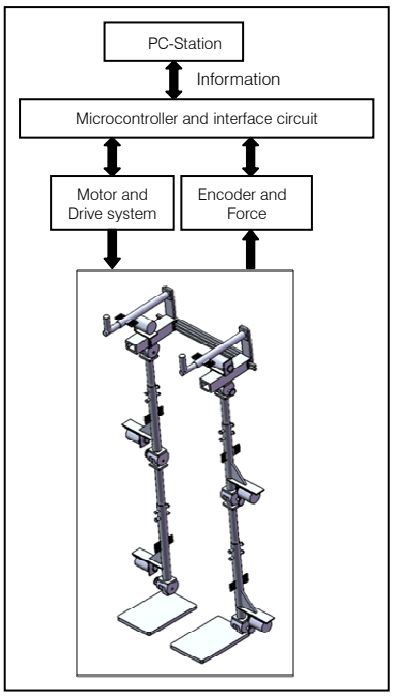

Figure 1 : The exoskeleton suit model.

III. Analysis for Leg-Exoskeleton Suit

The force analysis of mechanism is firstly considered to design the leg-exoskeleton suit. For the hip joint, torque generated by motor $(M_{1,4})$ must be more than torque generated by total weight of leg exoskeleton suit plus human leg.

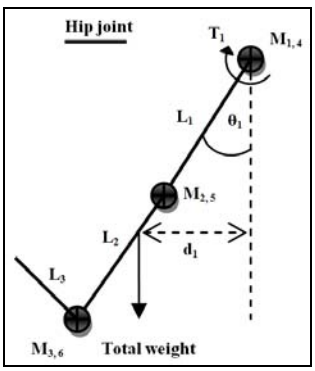

Figure 2 : Forces act at hip joint.

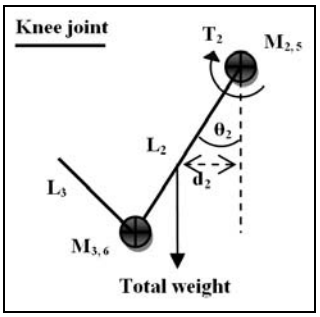

Figure 3 : Forces act at knee joint.

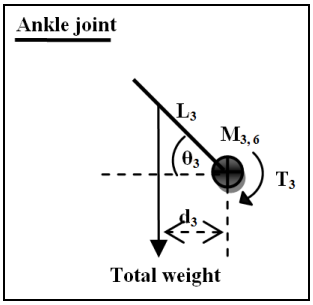

Figure 4 : Forces act at ankle joint.

For the knee joint, torque generated by motor $(M_{2,5})$ must be more than weight from knee joint to link 3.

Also, the torque by motor $(M_{3,6})$ can lift the ankle in desired position. The torque and total weight can be given as equation 1.

$$T_{hip/knee/ankle} = W_{hip/knee/ankle} \cdot d_{hip/knee/ankle} \quad (1)$$

which

$T_{hip/knee/ankle}$ = Total torque generated by motor at hip, knee and ankle joints

$W_{hip/knee/ankle}$ = Total weight of hip, knee and ankle

$d_{hip/knee/ankle}$ = The length from hip, knee and ankle joints to centre of mass due to vertica line

To calculate position of hip, knee and ankle, the Denavit-Hartenberg method is used. From the figure 5 the parameter for Denavit-Hartenberg equation must be specified due to reference coordinate.

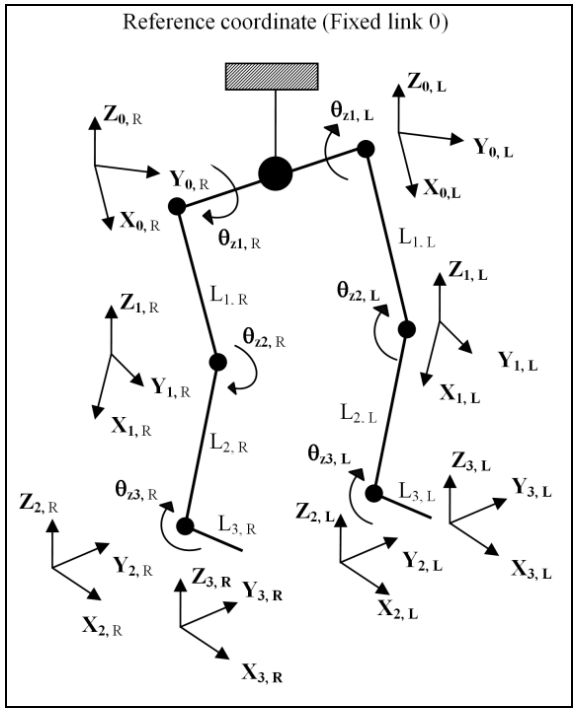

Figure 5 : Coordinates of leg-exoskeleton suit.

All parameters of left leg-exoskeleton suit are given shown in table 1.

Table 1 : Parameters of left leg for Denavit-Hartenberg equation.

Left link	$\theta_{i,L}$	α_i	a_i	d_i
1	$\theta_{1,L}$	0	$L_{1,L}$	0
2	$\theta_{2,L}$	0	$L_{2,L}$	0
3	$\theta_{3,L}$	0	$L_{3,L}$	0

For the right leg-exoskeleton suit, the parameters are given in table 2

Table 2 : Parameters of right leg for Denavit-Hartenberg Equation.

Right link	$\theta_{i,R}$	α_i	a_i	d_i
1	$\theta_{1,R}$	0	$L_{1,R}$	0
2	$\theta_{2,R}$	0	$L_{2,R}$	0
3	$\theta_{3,R}$	0	$L_{3,R}$	0

From general equation of transformation matrix from coordinate i-1 to coordinate i can be given below (McKerrow [12]).

$$ {}^{i-1}_{i}T = \begin{bmatrix} \cos\theta_i & -\sin\theta_i\cos\alpha_i & \sin\theta_i\sin\alpha_i & a_i\cos\theta_i \\ \sin\theta_i & \cos\theta_i\cos\alpha_i & -\cos\theta_i\sin\alpha_i & a_i\sin\theta_i \\ 0 & \sin\alpha_i & \cos\alpha_i & d_i \\ 0 & 0 & 0 & 1 \end{bmatrix} $$

The parameters of left leg-exoskeleton can be substituted in coordinate transformation matrix. For left hip joint, the transformation matrix with $i = 1$is given.

$$ {}^{0}_{1}T_L = \begin{bmatrix} \cos\theta_1 & -\sin\theta_1 & 0 & L_{1,L}\cos\theta_1 \\ \sin\theta_1 & \cos\theta_1 & 0 & L_{1,L}\sin\theta_1 \\ 0 & 0 & 1 & 0 \\ 0 & 0 & 0 & 1 \end{bmatrix} $$

The transformation matrix for left knee joint with $i = 2$ is Given

$$ {}^{1}_{2}T_L = \begin{bmatrix} \cos\theta_2 & -\sin\theta_2 & 0 & L_{2,L}\cos\theta_2 \\ \sin\theta_2 & \cos\theta_2 & 0 & L_{2,L}\sin\theta_2 \\ 0 & 0 & 1 & 0 \\ 0 & 0 & 0 & 1 \end{bmatrix} $$

And the transformation matrix with $i = 3$ for left ankle joint is given.

$$ {}^{2}_{3}T_L = \begin{bmatrix} \cos\theta_3 & -\sin\theta_3 & 0 & L_{3,L}\cos\theta_3 \\ \sin\theta_3 & \cos\theta_3 & 0 & L_{3,L}\sin\theta_3 \\ 0 & 0 & 1 & 0 \\ 0 & 0 & 0 & 1 \end{bmatrix} $$

As same consideration of the left leg-exoskeleton suit, the coordinate transformation matrixes of right legexoskeleton suit are given.

$$ {}^{0}_{1}T_R = \begin{bmatrix} \cos\theta_1 & -\sin\theta_1 & 0 & L_{1,R}\cos\theta_1 \\ \sin\theta_1 & \cos\theta_1 & 0 & L_{1,R}\sin\theta_1 \\ 0 & 0 & 1 & 0 \\ 0 & 0 & 0 & 1 \end{bmatrix} $$

$$ {}^{1}_{2}T_R = \begin{bmatrix} \cos\theta_2 & -\sin\theta_2 & 0 & L_{2,R}\cos\theta_2 \\ \sin\theta_2 & \cos\theta_2 & 0 & L_{2,R}\sin\theta_2 \\ 0 & 0 & 1 & 0 \\ 0 & 0 & 0 & 1 \end{bmatrix} $$

$$ {}^{2}_{3}T_R = \begin{bmatrix} \cos\theta_3 & -\sin\theta_3 & 0 & L_{3,R}\cos\theta_3 \\ \sin\theta_3 & \cos\theta_3 & 0 & L_{3,R}\sin\theta_3 \\ 0 & 0 & 1 & 0 \\ 0 & 0 & 0 & 1 \end{bmatrix} $$

To control position by left or right motors at hip joints (M_1 or M_4) due to reference coordinate, the transformation matrixes can be directly calculated by ${}^{0}_{1}T_{R,L}$. Then the transformation matrixes by left or right motors at knee joints (M_2 or M_5) due to reference coordinate can be calculated.

$$ {}^{0}_{2}T_{R,L} = {}^{0}_{1}T_{R,L} \cdot {}^{1}_{2}T_{R,L} \quad (2) $$

And the transformation matrixes due to reference coordinate by left or right motors ($M_{3,6}$) can be calculated.

$$ {}^{0}_{3}T_{R,L} = {}^{0}_{1}T_{R,L} \cdot {}^{1}_{2}T_{R,L} \cdot {}^{2}_{3}T_{R,L} \quad (3) $$

IV. Control Algorithm for Leg - Exoskeleton Suit

The principle of physical rehabilitation involves exercising and manipulating the body. It can restore joint and muscle function such as helping for people stand, body balance, walk, and climb stairs better. There are main three types of physical rehabilitation, which are active, active-assistive and passive motion. In order to leg-exoskeleton suit can operate following all types of rehabilitations, the control algorithm should be designed to cover all operations. In this work six DC motors are mainly used to drive leg-exoskeleton suit at hip, knee and ankle joints. In design of control algorithm the DC motor model is firstly considered. To control DC motor in desired position, the model of DC motor must be considered.

From mechanical torque applied to motor shaft, it equals torque constant K_T multiply by electrical armature current I_a.

$$ T_{motor} = K_T \cdot I_a \quad (4) $$

And the applied torque generates angular velocity ω according to the inertia J and friction B of motor with load.

$$ T_{motor} = J \cdot \dot{\omega} + B \cdot \omega \quad (5) $$

From equation 4 and 5, the acceleration can be given as

$$ \dot{\omega} = \frac{T_{motor}}{J} + \frac{B}{J} \cdot \omega \quad (6) $$

And the equivalent block diagram can be shown in figure 6.

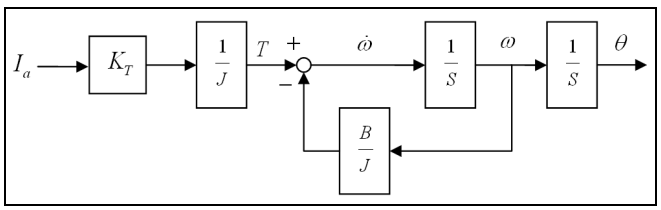

Figure 6 : DC motor block diagram.

For active motion, patients can exercise muscle or joint by themselves. In this mode the patients can move their hip, knee and ankle joints in desired position. In order to the leg-exoskeleton suit can sense patient's force, the rows of force sensor are attached under belts at link 3 (L_3), link 2 (L_2) and link 1 (L_1).

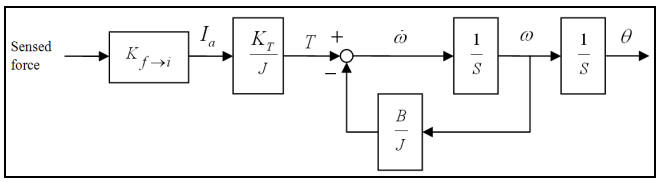

Figure 7 : Block diagram of active mode for rehabilitation.

When patient move left or right joint at hip, knee or ankle, the sensor will detect level of force and send the data to the central control unit (CCU). Then the CCU will execute and send the correspondence information to motor drive system. The resultant vector of sensed force at each joint both sides is used to adjust the current by gain $K_{f\to i}$, which will send the position as output shown in figure 7. In the passive motion mode, patients cannot actively participate in rehabilitation. Patient legs will be driven by exoskeleton suit. No effort is required from them. The desired positions of each joint must be firstly specified. In this mode, the CCU will execute the desired position as input and then sent information to motor drive system. To keep the precision in position control, the encoders are used for measuring the angle of all joints as position feedback in closed loop by using classical PID control.

$$ \delta\theta = \theta_d - \theta_a \quad (7) $$

The different angle between desired and actual angle is fed to PID loop.

$$ u = k_p \cdot (\delta\theta) + k_i \cdot \int (\delta\theta) \cdot dt + k_d \cdot \frac{d(\delta\theta)}{dt} \quad (8) $$

Where,
θ_d is the desired angle,
θ_a is the actual angle,
$\delta\theta$ is the difference between desired and actual angle,
k_p, k_i, k_d are gain parameters of PID.

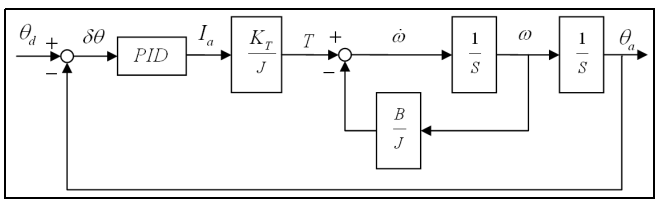

Figure 8 : Block diagram of passive mode for rehabilitation.

For the last active-assistive mode, this mode is the combination of active and passive motion for patients who can move their muscles with a little help or who can move their joints but feel pain when they do. The active-assistive mode is used when patients has capability to move their joints but has not reached the

desired level. For this mode, the patients will move joints actively at start with active motion. When the patients cannot move their joints and need help, the passive motion can be suddenly activated by patients and then the joints will be continue driven by leg-exoskeleton suit to desired point. From the block diagram, when patients start with active motion to move any joint, the information of sensed force will be sent to motor drive system.

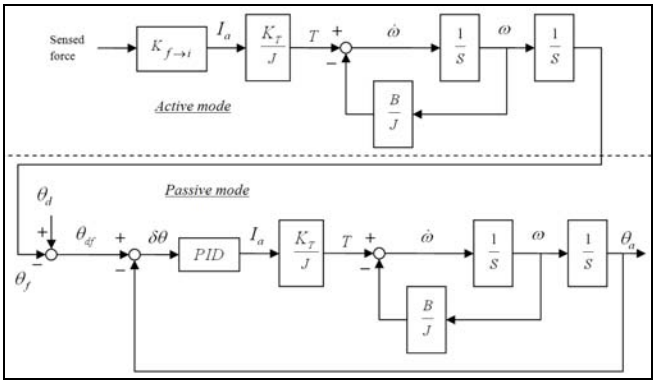

Figure 9 : Block diagram of active-assistive mode for rehabilitation.

The output θ_f from active mode will be compared with desired position θ_d . If position from active mode θ_f cannot reach to desired position θ_d , the different position θ_{df} is sent to passive mode and then the joints will be driven by leg-exoskeleton suit until its positions reach to desired position.

V. Simulation and Results

The kinematic motion of leg-exoskeleton suit is analyzed and simulated by MATLAB software. Firstly, we designed the structure of leg-exoskeleton suit by using robotic toolbox release 7.1 by Corke[13]as shown in figure 10. The hardware consists of main three joints in left and right sides and the parameters relationship between the links and joints is described based on Denavit and Hartenberg method. In simulation, the revolute, speed and acceleration of joints are studied how is the possible motion of the leg-exoskeleton suit. The results of simulation are considered to design and construct the real hardware. Many types of motion are simulated to test the conflict of links and joints. The angles of left hip, knee and ankle joints are specified in simulator with started position (0, 0, 0) and the final angle of joints are position (-90^o, 45^o, 90^o) in 10 second. The revolute angle, velocity and acceleration of hip, knee and ankle joints are shown in figure 11-13 respectively.

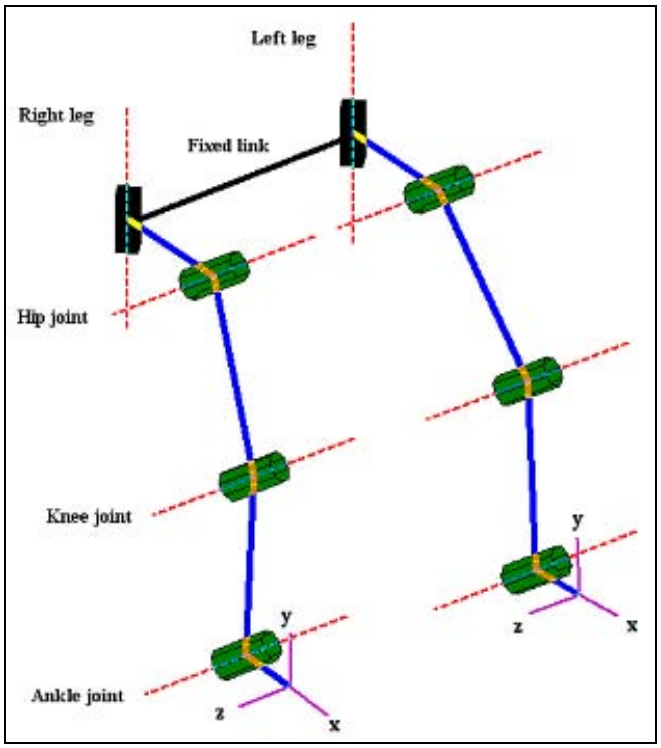

Figure 10 : The structure of leg-exoskeleton suit is designed by robotic toolbox.

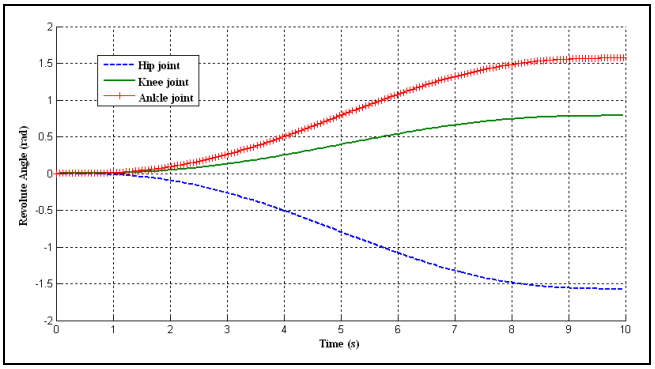

Figure 11: The revolute angle of hip, knee and ankle joints.

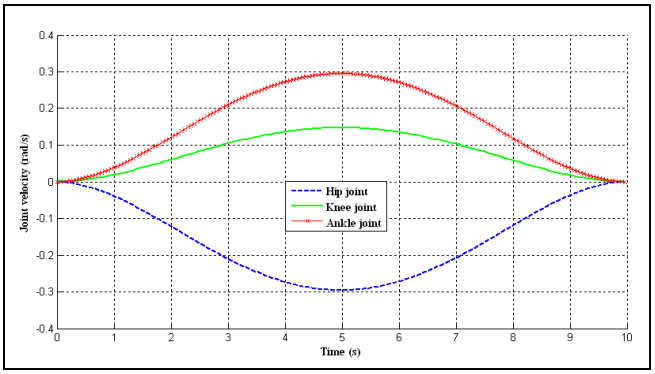

Figure 12 : The velocity of hip, knee and ankle joints.

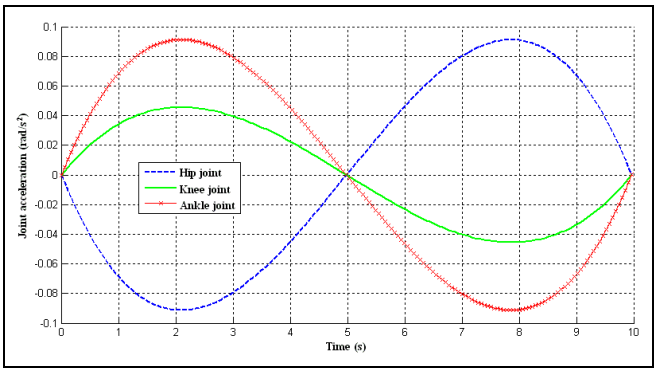

Figure 13 : The acceleration of hip, knee and ankle joints.

VI. Conclusions

The study of possible motion of leg-exoskeleton suit is focused to construct the real hardware. The design of leg-exoskeleton suit is achieved by Solid work software. By this software, the possible motion and conflict between links and joints can be simulated. In this work, three types of physical rehabilitation, which are active, active-assistive and passive motion, are analyzed and simulated. In order to leg-exoskeleton suit can operate following all types of rehabilitations, the control algorithm is designed to cover all types. In simulation, the active motion is only accomplished. The sensed force is used as input in control algorithm and the output as revolute angle, velocity and acceleration of hip, knee and ankle joints is given. The revolute, velocity and acceleration of each joint in left leg are simulated by MATLAB robotic toolbox. The leg-exoskeleton suit is specified at position of hip, knee and ankle joint angle (0, 0, 0) and the end of trajectory is position (-90^o, 45^o, 90^o).

VII. Acknowledgement

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2. Ethical Guidelines,

3. Submission of Manuscripts,

4. Manuscript's Category,

5. Structure and Format of Manuscript,

6. After Acceptance.

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Introduction:

The **Introduction** should "introduce" the manuscript. The reviewer should be presented with sufficient background information to be capable to comprehend and calculate the purpose of your study without having to submit to other works. The basis for the study should be offered. Give most important references but shun difficult to make a comprehensive appraisal of the topic. In the introduction, describe the problem visibly. If the problem is not acknowledged in a logical, reasonable way, the reviewer will have no attention in your result. Speak in common terms about techniques used to explain the problem, if needed, but do not present any particulars about the protocols here. Following approach can create a valuable beginning:

- Explain the value (significance) of the study
- Shield the model - why did you employ this particular system or method? What is its compensation? You strength remark on its appropriateness from a abstract point of vision as well as point out sensible reasons for using it.
- Present a justification. Status your particular theory (es) or aim(s), and describe the logic that led you to choose them.
- Very for a short time explain the tentative propose and how it skilled the declared objectives.

Approach:

- Use past tense except for when referring to recognized facts. After all, the manuscript will be submitted after the entire job is done.
- Sort out your thoughts; manufacture one key point with every section. If you make the four points listed above, you will need a least of four paragraphs.
- Present surroundings information only as desirable in order hold up a situation. The reviewer does not desire to read the whole thing you know about a topic.
- Shape the theory/purpose specifically - do not take a broad view.
- As always, give awareness to spelling, simplicity and correctness of sentences and phrases.

Procedures (Methods and Materials):

This part is supposed to be the easiest to carve if you have good skills. A sound written Procedures segment allows a capable scientist to replacement your results. Present precise information about your supplies. The suppliers and clarity of reagents can be helpful bits of information. Present methods in sequential order but linked methodologies can be grouped as a segment. Be concise when relating the protocols. Attempt for the least amount of information that would permit another capable scientist to spare your outcome but be cautious that vital information is integrated. The use of subheadings is suggested and ought to be synchronized with the results section. When a technique is used that has been well described in another object, mention the specific item describing a way but draw th

principle while stating the situation. The purpose is to text all particular resources and broad procedures, so that another person may use some or all of the methods in one more study or referee the scientific value of your work. It is not to be a step by step report of the whole thing you did, nor is a methods section a set of orders.

Materials:

- Explain materials individually only if the study is so complex that it saves liberty this way.
- Embrace particular materials, and any tools or provisions that are not frequently found in laboratories.
- Do not take in frequently found.
- If use of a definite type of tools.
- Materials may be reported in a part section or else they may be recognized along with your measures.

Methods:

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

Approach:

- It is embarrassed or not possible to use vigorous voice when documenting methods with no using first person, which would focus the reviewer's interest on the researcher rather than the job. As a result when script up the methods most authors use third person passive voice.
- Use standard style in this and in every other part of the paper - avoid familiar lists, and use full sentences.

What to keep away from

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

Results:

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

The page length of this segment is set by the sum and types of data to be reported. Carry on to be to the point, by means of statistics and tables, if suitable, to present consequences most efficiently.You must obviously differentiate material that would usually be incorporated in a study editorial from any unprocessed data or additional appendix matter that would not be available. In fact, such matter should not be submitted at all except requested by the instructor.

Content

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

What to stay away from

- Do not discuss or infer your outcome, report surroundings information, or try to explain anything.
- Not at all, take in raw data or intermediate calculations in a research manuscript.

- Do not present the similar data more than once.
- Manuscript should complement any figures or tables, not duplicate the identical information.
- Never confuse figures with tables - there is a difference.

Approach

- As forever, use past tense when you submit to your results, and put the whole thing in a reasonable order.
- Put figures and tables, appropriately numbered, in order at the end of the report
- If you desire, you may place your figures and tables properly within the text of your results part.

Figures and tables

- If you put figures and tables at the end of the details, make certain that they are visibly distinguished from any attach appendix materials, such as raw facts
- Despite of position, each figure must be numbered one after the other and complete with subtitle
- In spite of position, each table must be titled, numbered one after the other and complete with heading
- All figure and table must be adequately complete that it could situate on its own, divide from text

Discussion:

The Discussion is expected the trickiest segment to write and describe. A lot of papers submitted for journal are discarded based on problems with the Discussion. There is no head of state for how long a argument should be. Position your understanding of the outcome visibly to lead the reviewer through your conclusions, and then finish the paper with a summing up of the implication of the study. The purpose here is to offer an understanding of your results and hold up for all of your conclusions, using facts from your research and generally accepted information, if suitable. The implication of result should be visibly described. Infer your data in the conversation in suitable depth. This means that when you clarify an observable fact you must explain mechanisms that may account for the observation. If your results vary from your prospect, make clear why that may have happened. If your results agree, then explain the theory that the proof supported. It is never suitable to just state that the data approved with prospect, and let it drop at that.

- Make a decision if each premise is supported, discarded, or if you cannot make a conclusion with assurance. Do not just dismiss a study or part of a study as "uncertain."
- Research papers are not acknowledged if the work is imperfect. Draw what conclusions you can based upon the results that you have, and take care of the study as a finished work
- You may propose future guidelines, such as how the experiment might be personalized to accomplish a new idea.
- Give details all of your remarks as much as possible, focus on mechanisms.
- Make a decision if the tentative design sufficiently addressed the theory, and whether or not it was correctly restricted.
- Try to present substitute explanations if sensible alternatives be present.
- One research will not counter an overall question, so maintain the large picture in mind, where do you go next? The best studies unlock new avenues of study. What questions remain?
- Recommendations for detailed papers will offer supplementary suggestions.

Approach:

- When you refer to information, differentiate data generated by your own studies from available information
- Submit to work done by specific persons (including you) in past tense.
- Submit to generally acknowledged facts and main beliefs in present tense.

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

INDEX

R

S

T

U

V

Z

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