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Aerial Pollutant Gases Concentrations in Tropical Pig Pen Environment in Nigeria

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ABSTRACT: Research on the concentrations of aerial pollutant gases in tropical livestock buildings is needed in order to establish baselines for exposure limits in context of animal and human welfare in the tropical environments. The concentrations of aerial ammonia, nitrous oxide, methane, carbon monoxide, hydrogen sulfide and sulfur dioxide in selected pigpens in the Owerri area of Imo State, Nigeria were measured during the month of August 2002. These were thereafter correlated with pig building measurements in order to determine the possible influence of building measurements on concentration of the gases. Overall mean aerial concentrations of carbon monoxide CO (2.7 ± 0.34 ppm) was the highest mean value recorded and was followed by the 1.4 ± 0.0 ppm and 0.07 ± 0.14 ppm recorded for flammable gas (methane) and hydrogen sulfide respectively while the 0.0 ± 0.0 ppm recorded for ammonia was lowest. The average length of the buildings was 64.20 ft., while 21.40 ft., 11.60 ft and 4.35 ft. were obtained for width, height and sidewalls respectively. In all pens, the simple linear regression was not significant ($P<0.05$), with the coefficient of determination ranging from 0.001 (SO_2) to 0.364 (CO). For the different aerial pollutant gases, the best linear predictor was for CO using height of building followed by SO_2 using height and so on based on the R^2 values. Even though this study returned low levels of gases for the area during the month of August, there is the need to extend the study to other periods of the year especially the dry season months in other to further elucidate the effects of inclement whether on gas concentrations in pig pens. [Nature and Science. 2006;4(4):1-5].

Key words: Pig, pollutant gases, pigpen environment, tropical, Nigeria

INTRODUCTION

Livestock production in the tropics is essentially categorized into extensive, semi-intensive and intensive production systems. The intensive system usually involves commercial production of high performance exotic breeds of livestock. This system is resource driven and requires the operator to be in control of the housing, nutritional and health needs of the livestock (Williamson and Payne, 1978). The relative success of commercial pig and poultry production in the tropics (Delgado et al., 1998; FAO, 2000) has made these livestock business ventures very attractive in most developing countries.

In Nigeria, most livestock buildings used in intensive production are open sided affairs, roofed with corrugated iron sheets. While these open sided designs promote natural ventilation and are cheap to construct, non-insulated corrugated iron roofs may transmit considerable amount of incoming solar radiation into the livestock pen and may thus cause heat buildup. This constitutes a serious production problem especially in the warm humid tropical environment (Williamson and Payne, 1978; Oluyemi and Roberts, 2000). Indeed, there is hardly any attempt at enforcing standards in livestock building design and construction in Nigeria either for the benefit of the health of the operators or for that of the welfare of the animals. For example in the warm tropical environment of southeastern Nigeria, humidity of air is high and air movement is usually slow (Harry, 1978). There is the need to consider these while designing and constricting of livestock pens.

One of the untoward effects of poor livestock housing design and construction is aerial pollutant gases buildup in livestock buildings. The concentrations of these pollutants in livestock houses and their emission rates have been studied extensively in developed economies and several comprehensive reviews have been published (Harry, 1978; O'Neil and Philips, 1992; Wathes et al., 1997). These studies have shown that the potential effects of air quality on livestock productivity involve complex interactions between physiological behavior and disease (Wathes, et al., 1983; Wathes, et al., 1998). There is also very strong evidence for occupational respiratory disease in those who work

with livestock (Donhann et al., 1995; Reynolds et al, 1996). This is believed to arise from chronic exposure over several years to complex mixtures of aerial pollutants in livestock pens. For example, the physiological and psychological effects of extended ammonia exposure on man may include nausea, headache, depression and dizziness (Donhann et al., 1995; Borgers et al., 1997).

Furthermore, there is evidence that poor air quality influence the incidence and severity of common endemic respiratory diseases of pigs (Muirhead and Alexander, 1997; Wathes, 2001). Such information are however lacking for most tropical farming environments. Research on the concentrations and emission rates of aerial pollutant gases in tropical livestock buildings is therefore needed in order to establish baselines for exposure limits in context of animal and human welfare in the tropical environments.

This study reports recent field measurements of the concentrations of aerial ammonia, nitrous oxide, methane, carbon monoxide, hydrogen sulfide and sulfur dioxide in selected pig pens in the Owerri area of Imo State during the month of August 2002 and their correlation with livestock building measurements.

MATERIALS AND METHODS

Study area: Imo state is situated in the southern rain forest vegetational belt of Nigeria. It lies between latitude 5^0 and 6^0 3'N and longitude 6^0 15' and 7^0 34' E. The area is dominated by plains 200m above sea level except for elevations associated with the Okigwe uplands (Ofomata, 1975). It has an annual rainfall of about 1700 mm to 2500 mm, which is concentrated almost entirely between March and October. Average relative humidity is about 80% with up to 90% occurring during the rainy season. The mean daily maximum air temperature range from 28^0c to 35^0c while the mean daily minimum range from 19^0c to 24^0c.

In this rainforest zone, smallholder livestock production predominate with over 80% of rural families keeping west African Dwarf (WAD) ruminants and mixed breeds of local and exotic chicken (Molokwu, 1982; Ejiogu, 1990) primarily as source of investment, manure and meat at home or during festivals. In most parts of the state livestock are allowed to roam throughout the seasons, with little supplementation from kitchen wastes (Okoli et al., 2003). Prophylactic management of common infectious diseases is rarely practiced.

Pollutant gases and pig house measurements: Measurements of the concentrations of aerial ammonia NH_4, nitrous oxide (NO_2), Flammable gas (methane, CH_4), carbon monoxide (CO), hydrogen sulfide (H_2S) and sulfur dioxide (SO_2) were made in five intensive pig farms located in the Owerri area of Imo state during the month of August 2002. Average number of pig per pig house was 58 pigs. The measurements were taken between 10^{th} and 20^{th} August 2002 and during morning (9-11am.) and afternoon (1-3pm) hours. Each house was monitored once over a period of 12 hours. The procedure described by Wathes et al. (1997), which involves taking representative readings at different locations in a pen, was adopted. The factors considered included proximity to the open sided wall or middle of the pen as well as the sampling height. Such representative readings from each pen were later pooled to obtain the mean for each pen.

Concentrations of gases were measured in parts per million (ppm) as well as lower emissible limit (LEL) in the case of flammable gas (Methane) using the Gasman hand held personal gas detector (Crowcon, Instruments Ltd. England) that employs a catalytic bead sensor for flammable gas and electrochemical sensors for other gas measurements. During the gas measurements, these hand held equipment were held at about 2.5 feet above the litter level and the readings were recorded within 10 seconds. All analyses were calibrated for zero and span before and after reading.

In each farm, both inside and outside air temperature of the pens and relative humidity were also recorded. The length, width and height (floor to ridge) as well as the height of the sidewalls of the pens were measured in feet. The husbandry system practiced in these farms and the type of pig breeds kept were equally recorded.

Data analysis: Data generated were subjected to statistical analysis such as simple averages and analysis of variance (ANOVA). Where significant differences were observed, means were separated using the Duncan's multiple range test method, (Steel and Torrie, 1980). Furthermore, simple linear regression statistics was used to determine the possible influence of building measurements on concentration of the gases.

RESULTS

General observation: All the five farms studied were involved in commercial pock production with replacement stock being also being produced. The major breeds reared were large white, landrace and landrace x large white crosses (Table 1). Mean daily air temperature during the period of study was 26.35^0C while mean relative humidity was 88%. Daily removal of dung was practiced in all the farms. Roofing materials

utilized were mainly corrugated iron and asbestos sheets.

Aerial pollutant gases concentrations: Overall mean aerial concentrations of carbon monoxide CO (2.7 ± 0.34ppm) was the highest mean value recorded and was followed by the 1.4 ± 0.0 ppm and 0.07 ± 0.14 ppm recorded for flammable gas (methane) and hydrogen sulfide respectively while the 0.0 ± 0.0 ppm recorded for ammonia was lowest (Table 2).

The range of 3.5 ± 1.7 ppm to 4.0 ± 0.0 ppm of CO obtained in PA, PC and PE were significantly higher ($P<0.05$) than those of the other pens. The 2.0 ± 0.0 ppm of FM was obtained in PC and PD and was significantly higher ($P<0.05$) than those of the other pens. Similarly, the 0.15 ± 0.3 ppm H_2S obtained in PC was significantly higher than those of other pens (Table 2).

The building measurements: The average length of the buildings was 64.20ft., while 21.40ft., 11.60ft and 4.35ft were obtained for width, height and sidewalls respectively. The highest length value was the 114 ft. obtained in PB and PC while 8ft was recorded as the lowest measurement and was obtained in PE. The highest width value recorded was 32 ft. (PB) while the lowest was the 7ft obtained in PE (Table 3).

Correlation of aerial pollutants and pig house measurements: In all pens, the regression was not significant ($P<0.05$), coefficient of determination ranging from 0.001 (SO_2) to 0.364 (CO). For the different aerial pollutant gases, the best linear predictor was for CO using height of building followed by SO_2 using height and so on based on the R^2 value of the regression (Table 4).

Table 1. Husbandry methods employed in the various pig farms.

Pens	Floor type	Breed of pig	Dung Mgt.	Flock size	Roofing method	Ambient Temp (^{0}C)	Relative humidity (%)
PA	CF	LW	DRD	85	Asbestos Sheets	26.7	87.0
PB	CF	L x LW	DRD	123	Iron Sheets	26.5	88.0
PC	CF	LW	DRD	114	Iron sheets	26.2	88.5
PD	CF	L x LW	DRD	45	Iron Sheets	26.5	85.8
PE	CF	LW	DRD	8	Iron sheets	26.5	87.0

P = Pigpen, CF = Concrete floor, LW = Large white, L = Landrace, DRD = Daily removal of dung.

Table 2. Concentration of aerial pollutant gases in selected pigpens.

Pens	FM (LEL)	NH_3 (ppm)	CO (ppm)	SO_2 (ppm)	H_2S (ppm)	NO_2 (ppm)
PA	1.0±0.0	0.0±0.0	3.5±1.7^a	0.05±0.3	0.2±0.4	0.1±0.0
PB	1.0±0.0	0.0±1.0	2.0±1.0^b	0.1±0.0	0.0±0.0	0.2±0.0
PC	2.0±0.0^a	0.0±0.0	4.0±1.0^a	0.1±0.0	0.15±0.3	0.15±0.3
PD	2.0±0.0^a	0.0±0.0	1.0±1.0	0.0±0.0	0.0±0.0	0.1±0.0
PE	1.0±1.0	0.0±0.0	3.0±0.0^a	0.1±0.0	0.0±0.0	0.15±0.3
Total	7.0	0.0	13.5	0.35	0.35	0.5
Mean	1.4±0.0	0.0±0.0	2.7±0.34	0.07±0.14	0.07±0.14	0.1±0.08

ab, means in the same column with different superscript are significantly different ($P< 0.05$).

Table 3. Structural measurements of pig houses used for aerial pollutant gases measurements.

Pens	Length (Ft)	Height (Ft)	Width (Ft)	Sidewall (Ft)
PA	38.00	11.00	23.00	3.25
PB	114.00	15.00	32.00	5.00
PC	114.00	11.00	25.00	4.00
PD	47.00	14.00	20.00	5.00
PE	8.00	7.00	7.00	4.50
Total	321	58	107	21.75
Mean	64.20	11.60	21.40	4.35

Table 4. Prediction equations for concentrations of aerial pollutants gases using pig house measurements.

Aerial pollutants	Prediction equation	R^2	Standard Error	Significance
CO	CO = 22.983 – 5.860W	0.006	10.571	NS
	CO = 15.836 – 1.569H	0.364	2.882	NS
SO_2	SO_2 = 20.875 + 7.500W	0.001	10.595	NS
	SO_2 = 13.438 + 26.250H	0.141	3.351	NS
H_2S	H_2S = 19.816 + 22.632W	0.058	10.291	NS
	H_2S = 11.987 – 5.526H	0.032	3.561	NS
NO_2	NO_2 = 11.000 + 74.286W	0.115	9.976	NS
	NO_2 = 10.000 + 11.427H	0.023	3.572	NS

NS = Not significant; **W** = Width pen; H = Height of roof

DISCUSSION

Temperatures in the pens during the period of measurements were within the optimal range of 26 - 27^{O}C, while the mean humidity of 88.0% was much higher than the desirable 60-70% (Ferguson, 1986). This very high relative humidity is attributed to the period of the year (August), which is usually a heavy rainfall period. High temperature and humidity causes wet litter, which enhances the growth and multiplication of fungal, protozoa and bacterial pathogens in the tropics (Oluyemi and Roberts, 2000).

The present results revealed that the concentrations of the various aerial pollutant gases in the pigpens were relatively low during the month of August. For example, the mean concentration of ammonia in this study was 0.0ppm, while that of CO was 2.7 ppm. These figures are much lower than the current exposure limits recommended for animal welfare in Europe or the averages of 12.3 ppm and 24.2 ppm obtained in poultry houses in the UK during winter and summer month (Wathes et al., 1997; CIGR, 1992). It would seen from this study that the relatively clement weather conditions associated with mild temperatures and increased air movement during this period of the year may have helped in moving gases generated inside the pig pens to the outside. These measurements probably demonstrate relatively high standard of air quality in the pens. It would seem from the present result that the tropical open sided pens being used in the study area are well suited for pig production during the month of August. Because of the low levels of gases obtained during the period of study, there is the need to extend the study to other periods of the year especially the dry season months in order to further elucidate the effects of inclement whether on gas concentrations.

The study revealed that there is lack of standardization in the construction of pigpens in the study area. Some of the houses were either very wide or very low. The optimal width of pigpens in the humid tropics has been shown to be 10m wide, with 1.2m internal passages (Williamson and Payne, 1978). These help for more effective ventilation and reduction of heat irradiation from the roof especially during the hot periods of the year.

The study equally revealed the relative contribution of pig house dimension to the concentration of aerial pollutants and tried to explain the rate of increases of these pollutants due to changes in house measurements. The relative magnitude of the regression coefficient obtained was highest and positive for NO2 (74.286) and lowest and negative for CO (1.569). These indicate the average rate of increase or decrease of aerial pollutant gases concentrations with respect to the building dimensions

CONCLUSIONS

Even though this study returned low levels of gases for the area during the month of August, there is the need to extend the study to other periods of the year especially the dry season months in other to further elucidate the effects of inclement whether on gas concentrations in pig pens.

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Single Nucleotide Polymorphisms associated with the Intronic *Cis* Regulatory Regions of *PAX7*: A Potential Linkage to Increased Tumorigenesis of Rhabdomyosarcoma elucidated via *In Silico* Biology and Pyrosequencing™

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Abstract: Motivation. Single nucleotide polymorphisms (SNPs) exist as a type of genetic variation that can be either pathogenic or non pathogenic based on their influence on phenotype. At the date of the writing of this paper, there are 402 single nucleotide polymorphisms associated with intronic regions of human *PAX7*, which is found on chromosome one. Of these 75 are present in the intronic gene region of *PAX7* associated with alveolar rhabdomyosarcoma (ARMS) mainly found in the 3 prime regions of introns 5,6,7 and 8. The aim of this research is to identify those SNPs most likely to affect *PAX7* mediated tumorigenicity, particularly for alveolar rhabdomyosarcoma with which *PAX7* is associated. The research elucidated in this paper also examines the efficacy and ease of using *in silico* biological methods *prior* to the commencement of bench work to increase the success rate of SNP detection and allele quantification. Methods. *In Silico* Biology and Pyrosequencing™ are employed to confirm results obtained by conventional PCR gel electrophoresis and Sanger sequencing. Results. *In Silico* Biology and Pyrosequencing technologies are shown to be faster, more cost-efficient than conventional PCR and Sanger sequencing and provide excellent adjunct techniques to identify alterations in gene sequence. For *PAX7*, seventy-five intronic SNPs were identified to be within close vicinity (50 - 100 nucleotides) of intronic *cis* regulatory elements and some of these remain within the *PAX7* gene region present in the *PAX7-FOX1* fusion gene associated with alveolar rhabdomyosarcoma. Of these seventy-five, five were found to be different in alveolar rhabdomyosarcoma patients, both by conventional PCR, Sanger sequencing and Pyrosequencing. Changes due to LOH were revealed by the current genotyping results obtained by *in silico* means from the NCBI website and thus may provide an indication of altered sequences that contribute substantially to increased tumorigenicity. Moreover, six SNPs were within a conserved sequence in intron 5 & 8 of *PAX7* that is highly conserved in homologous *Pax7* genes of several animal phyla as well as in the exon/intron 23 regions of *NF-1* and intron 7 of *PAX3*. [Nature and Science. 2006;4(4):6-20].

Keywords: Single Nucleotide Polymorphisms; *PAX7;* Rhabdomyosarcoma; pyrosequencing, sequencing; ERMS, ARMS.

Abbreviations and notations: ARMS, alveolar rhabdomyosarcoma; ERMS, embryonal rhabdomyosarcoma cell lines; CNP, Copy Number Polymorphism; AQ, Allele Quantification; PSQ, Pyrosequencing; RD, Rhabdomyosarcoma; SNP, Single Nucleotide Polymorphism; TSS, transcription start site

1. INTRODUCTION

Single nucleotide polymorphisms (SNPs) exist as a type of genetic variation that can be either pathogenic or non-pathogenic based on their influence on phenotype. Up to the date of writing this paper, 402 single nucleotide polymorphisms have been identified within the intragenic region of the human *PAX7* gene locus, on chromosome 1. In a previous study [Barr et al., 2002], exonic sequences were examined for the presence of SNPs that could be associated with *PAX7* mediated pathogenesis. In this paper we have searched the intronic chromosomal sequences of *PAX7* for the presence of SNPs that may be associated with increased tumorigenesis. While it is well known that SNPs within the exonic regions of a gene may change the protein structure and affect the functionality of the resultant protein, SNPs within intronic regions are less commonly associated with changes in functionality. However changes to intronic sequences may affect gene

expression levels or mRNA stability and/or gene splicing which may also lead to increased pathogenesis and tumorigenesis. The research elucidated in this paper identifies intronic SNPs that may be associated with *PAX7* mediated tumorigenicity in the childhood tumour alveolar rhabdomyosarcoma. Here we also examine the efficacy and ease of using *in silico* biological methods *prior* to the commencement of bench work to increase the success rate of SNP detection and allele quantification. Our findings show that mutations do indeed occur within the immediate vicinity (50 – 100 nucleotides) of the given *cis-acting* elements found in previous research (Mitchell et al., 2006).

2. MATERIALS AND METHODS

In Silico Investigation

The introns of *PAX7* were scrutinized with five programs (three online, two offline and saved on PCs) which denote *cis* regulatory regions and/or transcription start sites (TSS) in submitted sequences. In our recent paper (Mitchell et al., 2006) we identified several *cis* regulatory elements in intronic regions of *Pax7*. The focus of the research for this paper is centered on identification of polymorphisms in the intronic regions of *Pax7* that may be associated with these *cis* regulatory elements. To do this the National Center for Biotechnology Information (NCBI) database for SNPs, incorporated into NCBI's Entrez system was queried to identify SNPs. Similarly Entrez databases, PubMed and GenBank were also queried. The Boolean advanced query allows one to use limits to restrict your search by search field, chromosome, and percentage of heterozygosity. To date (June 2006), we have identified four hundred-two SNPs associated with *PAX7* introns. After refining the search further based on the location of the SNPs (exon vs. intron) within the NCBI SNP database, seventy-five SNPs were identified as being associated with *PAX7* intronic regions and rhabdomyosarcoma, that is they remain within the *PAX7* region of the *PAX7-FOX1* fusion gene that is associated with alveolar rhabdomyosarcoma and are present within the intronic regions of *PAX7* found aberrantly expressed in some embryonic rhabdomyosarcomas. Furthermore, the seventy-five SNPs of introns 5, 7 and 8 were chosen for further investigation since the SNP locale statistics show that these three intronic regions had the highest concentration of single point polymorphisms. The list of seventy-five SNPs were analyzed relative to the results obtained by *in silico* methods to identify those polymorphisms close to previously identified *cis* regulatory regions (Mitchell et al., 2006). The finalized list contained thirty-six SNPs associated with the intronic regions of *PAX7* and rhabdomyosarcoma and are included in the transcript variants of PAX7.

Tumor Samples and Control Samples

DNA from five ARMS samples was analyzed; all five samples were from tumours previously identified as having the *PAX7-FKHR* (1:13) translocation associated with ARMS (Tang et al., 1999) and were obtained from the Biomaxx,Inc. One of the DNA samples was isolated from an ARMS cell line (CW9019), and four were DNA isolated from patient tumour samples. In addition DNA was isolated from ERMS cell line ATCC#: CCL 136. In addition, DNA was isolated from buccal swabs of five normal volunteers.

DNA Isolation

DNA from cell lines and tumour samples was isolated using Gentra Autopure LS; the volunteer samples were extracted with the BuccalAmp™ DNA extraction kit, QuickExtract™ DNA extraction solution, and Catch-All™ sample collection swabs (EPICENTRE® Biotechnologies http://www.epibio.com) were used for the DNA isolation of the five normal samples submitted by volunteers.

Primer & Assay Design

The primer design for Pyrosequencing was performed by utilizing the Biotage AB Assay Design Software version 1.0.6. Thirty to sixty base pairs 5' and 3' of the target polymorphisms were entered into the Assay Design program to identify the best forward, reverse, and sequencing primers. The automated program has as its output a list of scored (100=best, 60 or below passed to poor) primers for the target SNP(s).

PCR Analysis / Gel Electrophoresis

Because of the limited quantity of DNA available on the ARMS cases for this study, a DNA dilution–based PCR assay was used for allele quantification and SNP detection. The following DNA concentrations (serial dilutions) were examined for the five cases; 2, 1, 0.5, 0.25, 0.125 ng/ul. One ul of each dilution was used as the DNA template for the PCR reactions. PCR was performed in a volume of 25 ul with the presence of 100 uM of each dNTP, 10 mM Tris-HCl (pH 8.3), 2 mM magnesium chloride, 50 mM potassium chloride, 0.75 unit of Taq DNA polymerase, and 15 pmoles of each custom primer. The following reaction conditions were employed: denaturation at 96°C for 5 min,

followed by 60 cycles each of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C, with a final extension of 5 min at 72°C. Sixty cycles were used due to the small size of the fragment and the low concentration of the isolated patient DNA. Subsequently, aliquots of the reaction were resolved on 2% / 4% Invitrogen™ commercial pre-cast agarose E-gel, and the products were visualized by Sybr-Safe Green staining.

Pyrosequencing

Twenty microliters of the biotinylated PCR products was immobilized on streptavidin-coated magnetic beads (10 µl of Dynabeads M-280 streptavidin solution [Dynal, Oslo, Norway]) in 25 µl of 2x binding-washing buffer II (10 mM Tris-HCl, 2 M NaCl, 1 mM EDTA, and 0.1% Tween 20 [pH 7.6]) at 65°C for 5 min in a shaking mixer (1,400 rpm). The PCR product-Dynabeads complexes were captured with a PSQ 96 Sample Prep Tool (Pyrosequencing AB, Uppsala, Sweden) and transferred to a 96-well PSQ 96 SQA microtiter plate containing 0.5 M NaOH (50 µl per well), and single-stranded DNA was obtained through incubation for 4 min.

DNA Sanger Sequencing Big Dye Terminator Reaction

Sanger Sequencing has been around since 1977 [Rosen et al., 2000; Murphy et al.,2005]. Many advances have been made since then in terms of technology and the race to completely sequence the human genome. It was quickly realized that there are many SNPs and tandem repeats throughout the genome. Sanger sequencing is a very expensive, time consuming process requiring hours to review data and create contiguous traces using Phred and Phrap. The results of the combined procedure (Sanger and Pyrosequencing) are seen below in figures 5. After the Big Dye Terminator reaction of the samples, ethanol precipitation was used to remove excess dye. Twenty microliters of Hi-Di formamide was used and then the samples were denatured for four minutes at 98°C. Then, the sequencing samples were allowed to cool for 5 minutes. Run time of the samples in the 3730XL DNA Sequencer (Applied Biosystems©) was 1 hour. Unfortunately, without performing PCR and fragment analysis, allele quantification is not possible from standard Sanger sequencing.

3. RESULTS OF *IN SILICO* BIOLOGY

In silico methods were used to scan intronic regions of *PAX7* for SNPs (this paper) and *cis* regulatory regions (Mitchell et al., 2006). SNPs (three) located within or close to *cis* elements were identified for further investigations *in vitro* (Figs. 1-3).

***In Silico* PCR Amplification** (http://insilico.ehu.es/) & (http://genome.ucsc.edu/cgi-bin/hgPcr)

In-Silico PCR amplification was performed on the three SNPs identified by *in silico* methodology described above. *In-Silico* PCR amplification provides the user a systematic approach to prescreen the custom-designed primers, before purchase for the given template. It also elucidates the prediction of probable PCR products and search of potential mismatching location of the specified primer(s). The output of these programs allow for quick and easy changes to primer design or template choices. This process was employed for every custom-designed primer created by the PSQ software prior to purchase. The two websites compare the user's template and primers.

4. RESULTS OF *IN VITRO* STUDIES BASED ON RESULTS OF *IN SILICO* METHODS

Standard PCR Analysis/ Gel electrophoresis

Standard PCR assays performed on all seven samples revealed amplification of the three selected SNPs found in the intronic regions of the *PAX7* gene (**Figure 4**). The gene-specific fragment was visualized at all dilution levels for all these cases. **Figure 4** depicts positive PCR amplification for the control sample using primers 23, 24, and 40.

Pyrosequencing™ for allele quantification and confirmation of SNP detection

Pyrosequencing™ by Biotage©, although first initiated in 1993 (Sanger et al., 1977; Nyren et al., 1993) is based on the Sanger sequencing by synthesis method. Data from the Pyrosequencing method is a quantitative measure of each detected nucleotide and is used for measuring the amounts of alleles. This property allows the quantification of heterozygosity, multi-copy genes, pooled DNA samples, and mixed genotypes in heterogeneous samples (i.e. tumor and normal cells). In essence the user can get results from one Pyrosequencing plate that would equal four separate processes (RT-PCR, Big Dye Terminator cycle sequencing, and fragment analysis). Due to the small size of the fragment, twenty microliters of PCR product was used in the pyrosequencing reactions performed here. The biotinylated primers identified by *in silico* methods shown above were used to process the samples. Results are shown below. The total processing time of the samples was eight minutes, confirming the dramatic decrease in processing time over conventional Sanger sequencing.

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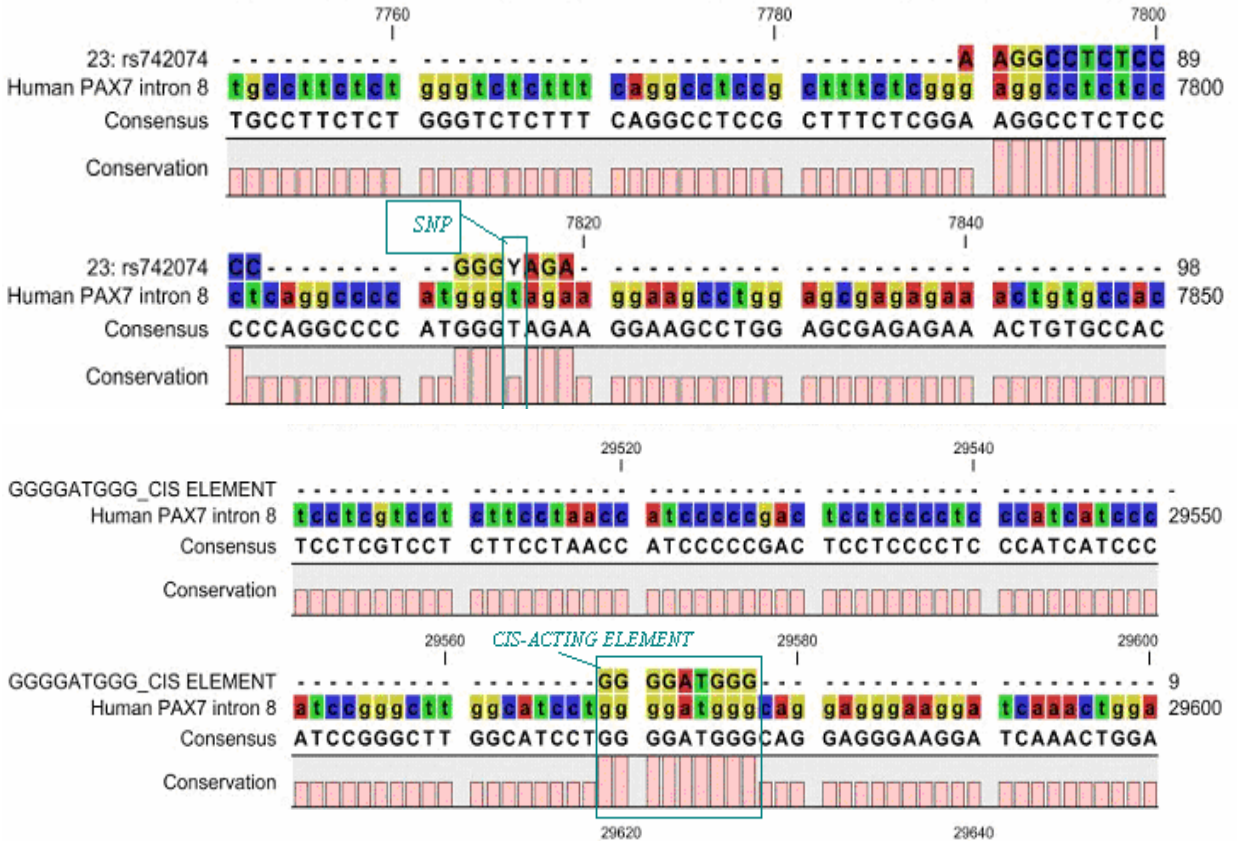

***Figure 1. Cis element(GGGGATGGG** indicated in teal; Mitchell and Ziman 2006) **found in Human PAX7 intron 8 located near SNP rs742074 (Y = IUPAC code* for (C/T)).**

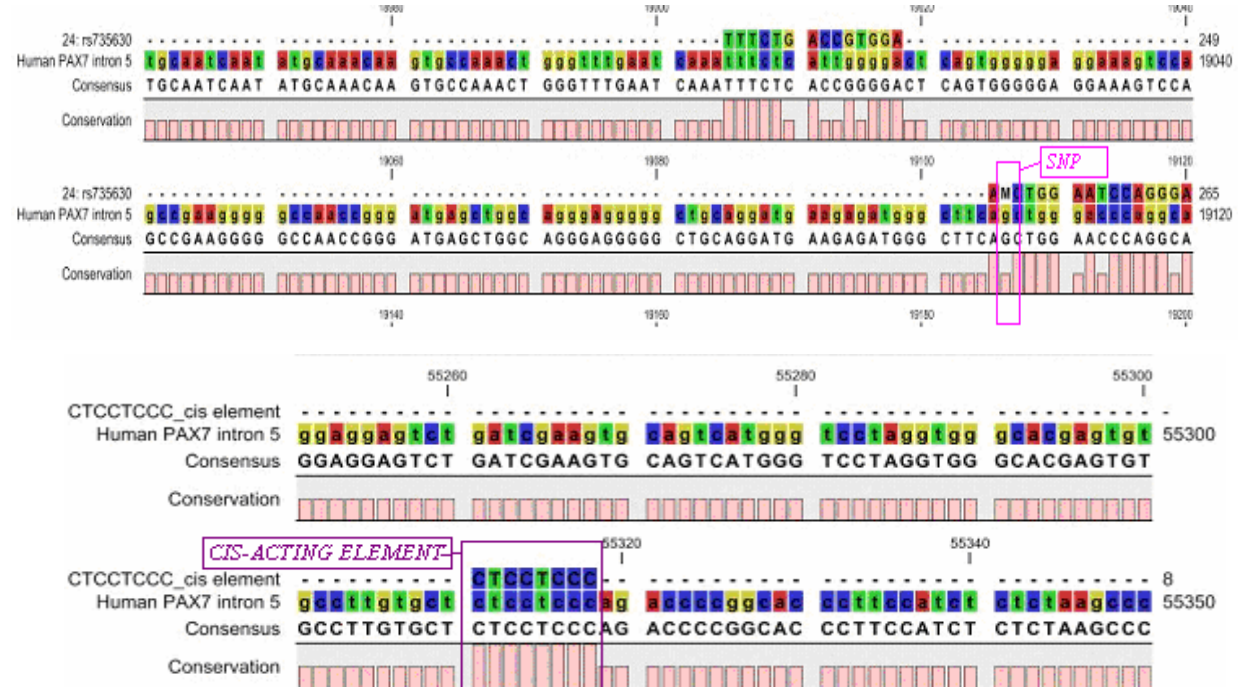

***Figure 2. Cis element CTCCTCCC** indicated in pink (Mitchell and Ziman 2006) found in Human PAX7 intron 8 located before SNP rs735630 (M = IUPAC code for C/A).*

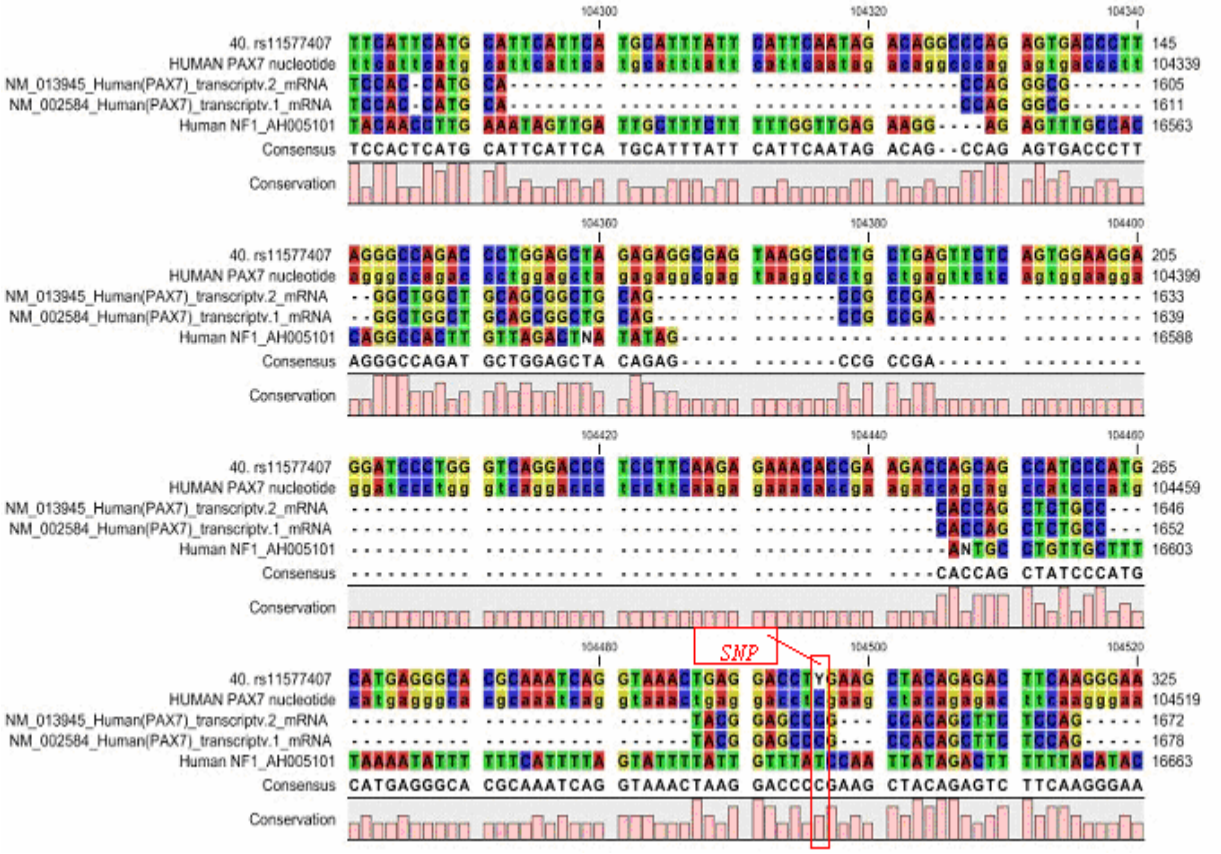

Figure 3. Multiple alignment of PAX7 transcript vqriants (NM_013945 and NM_002584 respectively), Human NF1, and the single nucleotide polymorphism found in the 3' end of Human PAX7 intron 8 located before SNP rs11577407 (Y = IUPAC code for C/T) indicated in orange.

***Cis* –acting element: GGGGATGGG**

Primer 23: rs742074 [*Homo sapiens*] Intron 8 chromosome position: chr1:18802796-18802962

Genotype Detail

T: 0.955

C: 0.045

Primer	**Sequence**
Forward PCR Primer	TAATCCACCCCTCTCAAGAATG
Reverse PCR Primer	TGAGATTTGCCTTCAGATAAAACC
Sequencing Primer	ACCAGGAAAAAAGTAAAAT

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***Cis* –acting element: CTCCTCCC**

Primer 24: rs735630 [*Homo sapiens*] Intron 5 chromosome position: chr1:18726993-18727052

Genotype Detail

A: 0.950

C: 0.050

Primer	**Sequence**
Forward PCR Primer	CGCAGCCCTTGTTTCTGA
Reverse PCR Primer	CAGGGGCCAGAAGCTGGT
Sequencing Primer	CTTGTTTCTGACCGTG

NF1 associated Primer 40: rs11577407 [Homo sapiens] Intron 8 chromosome position: chr1:18807266-18807391

Primer	**Sequence**
Forward PCR Primer	ATGAGGGCACGCAAATCAG
Reverse PCR Primer	ACCCCAGAGACACGAGCAC
Sequencing Primer	AATCAGGTAAACTGAGGAC

Genotype Detail

C:0.998

T:0.002

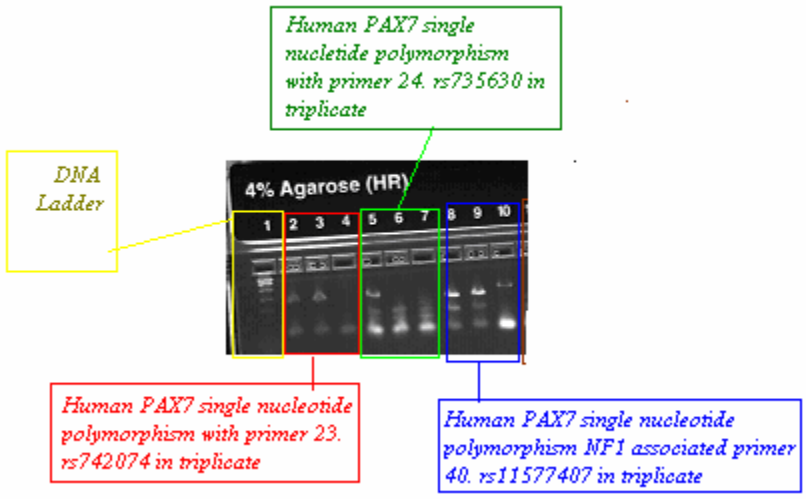
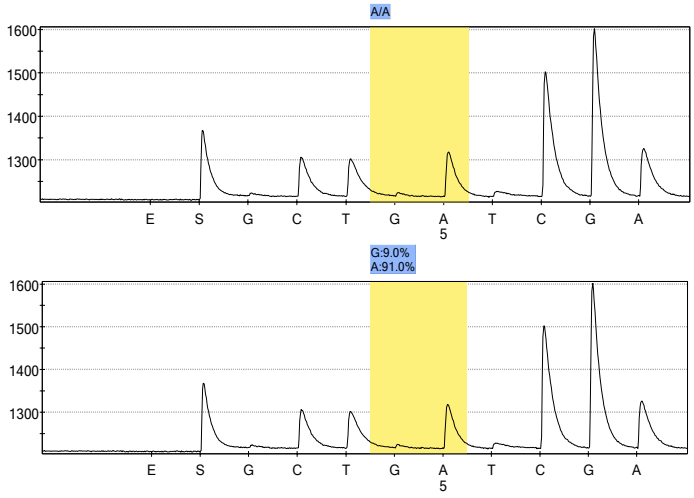


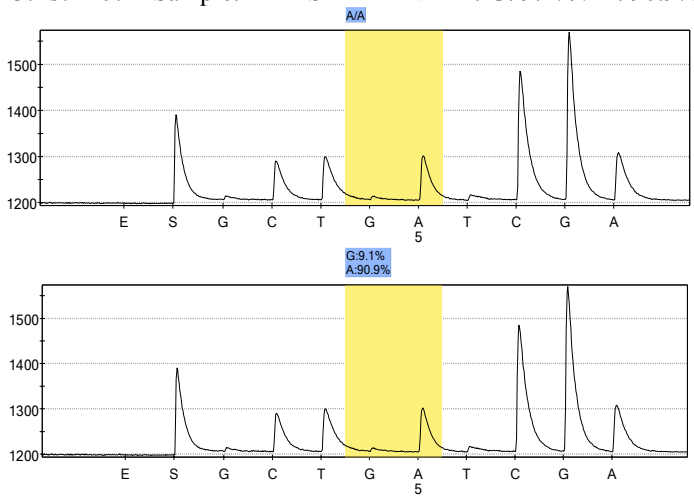
Figure 4. Invitrogen™ 4% precast Agarose Gel electrophoresis showing serial dilution for DNA isolated from volunteer buccal swab. Sybr-Safe staining resolved on Bio-Rad UV Gel Doc 2000. Gel electrophoresis of DNA isolated from ATCC #136 ERMS Cell line (not shown) , ARMS patients(1-5; (not shown)) and control sample (shown below) showed similar results with the biotinylated primers 23,24 and 40.

Figure 5 shows the results of the Biotage© Pyrosequencing™ PSQ HS 96A & Sanger sequencing for Primers 23, 24 & 40 for ARMS, ERMS and Control Samples （Figure 5）.

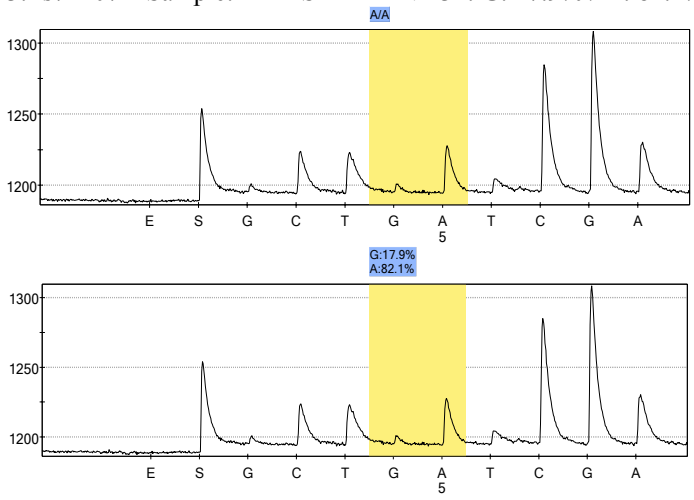
23: rs742074 Sample: ARMS PATIENT 1: G: 9.0% / A: 91.0%



23: rs742074 Sample: ARMS PATIENT 2 : G: 9.1% / A: 90.9%



23: rs742074 Sample: ARMS PATIENT 3 : G: 17.9% / A: 82.1%



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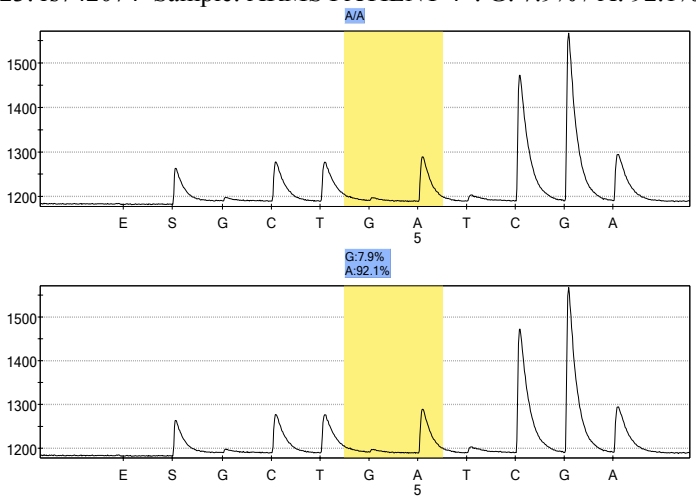
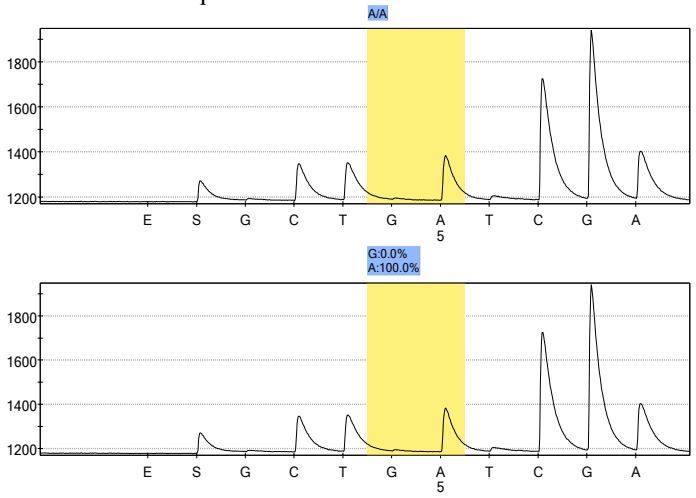
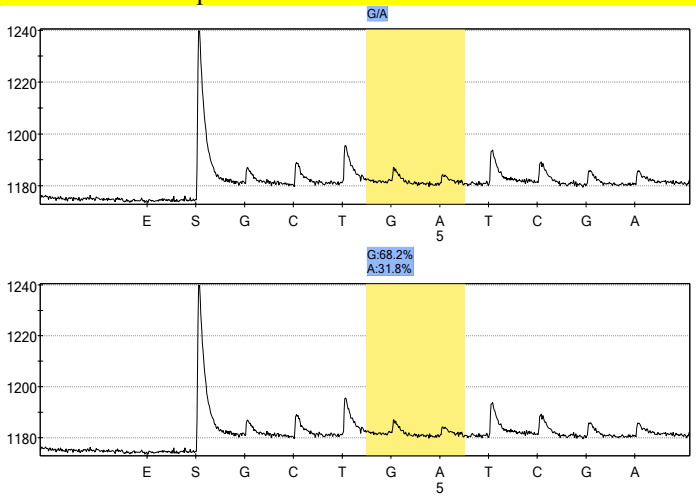
23: rs742074 Sample: ARMS PATIENT 4 : G: 7.9% / A: 92.1%



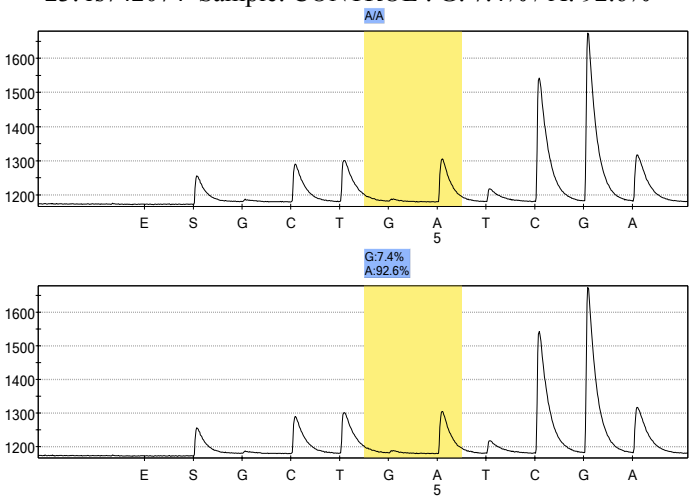
23: rs742074 Sample: ARMS PATIENT 5: G: 0.0% / A: 100.0%



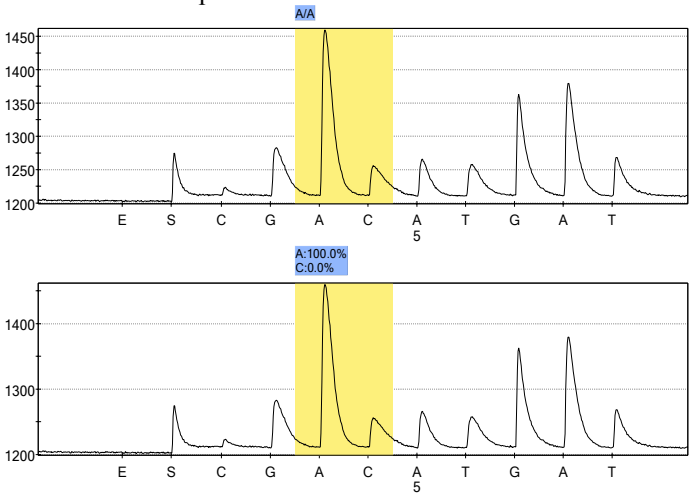
23: rs742074 Sample: ERMS CELL LINE : G: 68.2% / A: 31.8%



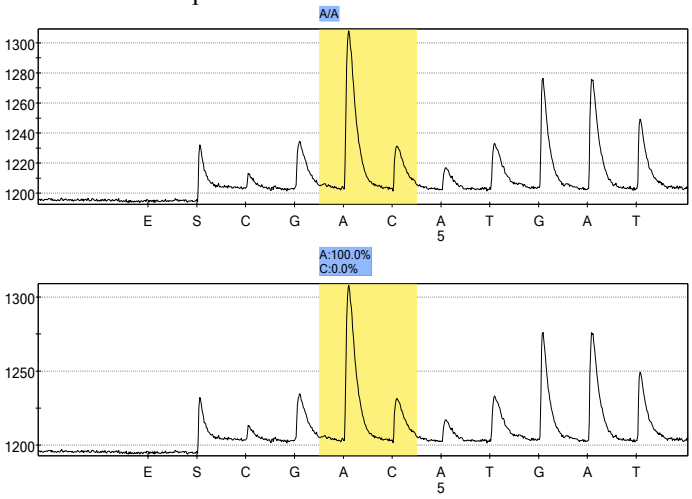
23: rs742074 Sample: CONTROL : G: 7.4% / A: 92.6%



24: rs735630 Sample: ARMS PATIENT 1: A: 100.0% / C: 0.0%



24: rs735630 Sample: ARMS PATIENT 2 : A: 100.0% / C: 0.0%



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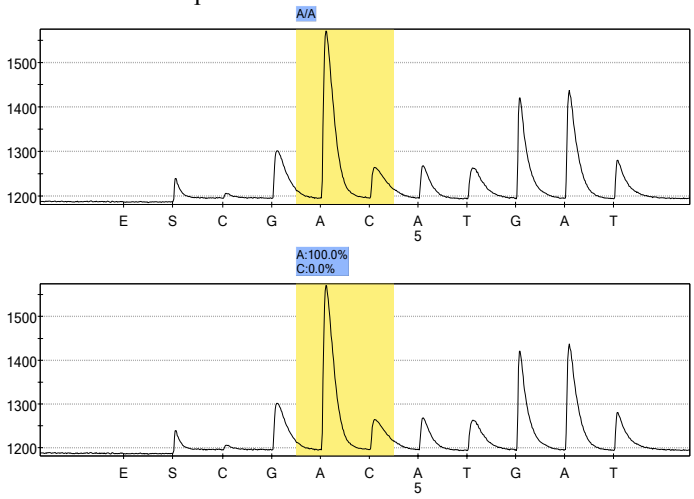
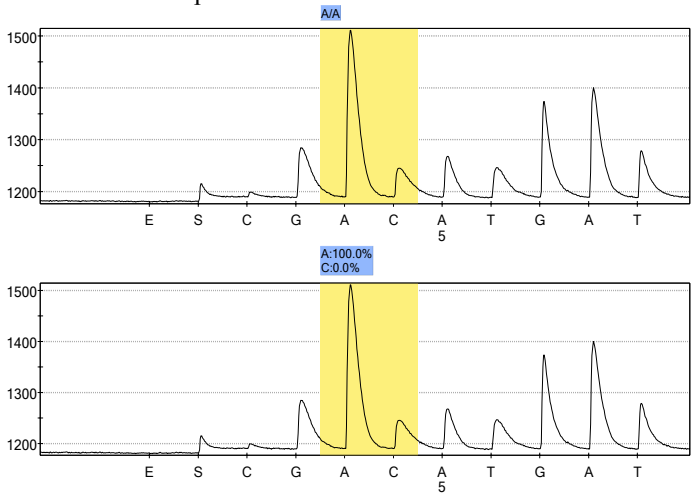
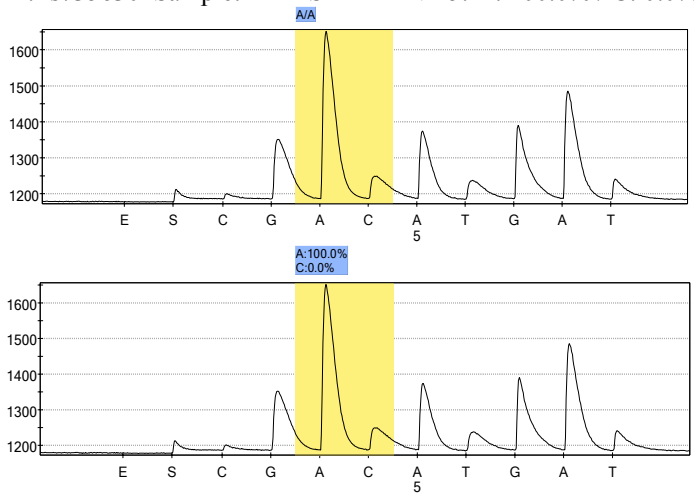
24: rs735630 Sample: ARMS PATIENT 3 : A: 100.0% / C: 0.0%



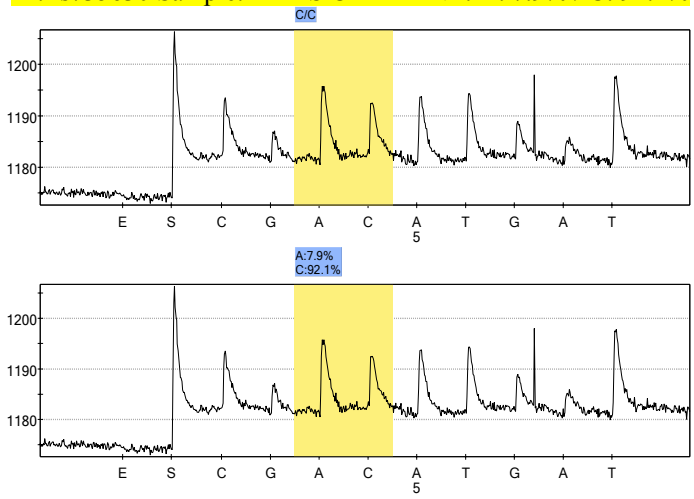
24: rs735630 Sample: ARMS PATIENT 4 : A: 100.0% / C: 0.0%



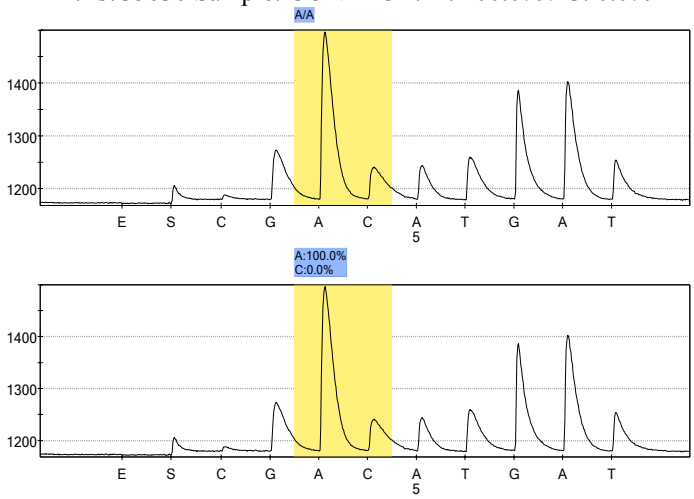
24: rs735630 Sample: ARMS PATIENT 5: A: 100.0% / C: 0.0%



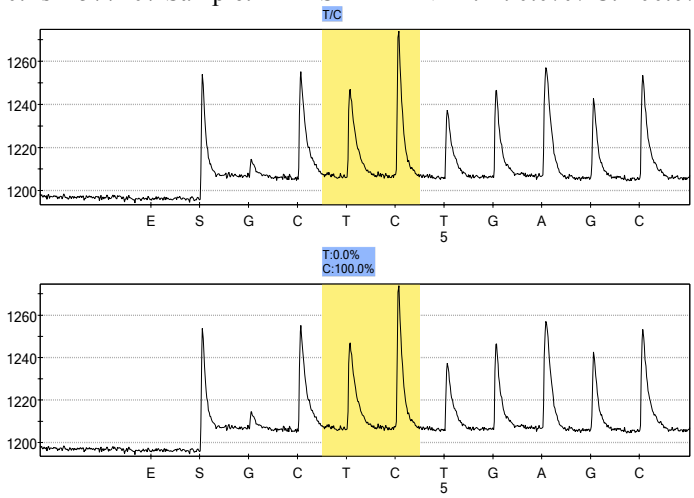
24: rs735630 Sample: ERMS CELL LINE: A: 7.9% / C: 92.1%



24: rs735630 Sample: CONTROL: A: 100.0% / C: 0.0%



40. rs11577407 Sample: ARMS PATIENT 1: T: 0.0% / C: 100.0%



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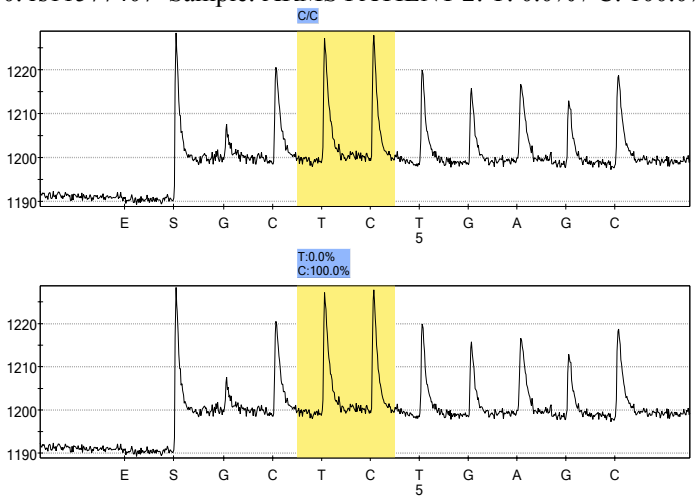
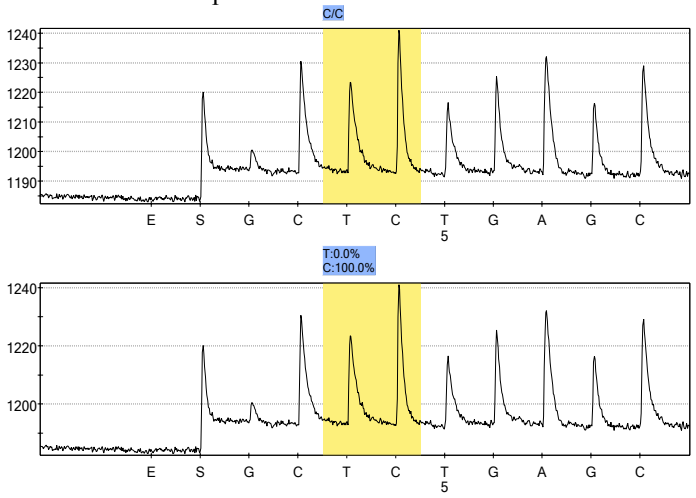
40. rs11577407 Sample: ARMS PATIENT 2: T: 0.0% / C: 100.0%



40. rs11577407 Sample: ARMS PATIENT 3: T: 0.0% / C: 100.0%



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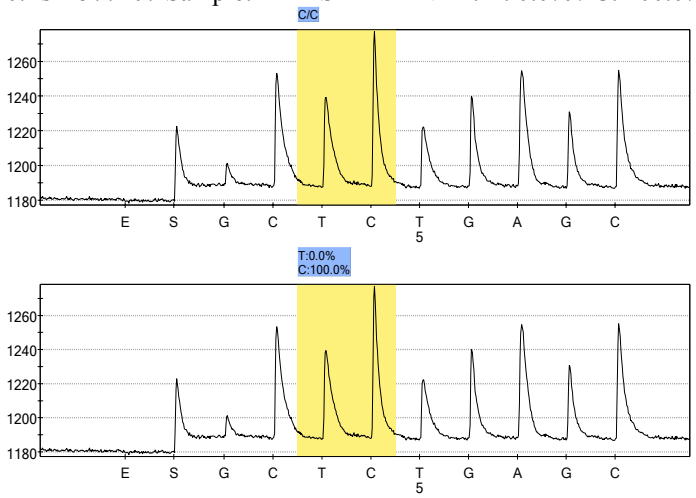
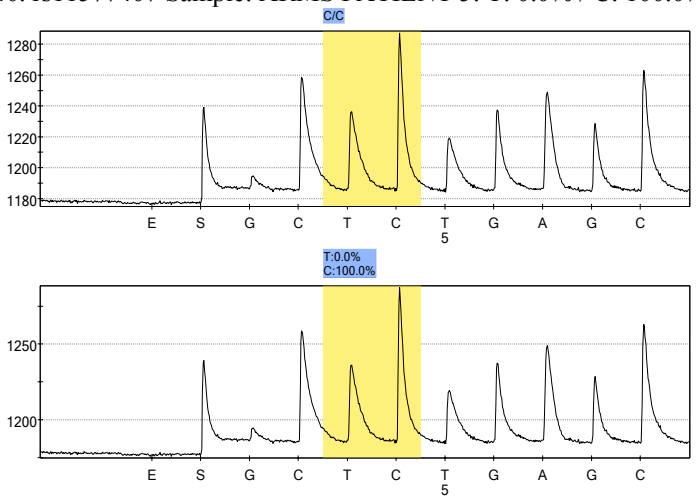
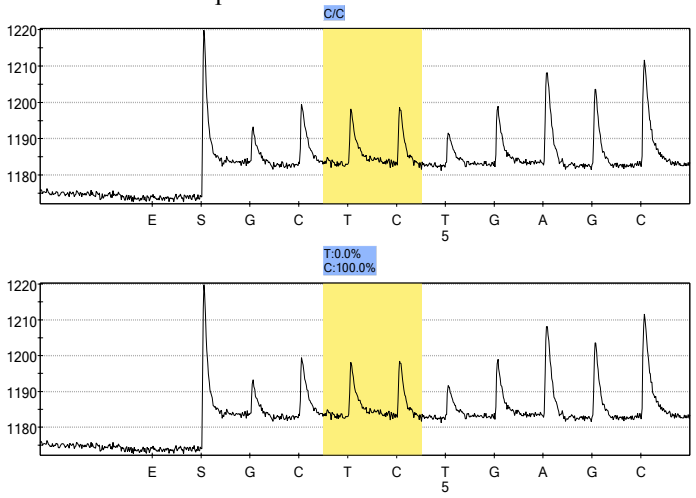


40. rs11577407 Sample: ARMS PATIENT 5: T: 0.0% / C: 100.0%



40. rs11577407 Sample: ERMS CELL LINE: T: 0.0% / C: 100.0%



40. rs11577407 Sample: CONTROL: T: 0.0% / C: 100.0%

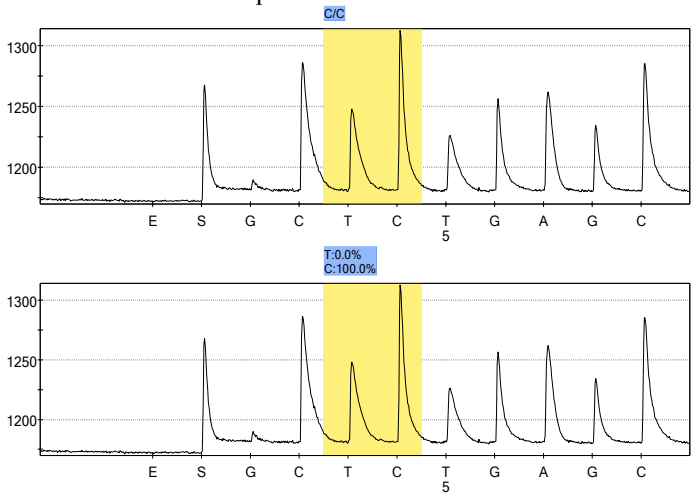


Figure 5. The results of the Biotage© Pyrosequencing™ PSQ HS 96A & Sanger sequencing for Primers 23, 24 & 40 for ARMS, ERMS and Control Samples.

5. DISCUSSION

Comparison of *In silico* PCR Analysis and Pyrosequencing to conventional *in vitro* PCR Analysis/ Gel electrophoresis

Any new process introduced into the scientific field must undergo rigorous testing. It takes many publications to initiate the establishment of new methods. The methods applied in this paper while not novel technology, are not common place in research laboratories. *In silico* biology PCR analysis provides one with a prescreening of the functionality of the primers chosen. It does not ensure that your PCR reaction will work. Those issues are resolved at the bench where the constraints on success range from template quality to varying concentrations of primers to thermocycler parameters. The programs do however give some assurance to the scientist during the ordering process of primers and reagents. The programs also decrease the time spent on trial and error of the selection of primer. What normally would have taken two weeks to perform was reduced to one week. *In silico* PCR also provided a troubleshooting method of where the primer (s) would anneal their $\mathbf{T_M}$, and possible creation of secondary structures within the oligo itself or with the given template. The size of the fragment from the use of paired primers also helps the scientist to select the proper concentration of agarose gel to use (0.8%, 1.0%, 2.0% and so on....) and the number of cycles for needed to amplify the target sequence.

The results observed when comparing the two processes were very helpful in highlighting the cost aspects associated with development of the genotyping assay itself. The time taken from ordering the oligos to Sanger sequencing was three days. Two additional days were required to process and screen for the Pyrosequencing to confirmation the results. This procedure may become a new way to reduce the time downstream of all SNP detection, discovery and allele quantification algorithms .

Pyrosequencing Results

Results presented here show that in DNA isolated from the ERMS cell line, two variants in genotype and allele quantification were identified, one for primer 23 A→G in intron 8 region, and for primer 24 C→A in intron 5 of Human PAX7. This may be an anomaly of cell culture yet no changes were identified in the ARMS cell line. The pyrosequencing method used for studying this gene was selected to minimize the risk of missing a mutation and for accurate allele quantification. Therefore it is possible that with this new technology and short processing time, has revealed previously unidentified changes in allele frequency associated with ERMS. The importance of the findings presented here should be viewed in the light of the significance of finding changes in allele frequency associated with any form of cancer. If LOH is detected it may well indicate disease severity or susceptibility to the disease. It is therefore important to investigate all SNPS for their association with tumorigenicity allowing physicians to refine the care and treatment of patients with these diseases. Further research is required to assess the number of cases of ERMS that show similar allele changes. Our studies highlight the ease and speed with which these studies may be undertaken.

Interestingly there were no changes in SNP frequency observed in the ARMS samples relative to the expected allele frequency at the selected SNPs. Since only a few SNP sites were investigated in this paper, additional SNP analysis at other identified sites may reveal significant changes in allele frequency and LOH in ARMS patients. Our findings do however highlight the differences in the ARMS and ERMS sarcomas; ARMS is associated with a chromosomal translocation that produces a *PAX7-FOX1* fusion gene, whereas ERMS is associated with increased expression of *PAX7* (Robson *et al.,*2006). The increased expression of Pax7 in ERMS samples may be linked to the changes in allele frequency or LOH at the sites identified. Further research is required to confirm this association.

Acknowledgment

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Analysis on the Operational Structure of Green Food Enterprises in Heilongjiang Province

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Abstract: Green food industry in Heilongjiang has ranked top in China after 15 years of efforts. There is no doubt that further developments of green food industry will make much more contributions to provincial economy and to raise farmers' income. While, there are evidences of unbalance in industrial structure and clear defects which will be the main hindering factors for its further developments. Developing strategies were presented in this presentation, that is, favorable governmental polices, and stable base constructions, large scale cooperation, collective orientation, technological innovation and famous brands will be the only alternative for the development of green food industry. [Nature and Science. 2006;4(4):21-25].

Keywords: Heilongjiang Province; Green Food; Enterprises; Operational Structure

Introduction

In late 1990s, agricultural economy in China encountered the problems of low prices of agricultural products, increases in productivity with no increase in farmer's income, and serious defects of industrial structure. Though the total crop output reached 30 billion kilograms in Heilongjiang province, there was the problem of insufficient demands, which called for the strategy of "developing green food and taking specialized path". At present, there are 7 dominating green food series in Heilongjiang province, including, soybean, maize, rice, dairy, forest products, beverages and local products, and the industrial mode of connections of leading enterprises, producing bases and farmers has come into being.

1. Current situations and trend of green food industry in Heilongjiang province

1.1 Current situations of green food industry

Since 1990, Green food industry in Heilongjiang has made great progress and ranks top in Chin after 15 years of efforts. Comparing with 1999, planting areas of green food in Heilongjiang reaches 34.9 million mus, 10.8 times than that of 2.96 million mu; Total outcome of green food is 15.81 million tons, 13.9 times of than that of 1.06 million tons; Total production value is ￥40 billion yuan, 10.2 times than that of ￥3.56 billion yuan; Increased part of income is ￥1100 yuan per capita, 17.6 time than that of ￥59 yuan. Green food varieties are increased to 839 items of 14 dominating series, including breeding, dairy products, alcohol and beverages and bee products. About the details of green food production (Table 1).

1.2 Developing tendency of green food industry

According to the analysis of green food production in Heilongjiang province, there is a linear function between time and output, which will directly influence farmer's income. Based on tendency extension theory, we concluded following equation:

$$Y = a + bX \quad (1)$$

among:

Y is anticipation value; X is time ordinal; a, b are unknown coefficient

According to Least Square Theory, when ∑X=0,:

$$a = \sum Y / n,$$

$$b = \sum XY / X^2 \quad (2)$$

Based on the statistics of green food areas from 1999-2005, we get the anticipated areas in 2006. Then, a= 1602.3, b=486.4. then,

Y=a+bX=1602.3＋486.4×4=3547.9

Therefore, we get the result that green food production value will reach ￥77.1billion yuan , total output will reach 16.4million tons and farmers' income will increase ￥1107.9 yuan in year 2006.

According to above theory, the development tendency of green food in Heilongjiang province is predicted as Table 2.

2. Operational structure analysis of green food enterprises in Heilongjiang province

2.1 General structure of green food enterprises in Heilongjiang province

From year 2003 to 2005, there are totally 299 enterprises got green food authentications, with 90, 94 and 115 respectively in each year, among them, 159 grain producing enterprises, 39 vegetable enterprises and 3 aquatic product enterprises. About the structure of green food enterprises (Table 4).

2.2 Structure analysis of green grain enterprises in Heilongjiang province

Grain products enterprises share the lion proportions of green food enterprises in Heilongjiang. Rice planting bases reaches the area of 6.33million mu, soybean areas is 6.83million mu, maize areas is 4.2million mu,, with the respective weights of 27%, 29% and 18%. There are 5 main green food bases which occupies 70% of total green food production, among which, rice bases centered with Suihua, Harbin and Reclamation Head Bureau have rice areas of 4.292 million mu, 69.8% of total areas; soybean bases centered with Suihua, Harbin and Heihe have soybean areas of 4.04million mu, 58.8% of total areas; maize bases centered with Suihua and Qiqihar have maize areas of 3.52million mu, 83.8% of total. Operational structure of green food enterprises is as follows (Table 5).

2.3 Structure analysis of green meat enterprises in Heilongjiang province

Stockbreeding is the main industry in Heilongjiang, which occupies 40% of total production value. Even though green stockbreeding is still a small part, it has made great progress in a certain degree in recent years. Up to the end of 2004, there were 159 authentic green breeding enterprises, which increased by 89% than the preceding year. Green dairy output is 1.587million tons, which increased by 33% than preceding year, reached 42% of total output, and this percentage is increased by 3 points; Live pigs are 0.503million heads, increased by 3.5 times; Chicks are 2.682million, increased by 4.2 times. Green pork production takes Qiqihar and Suihua as its center, with the production of 0.419million heads in year 2004, which was 83.3% of total production. Green chicken productions are mainly in Mudanjiang and Harbin, with production of 2.57million in 2004, which was 95.8% of total output. About the detail of green meat production (Table 5).

2.4 Green food processing enterprises in Heilongjiang province

With the rapid growths in planting and breeding industries, green food processing industry also get great developments. There are 122 main enterprises with sales income over ￥30million, 42 over ￥100million and 12 enterprises are granted as national grade agricultural industrialization dragon-heads enterprises. At the end of year 2004, there were 215 green food processing enterprises, with total production value of ￥12.93billion yuan, increased by 21% than preceding year, with added value of ￥3.62billion yuan, increased by 16% than last year. There are 101 enterprises taking dairy, soybean and rice as raw materials with sales income of ￥8.79billion yuan, increased by 24.5%, and shared 66.8% of total sales of its industry. There are 18 dairy processing enterprises with total sales of ￥3.76billion yuan, increased by 46.3% than preceding year, 24 soybean processing enterprises with sales of￥1.12billion yuan, increased by 23%, 59 green rice processing enterprises with sales of ￥3.91billion yuan, increased by 9.2%. Joint-stock companies compose 54.7% of total number of green food processing enterprises. 84.8% of them are grade A companies. About details (Table 6).

Table 1. Green food production in Heilongjiang Province

	Authentication number	areas （10.000mu）	Production value （100 million yuan）	Output （10.000 tons）	Income increased （yuan）	Exportation (100 million yuan)
1999	145	296	35.6	106	59	0.4
2000	165	750	70	450	154	
2001	281	1035	21	508	206	5.9
2002	470	1452	177	600	09	12.4
2003	610	1813	190	820	60	19.2
2004	787	280	327	1110	866	21
2005	—	3490	400	1581	1100	—

Sources:Heilongjiangyearbook, http://greenfood.northeast.cn

Table 2. Heilongjiang Province organic foods development tendency

Year	Areas (10.000 mu)	Production Value (100 million yun)	Output (10.000 ton)	Income Increase (yuan)
2006	3548	771	1640	1108
2007	4100	797	1848	1330
2008	4697	887	2138	1570
2009	5280	1023	2420	1801
2010	5372	1202	2669	2014

Sources: Heilongjiangyearbook, http://greenfood.northeast.cn

Table 3. Structure of green food enterprises in Heilongjiang province (2003-2005)

	Grain	Vegetables	Dairy	Meat	Eggs
Number	159	39	25	16	4
Weights (%)	53.2	13	8.4	5.4	1.3
	fungus	beverages	alcohol	oil	others
Number	18	27	14	16	15
Weights (%)	6.0	9.0	4.7	5.4	5.0

Sources: collected from http://greenfood.northeast.cn.

(Notes: for the reason of various green food are produced in the same enterprises, statistical numbers are more than actual total number, therefore, the total weight is large than 100%).

Table 4. Structure of green grain enterprises in Heilongjiang province (2003-2005)

	Rice	Soybean	Maize	Wheat	Miscellaneous
Enterprises	96	37	8	5	13
weights（%）	60.4	23.3	5.0	3.1	8.2

Sources: collected from http://greenfood.northeast.cn.

Table 5. Heilongjiang Province organic foods meats production enterprise structure (2003 - 2005)

	Pork	Beef	Lamb	Chicken	Geese	Rabbit meat
Number of enterprises	2	4	3	3	3	1
Weights (%)	12.5	25	18.8	18.8	18.8	6.3

Sources: collected from http://greenfood.northeast.cn.

Table 6. Structure of green meat enterprises in Heilongjiang province (2003-2005)

	Processing quantity 10.000 ton	Number of enterprises	Enterprises over ￥100million	Taxes ￥100million	Production value ￥100million
1999	69	—	1	2	10.1
2000	—	—	6	4.7	24.4
2001	222	129	12	7.6	53.7
2002	—	—	20	11.6	84
2003	378	210	27	16.5	106.9
2004	468	215	36	20.1	129.3
2005	—	—	42	23.5	160

Sources: collected from http://greenfood.northeast.cn.

3. Problems in green food enterprises in Heilongjiang province

Even though green food industry in Heilongjiang grows rapidly and has a very significant position in China, there are still many problems.

3.1 Too many less known and inferior brands which intensifies internal competitions

According to statistics, there were 96 registered green rice and 37 green soybean enterprises in Heilongjiang in years 2003-2005. Too many unknown brands bring intensive internal competition, which

greatly undermines the composition of internal forces in competing in national and international markets.

3.2 Inferiority in quality weaken its competitiveness in international green food market

Rapid as green food industry grows, the products qualities just lingering on low levels. As we all know, green food in China is classified into Grade A and Grade AA, the later reaches the international organic food standards. At present, there is less than 10% of green food planting and organic food producing enterprise has granted Grade AA, and the areas are only 8.8% of total green food planting area, and bee products is the only one meets the international standards of organic food.

3.3 Green stock raising industry shows signs of decline in scale and has difficulty in achieving scale advantages

Heilongjiang is a big province in stock raising industry which shares half of its provincial revenue, while green stock raising develops in a small scale, some products even show the signs of decline, which makes it difficult to form scale advantage in market. There are only 19 counties have green stock raising products which is only small part of the whole industry. In 2004, green pork production was 0.285 million heads, only 2.6% of total; green beef, lamb and geese productions are respectively 0.045 million, 0.035 million and 1.333 million heads, and decreased respectively by 42%, 81% and 62% than preceding year. Further decline in production will incur economic drop in this industry.

3.4 Being in short of professionals undermines its developing potential

Green food production is in need of professionals to guarantee its products with high quality. In 2004, the total number of green food production staff was 62,000, increased by 15% than the preceding year, but there was only 3000 of them had the intermediate technical titles, which decreased by 13% than that in 2003. In short of professionals will be a great hindering factor of the whole industry.

3.5 Unreasonable benefits connection mechanism discourages industrial effectiveness

Unreasonable benefits connection mechanism and absence of statutory supervision is a common problem in national agricultural industrialization, and it is also a key blocking factor in the process of agricultural industrialization. In 2004, there were 215 food processing enterprises with green food production of 4.68million tons, while orders' quantity was 2.033million tons, decreased by 14%. Green food processing proportionate dropped from 55.7% to 43.4%. There were 45 green food processing enterprises in red or zero profits, which was 20.9% of the total green food processing enterprises.

4. Measures to promoting green food enterprises in Heilongjiang

4.1 Creating favorable environments with beneficial governmental polices

Green food enterprises, especially virgin enterprises are greatly in need of governmental supports, which make policy innovations of great imperative. According to practical investigations, governmental supports should focus on the following aspects: a. Adjusting tax system. By taking references from other countries' successful practices and cut down tax rates.b. Increasing government's financial supports. Even though provincial finance authority has transferred ￥0.275 billion yuan to green food industry in recent 5 years, there is still a great need of capitals. Government should give more supports to technology reform in heading enterprises, permitting low interest loans to new projects and increasing investments in base construction. c. Issuing favorable loan policy. At present, heading green food enterprises commonly lack of purchasing capitals, so we suggest commercial banks give more credit beneficial supports to them.

4.2 Constructing planting bases to ensure raw material supply to well-known brands.

Raw material supply is the essential condition for green food processing, which make it imperative to construct its planting bases. Taking scale and processing capacity into account, heading processing enterprises need to construct stable raw material supply bases and increase its technical contents, optimize well-bred species and promote well-bred live stocks, meanwhile, promote agricultural standardization system and organize farmers to carry on specialized production.

Reasonable benefits connection mechanism makes for a stable planting base. Processing enterprises need to establish benefit connection with farmers and form benefit and risk share community by contracts, paying purchase deposits, granting funds, issuing materials, offering serial services, and perform joint stocks and stock cooperatives companies, therefore, turn planting base into a workshop.

4.3 Promoting communications and realizing leaping style development in green food enterprises

To improve its competitiveness in domestic and international markets, green food enterprise can rely on its own power originated from its scale and strength. In this sense, taking its small scale and inferiority in techniques into consideration, green food enterprises in

Heilongjiang province should take the path of cooperative and affiliated operation, which includes: a. Cooperating with banks to start large projects; b. Introducing large foreign capital; c. Combining with large companies.

4.4 Taking the way of collective, industrialized and integrative managements

Large organic food companies in advanced countries, such as in the USA and west Europe, have experienced collective management stage from small and medium size and finally established their dominance in world markets. Similarly, greed food enterprises in Heilongjiang province need to put themselves into the world markets and global circulations. They need to organize into multiregional and multi-industrial group companies and take the strategy of high-tech, high benefits and collective managements. For green food industry in Heilongjiang, industrialization mode of production-research, planting-breeding-processing,
trade-industry-agriculture will be optimized choice for its rapid developments.

4.5 Improving quality through scientific and technological innovations

Scientific and technological innovation is of key importance for heading green food enterprises in performing their well-known brands strategies, therefore, they need to increase R&D funds, about 5% of their sales income, establish research center to form technology innovation system, insist on the combination of production and research, promote scientific findings transformation. Also, they need to keep in pace with newest techniques and introduce, digest, absorb, innovate and apply new findings to promote technological contents of theirs products. To recruit talents, green food enterprises need to offer good welfares, improve their working and living conditions, give enough incentives for those who make great contributions in technological and managing innovations. To maintain advance in technology, the proportion of technician and senior professionals need to reach respectively 30% and 10% of their staff.

4.6 Expanding markets shares by creating well-known brands

With the development of economic globalization, international competitions are introduced in domestic markets, which makes brands a most effective tools in the increasingly intensive competitions. With no exceptions, green food industry has to resort to famous brand strategy. Integration of various brands is the most effective method in rapid expanding manufacture scale and creating new famous brands, which can be achieved by introducing, extending and promoting brands' reputations.

5. Conclusions

Heilongjiang is a big green food production province, but it is still not a superb power in the market. There are still many defects within its structure which hinders its developments. Green food enterprises can only realize its predicted objects by finding a proper way which suit its development best.

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Clone and Sequence Analysis of Trehalose Synthesis from Pseudomonas Stutzeri

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Abstract: We have cloned Trehalose Synthesis gene using PCR from Pseudomonas Stutzeri. We linked this gene with pGEM-T-Easy Vector , analysis the whole gene sequence and transformation the recombinant gene into *Escherichia coli* JM109 .The result of sequence of this gene showed :cloned gene whole length is 2070 bp, has 96.66% homology compared with recorded sequence AF113617in GenBank, encoded 689 amino acids , has 99.71% homology compared with AF113617 . This cloned gene has been recorded by GenBank, accession number is DQ452614. [Nature and Science. 2006;4(4):26-31].

Keywords: Trehalose Synthesis; TreS gene; PCR clone; sequence analysis

Introduction

Trehalose is a deoxidizing disaccharide containing two glucoses which are combined with α，α−1，1 glucosidic linkage. It is abundant in animals, plants and microbe. Trehalose has an important function in protecting plant stress tolerance. It usually produced in the condition of intimidating. The content of the trehalose varys as the condition changes. Trehalose is a irritability metabolite. The reason that why some species performance stress tolerance in inclemency environment is trehalose can have the protection effect on biological giant molecule, such as biomembrane, protein and nucleic acid etc. So according to that, the species which are rich in trehalose can show the peculiar biological speciality. The function of the biological protection of trehalose is that it strongly binds up the water molecules, owning the bound water together with membrane lipid. Or it can instead the function of combining water with the membrane. Consequently preventing the denaturalization of biomembrane and membrane protein. Because of the potential applications of the trehalose on food, cosmetic, medicament and the biological production, the clone and expression of Trehalose synthesis gene become a hotspot of biological researching. In Pseudomonas Stutzeri, maltose is used as a substrate. With the help of Trehalose Synthesis, Trehalose changes the maltose which is combined with α，α−1，4 glucosidic linkage into Trehalose which is combined with α, α−1, 1 glucosidic linkage.

1. Materials

1.1 Bacteria and Plasmid

Pseudomonas stutzeri1.1803, from the Conservation of microorganism bacteria; E. coli JM109, from Bao Bioengneering Co. of Da Lian, China; pGEM-T-Easy, from Promega Co., USA

1.2 Reagent

Enzymes and IPTG, X-gal, dNTP, from Bao Bioengneering Co.of Da Lian ,China and Promega Co; T4DNA ligase from GIBCO Co.; UNTQ-10 Kit from Shang Hai Bioengineering Ltd. Co. Primer was synthesized by Bao Bioengneering Co. of Da Lian ,China; Gene sequence analysis was done by Shang Hai Boya Bioengneering Ltd. Co., USA.

2. Methods

2.1 Isolation plasmid

Inoculating the E. coli containing pGEM-T-Easy plasmid into LB liquid culture medium which has specific concentrations overnight. And then isolation plasmid with the method of alkaline lysis.

2.2. PCR Amplification of TreS gene

Based on the sequence of TreS gene on Genebank, the accession number is AF113617, we designed two primers for PCR reaction. And inserted the restriction enzyme sites of the BamH Ⅰ and Sac Ⅰ on the 5′ end and 3′ end.

P_1 (5' primer) 5'GGATCCATGAGCATCCCAGA CAACAC 3', BamH Ⅰ;
P_2 (3' primer) 5'GGAGCTCTCAGATCACCGGCGGCGCGG 3', Sac Ⅰ.

Isolating the whole DNA of *Pseudomonas stutzeri* as the template for PCR amplification. Conditions: 94 ℃ 5 min, 94℃ 30 s, 54℃ 30 s, 72℃ 1 min, 72℃ 7 min, 4 ℃ hold, 40 cycles. The PCR product was tested through 0.8% agarose gel electrophoresis, and purified and reclaimed by 0.8% agarose in order to use in next step.

2. 3 TreS gene clone

Using BamH Ⅰ and Sac Ⅰ sites on pGEM-T-Easy, T_4ligase link PCR product with pGEM-T-Easy vector. Transformating *Escherichia coli* JM109, screen positive clone on LB plate with Amp, IPTG and X-gal. Identifying the recombinant by digestion, and analyze the gene sequence.

2. 4 Sequence Analysis

Commission Shang Hai Boya Bioengneering Ltd., Co. sequence the target whole gene clone. Using BLAST and GeneBank data to analysis the homology of the target sequence.

3. Result

3. 1 Amplification of TreS gene

We use the whole DNA of *Pseudomonas stutzeri* as the template and the designed oligonucleotide as the primers to amplify the TreS gene. PCR product is 2.07 Kb, consistent with result of anticipate (Chart 1). And reclaimed by 0.8% agarose in support.

3. 2 Amplification product clone and analysis

Link the PCR amplification product purified by low melting point agarose with pGEM-T-Easy and then transform into *E. coli* JM109, then cut the recombinant plasmid with the restriction enzymes of BamH Ⅰ and Sac, and test it use PCR. (Chart 2.3) The result shows that the vector has been inserted the 2.07 kb DNA fragment.

3. 3 The result of sequence analysis of TreS gene and the homologous searches with the reported gene

After determining one sequence of the recombinant, the result shows that this fragment whole length is 2070 bp, encoding 331 amino acid. Comparing with the sequence on GeneBank AF113617 (Chart 4). It have 18 differences on 52、225、231、249、831、843、972、1038、1213、1299、1561、1563、1564、1569、1809、1824、1848、2031sites. The results show homologous rate of correspond region are almost 96.66% compared with AF113617.

3.4 Putative Amino Acid Sequence and the homologous analysis

Translating the ORF of TreS gene into amino acid, putative molecular weight 75.7KD, isoelectric point 5.14. Homologous rate of correspond region are almost 99.71% copare with AF113617. The differences between them only on 521 and 522 sites. Send TreS amino acids sequence to the server of NCBI, use BLASTP tool to homologous searches, the result shows the sequences which have high homologous sequence with TreS amino acids sequence (Chart 5). They are the amino acids sequence of glycosidase, putative and hypothetical protein. By using DNAMAN4.0 to compare homologous sequence of TreS gene with 3 items of TreS genes which were published in GeneBank, the result shows that the homologous rate of all of them are above 86.07% (Chart 6).

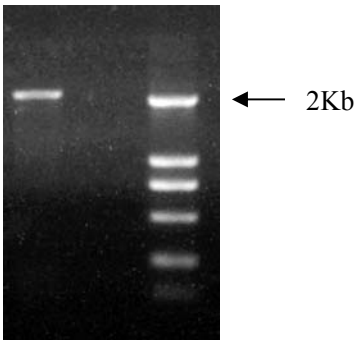


Chart 1. cDNA PCR amplification

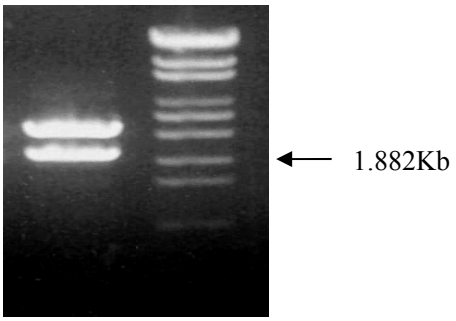


Chart 2. Recombinant of TreS gene plasmid was cutted by BamH Ⅰ and Sac Ⅰ

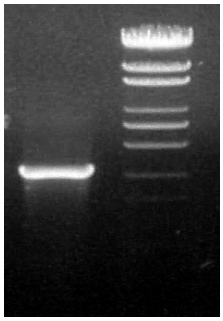


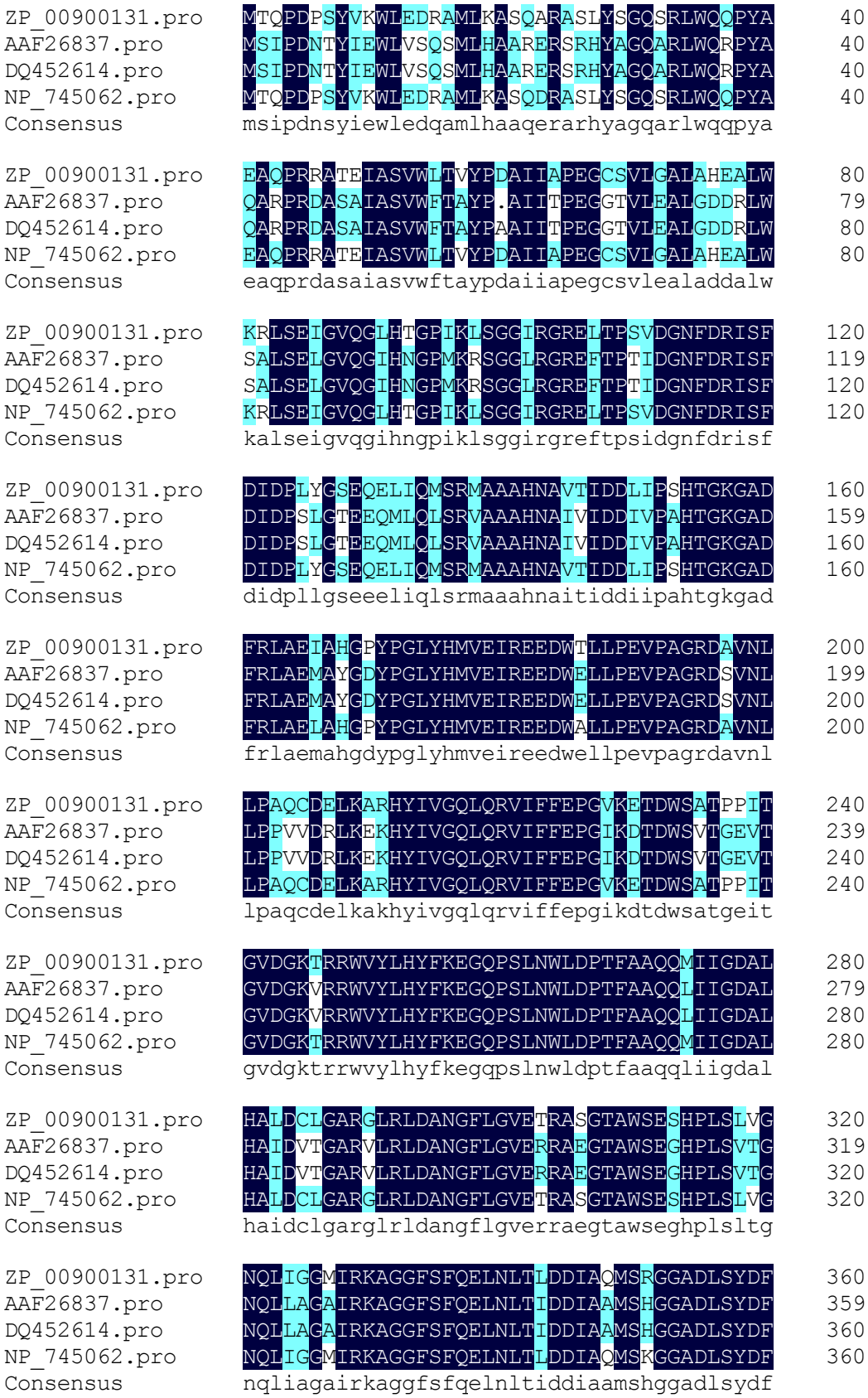
Chart 3. PCR identification of recombinant plasmid

1 ATG…ATGTTG…GGT…GAT…CTG…GCA…CAT….CTT…ATA…
2 ATG…ATGCTG…GGC…GAC…CTC…GCG…CAC…CTC…ATC…
1 …CTG…CTA…CCCGCCGAA…CCC…CCA…GAA…CTC
2… TTG…CTG…GCGCCCGAG…CCG…CCG…GAG…CTT

Chart 4. TreS gene sequence
1. DQ452614 bacteria 2. AF113617 bacteria

Sequences producing significant alignments:		(Bits)	Value
gi|6724082|gb|AAF26837.1|	trehalose synthase [Pseudomonas stutze	1387	0.0
gi|66045972|ref|YP_235813.1|	glycosidase, putative [Pseudomon...	1144	0.0
gi|71557609|gb|AAZ36820.1|	trehalose synthase [Pseudomonas sy...	1141	0.0
gi|28870130|ref|NP_792749.1|	glycosidase, putative [Pseudomon...	1139	0.0
gi|82737280|ref|ZP_00900131.1|	Trehalose synthase [Pseudomonas p	1056	0.0
gi|26989637|ref|NP_745062.1|	trehalose synthase, putative [Ps...	1055	0.0
gi|67987823|gb|EAM75610.1|	hypothetical protein KradDRAFT_254...	776	0.0

Chart 5. The result of the searches on GeneBank of TreS amino acids sequence


ZP_00900131.pro MTQPDPSYVKWLEDRAMLKASQARASLYSGQSRLWQQPYA 40
AAF26837.pro MSIPDNTYIEWLVSQSMLHAARERSRHYAGQARLWQRPYA 40
DQ452614.pro MSIPDNTYIEWLVSQSMLHAARERSRHYAGQARLWQRPYA 40
NP_745062.pro MTQPDPSYVKWLEDRAMLKASQDRASLYSGQSRLWQQPYA 40
Consensus msipdnsyiewledqamlhaaqerarhyagqarlwqqpya
ZP_00900131.pro EAQPRRATEIASVWLTVYPDAIIAPEGCSVLGALAHEALW 80
AAF26837.pro QARPRDASAIASVWFTAYP.AIITPEGGTVLEALGDDRLW 79
DQ452614.pro QARPRDASAIASVWFTAYPAAIITPEGGTVLEALGDDRLW 80
NP_745062.pro EAQPRRATEIASVWLTVYPDAIIAPEGCSVLGALAHEALW 80
Consensus eaqprdasaiasvwftaypdaiiapegcsvlealaddalw
ZP_00900131.pro KRLSEIGVQGLHTGPIKLSGGIRGRELTPSVDGNFDRISF 120
AAF26837.pro SALSELGVQGIHNGPMKRSGGLRGREFTPTIDGNFDRISF 119
DQ452614.pro SALSELGVQGIHNGPMKRSGGLRGREFTPTIDGNFDRISF 120
NP_745062.pro KRLSEIGVQGLHTGPIKLSGGIRGRELTPSVDGNFDRISF 120
Consensus kalseigvqgihngpiklsggirgreftpsidgnfdrisf
ZP_00900131.pro DIDPLYGSEQELIQMSRMAAAHNAVTIDDLIPSHTGKGAD 160
AAF26837.pro DIDPSLGTEEQMLQLSRVAAAHNAIVIDDIVPAHTGKGAD 159
DQ452614.pro DIDPSLGTEEQMLQLSRVAAAHNAIVIDDIVPAHTGKGAD 160
NP_745062.pro DIDPLYGSEQELIQMSRMAAAHNAVTIDDLIPSHTGKGAD 160
Consensus didpllgseeeliqlsrmaaahnaitiddiipahtgkgad
ZP_00900131.pro FRLAEIAHGPYPGLYHMVEIREEDWTLLPEVPAGRDAVNL 200
AAF26837.pro FRLAEMAYGDYPGLYHMVEIREEDWELLPEVPAGRDSVNL 199
DQ452614.pro FRLAEMAYGDYPGLYHMVEIREEDWELLPEVPAGRDSVNL 200
NP_745062.pro FRLAELAHGPYPGLYHMVEIREEDWALLPEVPAGRDAVNL 200
Consensus frlaemahgdypglyhmveireedwellpevpagrdavnl
ZP_00900131.pro LPAQCDELKARHYIVGQLQRVIFFEPGVKETDWSATPPIT 240
AAF26837.pro LPPVVDRLKEKHYIVGQLQRVIFFEPGIKDTDWSVTGEVT 239
DQ452614.pro LPPVVDRLKEKHYIVGQLQRVIFFEPGIKDTDWSVTGEVT 240
NP_745062.pro LPAQCDELKARHYIVGQLQRVIFFEPGVKETDWSATPPIT 240
Consensus lpaqcdelkakhyivgqlqrviffepgikdtdwsatgeit
ZP_00900131.pro GVDGKTRRWVYLHYFKEGQPSLNWLDPTFAAQQMIIGDAL 280
AAF26837.pro GVDGKVRRWVYLHYFKEGQPSLNWLDPTFAAQQLIIGDAL 279
DQ452614.pro GVDGKVRRWVYLHYFKEGQPSLNWLDPTFAAQQLIIGDAL 280
NP_745062.pro GVDGKTRRWVYLHYFKEGQPSLNWLDPTFAAQQMIIGDAL 280
Consensus gvdgktrrwvylhyfkegqpslnwldptfaaqqliigdal
ZP_00900131.pro HALDCLGARGLRLDANGFLGVETRASGTAWSESHPLSLVG 320
AAF26837.pro HAIDVTGARVLRLDANGFLGVERRAEGTAWSEGHPLSVTG 319
DQ452614.pro HAIDVTGARVLRLDANGFLGVERRAEGTAWSEGHPLSVTG 320
NP_745062.pro HALDCLGARGLRLDANGFLGVETRASGTAWSESHPLSLVG 320
Consensus haidclgarglrldangflgverraegtawseghplsltg
ZP_00900131.pro NQLIGGMIRKAGGFSFQELNLTLDDIAQMSRGGADLSYDF 360
AAF26837.pro NQLLAGAIRKAGGFSFQELNLTIDDIAAMSHGGADLSYDF 359
DQ452614.pro NQLLAGAIRKAGGFSFQELNLTIDDIAAMSHGGADLSYDF 360
NP_745062.pro NQLIGGMIRKAGGFSFQELNLTLDDIAQMSKGGADLSYDF 360
Consensus nqliagairkaggfsfqelnltiddiaamshggadlsydf

```
ZP_00900131.pro   ITRPAYQHALLTGDTEFLRLMLKEMHAFGIDPASLIHALQ   400
AAF26837.pro      ITRPAYHHALLTGDTEFLRMMLREVHAFGIDPASLIHALQ   399
DQ452614.pro      ITRPAYHHALLTGDTEFLRMMLREVHAFGIDPASLIHALQ   400
NP_745062.pro     ITRPAYQHALLTGDTEFLRLMLKEMHAFGIDPASLIHALQ   400
Consensus         itrpayhhalltgdteflrlmlkemhafgidpaslihalq

ZP_00900131.pro   NHDELTVELVHFWTLHAHDMYLYKGQTLPGSILREHIREE   440
AAF26837.pro      NHDELTLELVHFWTLHAYDHYHYKGQTLPGGHLREHIREE   439
DQ452614.pro      NHDELTLELVHFWTLHAYDHYHYKGQTLPGGHLREHIREE   440
NP_745062.pro     NHDELTVELVHFWTLHAHDMYLYKGQTLPGSILREHIREE   440
Consensus         nhdeltlelvhfwtlhahdhyhykgqtlpgghlrehiree

ZP_00900131.pro   IYERLSGEHAPYNLRFVTNGIACTTASLIAAALGIRDLEQ   480
AAF26837.pro      MYERLTGEHAPYNLKFVTNGVSCTTASVIAAALNIRDLDA   479
DQ452614.pro      MYERLTGEHAPYNLKFVTNGVSCTTASVIAAALNIRDLDA   480
NP_745062.pro     IYERLSGEHAPYNLRFVTNGIACTTASLIAAALGIRDLEQ   480
Consensus         iyerlsgehapynlkfvtngiacttasliaaalgirdlda

ZP_00900131.pro   IGATDIELIKKVHLLLVMYNAMQPGVVALSGWDLVGALPL   520
AAF26837.pro      IGPAEVEQIQRLHILLVMFNAMQPGVFALSGWDLVGALPL   519
DQ452614.pro      IGPAEVEQIQRLHILLVMFNAMQPGVFALSGWDLVGALPL   520
NP_745062.pro     IGVADIELIKKVHLLLVMYNAMQPGVVALSGWDLVGALPL   520
Consensus         igpadielikklhillvmfnamqpgvfalsgwdlvgalpl

ZP_00900131.pro   PAEAVAERMLDGDTRWIHRGGYDLAGLDPQAVASVRGMPR   560
AAF26837.pro      APEQVEHLMGDGDTRWINRGGYDLADLAPEASVSAEGLPK   559
DQ452614.pro      PAEQVEHLMGDGDTRWINRGGYDLADLAPEASVSAEGLPK   560
NP_745062.pro     PAEAVAERMLDGDTRWIHRGGYDLAGLDPQAEASVRGMPR   560
Consensus         paeavaelmgdgdtrwihrggydladlapeasasaeglpk

ZP_00900131.pro   ARSLYGSLDSQLDEGDSFACQVKKLLAVRQAYGIATSRQV   600
AAF26837.pro      ARSLYGSLAEQLQRPGSFACQLKRILSVRQAYDIAASKQI   599
DQ452614.pro      ARSLYGSLAEQLQRPGSFACQLKRILSVRQAYDIAASKQI   600
NP_745062.pro     ARALYGSLDRQLDESDSFACKVKKLLAVRQAYGIATSRQV   600
Consensus         arslygslaeqldepdsfacqlkkilavrqaydiaaskqi

ZP_00900131.pro   LVPEVRSPGLLVMVHELPAGRGIQITALNFGQEAIAEELL   640
AAF26837.pro      LIPDVQAPGLLVMVHELPAGKGVQLTALNFSAEPVSETIC   639
DQ452614.pro      LIPDVQAPGLLVMVHELPAGKGVQLTALNFSAEPVSETIC   640
NP_745062.pro     LVPEVSSPGLLVMVHELPAGRGIQITALNFGQDAIAEELL   640
Consensus         lipdvqapgllvmvhelpagkgiqitalnfgaeaiaeeic

ZP_00900131.pro   LTGFTPGPVVDMINETVEGDLTEDGRLMVNLDPYEALCLR   680
AAF26837.pro      LPGVAPGPVVDIIHESVEGDLTDNCELQINLDPYEGLALR   679
DQ452614.pro      LPGVAPGPVVDIIHESVEGDLTDNCELQINLDPYEGLALR   680
NP_745062.pro     LTGFTPGPVVDMINETVEGDLTEDGRLMVNLDPYEALCLR   680
Consensus         lpgfapgpvvdiihesvegdltddcelminldpyealalr

ZP_00900131.pro   IVNSSGHV.     688
AAF26837.pro      VVSAAPPVI     688
DQ452614.pro      VVSAAPPVI     689
NP_745062.pro     IVNSSGHV.     688
Consensus         ivnaaghvi
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Chart 6. Compare Deduce Amino Acid Sequence of TreS Gene with published Amino Acid Sequence of TreS Gene. The Accession Number of Genbank of Comparing Sequence: ZP_00900131、AAF26837、NP_745062

4. Discussion

Through the experiment analysis, the expression product of ORF sequence which is from *Pseudomonas stutzeri* is Trehalose Synthesis gene. The homologous rate of the DNA sequence and reported TreS geneAF113617 is very high. We only searches one TreS gene on GenBank through BLAST. We compared the deduce amino acid sequence with the published amino acid sequence, it has the almost same region with the unknown function amino acid sequence, indicant enzyme and TreS gene sequence. It is reported that these regions correlate to the catalyze of amylum enzyme family and the substrate bind sites. So it has the similar structure domain. It is shows that the differences between amino acid sequences are not the reason of the different functions. So it has the deeply significance to research the third dimensional structure of Trehalose Synthesis protein, and further knows the mechanism of Trehalose Synthesis gene recognizing the substrate and the energy coupling in transferring between two indicans in the process of catalyzing.

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Mathematical Modeling of Salt Water Transport and its Control in Groundwater

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ABSTRACT: A mathematical model is presented for simulating two-dimensional sub-surface transport of salt-water considering the porous medium to be homogeneous and isotropic under the influence of constant seepage velocity. The transport equation is solved numerically by using finite difference method of Alternate Direction Implicit (ADI) scheme. Model simulations were carried out when sub-surface barrier is either absent or present. The effectiveness of the sub-surface barrier to arrest salinity movement is studied by choosing its locations at $X_1 = 1.2$ and $X_1 = 2.8$. The time evolution of the zone of salt-water concentration is studied for two years, four years, six years and ten years period for all the situations. It is observed that due to the barrier location at $X_1 = 2.8$, the salinity plume at the upper boundary is restricted in comparison to the non-barrier case. However, due to barrier, location at $X_1 = 1.2$ salinity intrusion is controlled significantly. [Nature and Science. 2006;4(4):32-39].

Keywords: Mathematical model; Simulation; ADI scheme; Salt water intrusion; Subsurface transport

INTRODUCTION

Intrusion of salt-water is a major problem in the coastal regions of all over the world. The occurrence of saline water intrusion in groundwater represents a special category of pollution, making groundwater unfit for human consumption as well as for industrial use. As these processes are of sub-surface nature, it is often difficult to assess the extent of contamination. Todd (1974)[4] has studied salt water intrusion in an aquifer and its control by considering various alternatives with respect to the flow field. Suitable sub-surface barrier was proposed by Todd (1974)[4] which includes cement grout, sheet pile or pulled clay etc. to arrest salt-water intrusion. Mahesha and Babu (2002)[3] have studied the effectiveness of sub-surface barrier on salt-water intrusion for sudden draw down conditions. Although, determination of interface by knowing the flow field is an important aspect under draw down condition, the roll of concentration gradient for mixing in relatively calm water at great depth cannot be over emphasized.

Latinopoulos *et al.* (1988)[2] have obtrained analytical solution for chemical transport in two-dimensional aquifers. Das *et al.* (2000)[1] have studied solute transport in porous media with first order chemical reaction for various disposal schemes by using numerical method.

The present study describes the evolution of salt-water concentration in a homogeneous, isotropic aquifer by assuming constant seepage velocity. This study would by useful in making initial estimation on the extent of aquifer contamination when *in situ* information is lacking and also for choosing suitable location of sub-surface barrier to arrest salinity intrusion.

MATHEMATICAL FORMULATION

The transport of conservative type of material through a homogeneous, isotropic plane aquifer of unit thickness under constant seepage velocity can be described by advective-dispersive differential equation in Cartesian coordinate system as :

$$D_x \frac{\partial^2 f}{\partial x^2} + D_y \frac{\partial^2 f}{\partial y^2} - V \frac{\partial f}{\partial x} - S \frac{\partial f}{\partial t} = 0 \qquad (1)$$

where, x – axis is in the direction of the flow and y – axis is along the depth of the aquifer. The schematic diagram of sub-surface transport process is shown in Figure1. Assuming the depth of the aquifer (L) is orthogonal to the natural groundwater flow direction, a finite line source of length 'a' aligned along the y-axis is allowed to enter into the model from left boundary. D_x and D_y being longitudinal and transverse dispersion coefficients respectively, U being the constant seepage velocity and R being retardation factor. The corresponding initial and boundary conditions can be described as –

$$f(x, y, 0) = f_i$$

$$f(0, y, t) = f_{input} \quad y \le a, \ t > 0 \qquad (2)$$

$$= f_i \quad y > a, t < 0 \qquad (3)$$

$$\text{Lt} \ \frac{\partial f}{\partial y} = 0 \qquad (4)$$

$$y \to L$$

$$\text{Lt} \ \frac{\partial f}{\partial f} = 0 \qquad (5)$$

$$x \to \infty$$

where, f_i is the initial concentration of the aquifer and f_{input} is the boundary concentration. An impervious sub-surface barrier of unit thickness extending upto length 'a' from impermeable bottom is considered at a distance x_1 from the left boundary. The following internal boundary condition is imposed for impervious barrier.

$$\text{Lt} \ \frac{\partial f}{\partial x} = 0, \ y \le a, \ t > 0 \qquad (6)$$

$$x \to x_1$$

Writing the governing equation (1) in non-dimensional form as –

$$D_1 \frac{\partial F}{\partial X^2} + D_t \frac{\partial F}{\partial Y^2} - \frac{\partial F}{\partial X} - S \frac{\partial F}{\partial T} = 0 \qquad (7)$$

where, the dimensional quantities are given by –

$$X = \frac{x}{L}; Y = \frac{y}{L}; A = \frac{a}{L}; T = \frac{tU}{L}; F = \frac{f}{f_0}; F_1 = \frac{f_1}{f_0}; F_{input} \frac{F_{input}}{f_0}; D_1 = \frac{D_x}{LV}; D_1 = \frac{D_y}{LV}$$

Here, f_0 is the concentration of sea salinity. The corresponding initial and boundary conditions can be written in the dimensionless form as –

$$F(X,Y,0) = F_1 \qquad (8)$$

$$F(0,Y,T) = F_{input} \quad Y \leq A,\ T \geq 0 \qquad (9)$$

$$= F_1 \quad Y \leq A,\ T \geq 0$$

$$\text{Limit}\ \frac{\partial F}{\partial Y} = 0 \qquad (10)$$

$$Y \to 0$$

$$\text{Limit}\ \frac{\partial F}{\partial Y} = 0 \qquad (11)$$

$$Y \to \infty$$

The governing equation (7) described above is solved by using initial and boundary conditions (8) – (11) and employing finite difference scheme, specifically by using ADI method.

NUMERICAL COMPUTATION

In order to solve the governing equation (7) numerically, a well known. Alternate Direction Implicit (ADI) scheme of finite difference technique is adopted. This is based on implicit approach of the Douglas Rachford difference scheme. The details of the scheme and its finite difference representation can be obtain from Das *et al.* (2000)[1]. To get an insight in to the dispersion process, numerical computations have been made by assigning the values of various parameters as $D_1 = 0.5$, $D_t = 0.05$, $A = 0.6$. Initially the aquifer is free from salinity i.e., $F_i = 0$. The boundary salinity F_{input} is taken as unity due to non-dimensional formulation with respect to constant sea salinity i.e. f_0 = 34 ppt, considered in this study.

The model area covers non-dimensional distance varying from 0 to 5.0 along the length of the aquifer and 0 to 1.0 along the depth of the aquifer. A square grid cell with $\Delta X = \Delta Y = 0.1$ is considered for which the total number of grid cells along (X, Y) directions, become (50, 10). The model simulations were carried out for 3 years, 6 years, 9 years and 12 years period either due to the absence or presence of the sub-surface barrier. Further, effectiveness of the barrier is investigated by considering two locations for which non-dimensional distances are $X_1 = 2.8$ and $X_1 = 1.2$, which is nearly middle of the aquifer and closer to the left boundary respectively.

RESULTS AND DISCUSSION

To begin with, the model simulations were carried for non-barrier case. Figure 2(a) – 2(d) show the evolution of the zone of salt-water concentration for 3 years, 6 years, 9 years and 12 years respectively. To analyze the result, monitoring concentration has been chosen as 0.05, which is 5% of the input concentration, beyond which groundwater would not be useful, assumed in this study. It can be observed that the concentration plume progresses upto a distance of 24 grids (X = 2.5) along the longitudinal direction and 7 grids (Y = 0.7) along vertical direction (Figure 2a). The contour lines with higher values indicate higher concentration. At the end of four years simulation, concentration plume touches at the upper boundary, and the interface moves upto 36 grids along longitudinal direction (Figure 2b). The salt-water plume further progresses at the end of nine years simulation and covers nearly 85% the area of the aquifer (Figure 2c). Finally, after ten years of simulation whole groundwater becomes completely saline (Figure 2d).

To understand the effectiveness of the barrier and its location, the barrier is considered at a distance X_1 = 2.8 which is nearly middle of the aquifer length. Figure 3(a) – 3(d) show the evolution of the zone of salt-water concentration plume with various years of simulation. Figure 3a shows that the salinity contours progresses without any disturbance at the end of 3 years, similar to the non-barrier case. After 6 years of simulation it is observed that the interface just touches the barrier and dispersion takes place towards the upper boundary (Figure 3b). At the end of 9 years simulation, the corresponding concentration values are found to be lower in comparison to the non-barrier case. The concentration plume moves upto a distance of 37 grids as shown in Figure 3(c). Figure 3(d) shows that the contour with 0.05 attaches completely to the upper boundary. There is an overall reduction of salinity due to the presence of the barrier.

Figure 4(a) – 4(d) shows the movement of concentration plume when an impervious barrier is considered at a distance (X_1 = 1.2) from the left boundary. It can be seen that due to closeness of the barrier from left boundary, salt-water intrusion is arrested significantly (Figure 4a). However, due to the presence of the barrier the saline water disperses in the vertical direction due to concentration gradient. After four years of simulation, the concentration plume attaches to the upper boundary and spreads upto nearly 10 grids (Figure 4b). At the end of 9 years simulation, the spreading of salinity plume further increases at the upper boundary and covers upto 20 grids (Figure 4c). Figure 4(d) shows that the interface could move upto 39 grids along horizontal direction. Therefore, any possible withdrawal of groundwater after a longitudinal distance of 1.2 near the bed would indicate salinity, whereas, at higher depth it still remains unaffected. At the end of 12 years of simulation, the corresponding concentration values are found to be lower in comparison with earlier cases, indicating significant reduction in salinity. Model results indicate that the barrier closer to the sea face boundary reduces the salinity intrusion considerably.

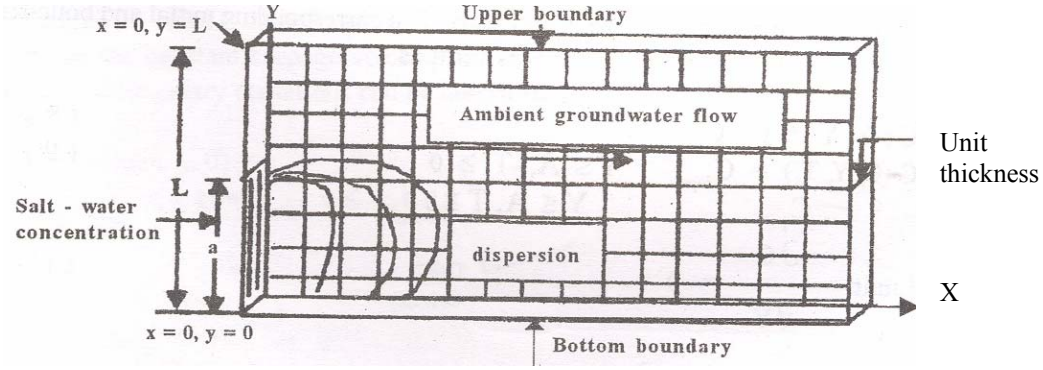

Figure 1. Schematic cross sectional representation of subsurface transport in an aquifer

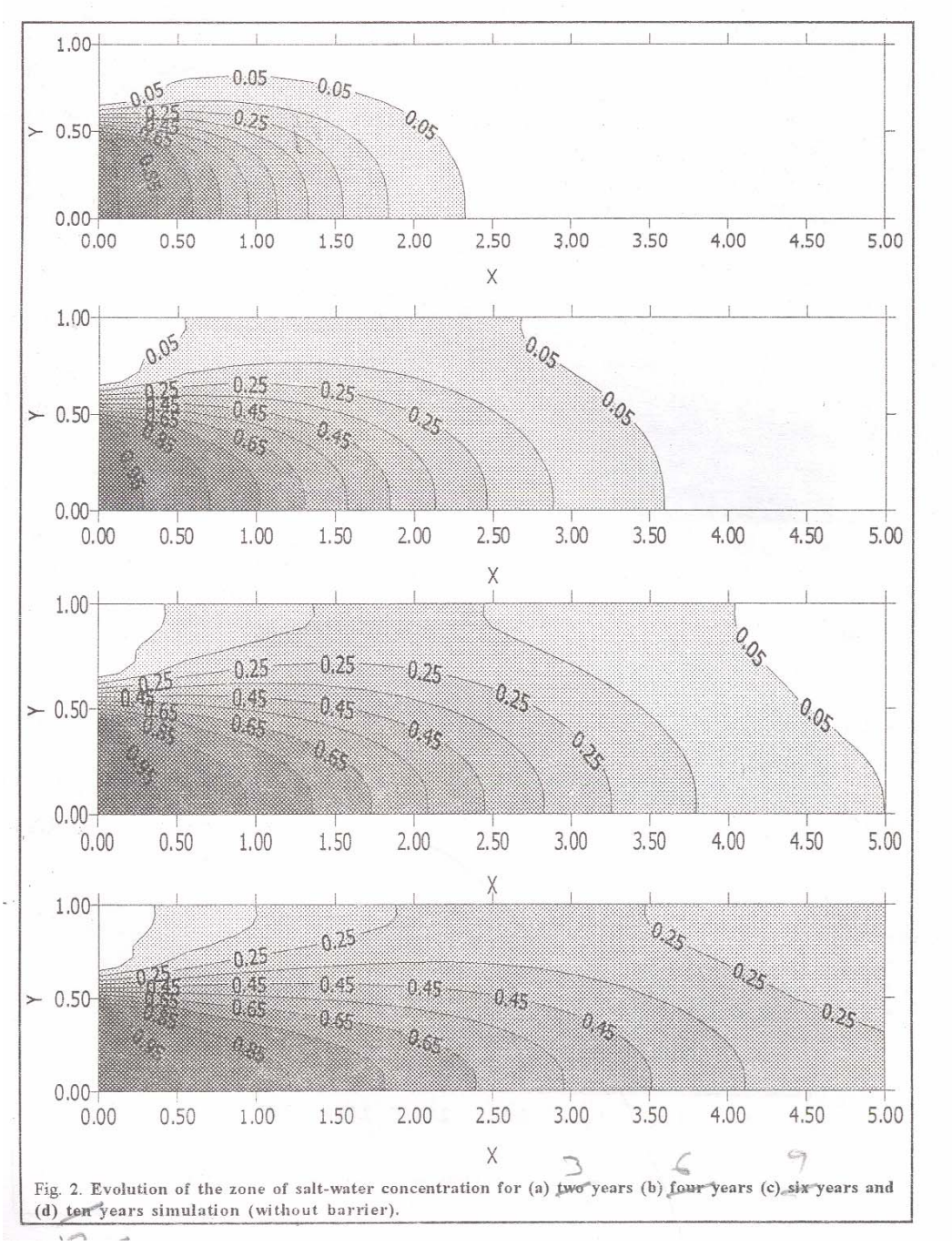

Fig. 2. Evolution of the zone of salt-water concentration for (a) two years (b) four years (c) six years and (d) ten years simulation (without barrier).

Figure 2. Evolution of the zone of salt-water concentration for (a) 3 years (b) 6 years (c) 9 years and (d) 12 years simulation (without barrier)

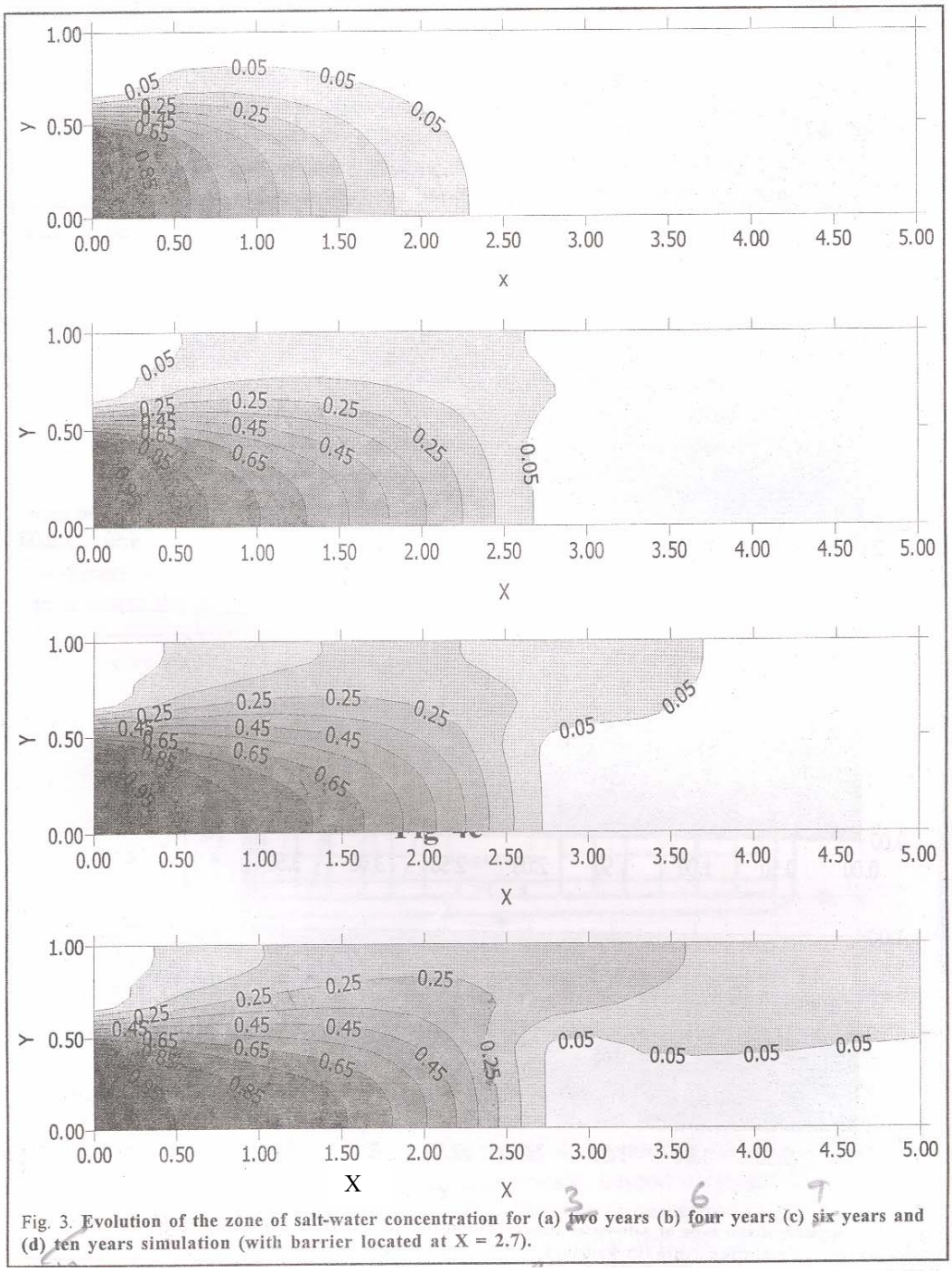

Fig. 3. Evolution of the zone of salt-water concentration for (a) two years (b) four years (c) six years and (d) ten years simulation (with barrier located at X = 2.7).

Figure 3. Evolution of the zone of salt-water concentration for (a) 3 years (b) 6 years (c) 9 years and (d) 12 years simulation (without barrier located at X = 2.8)

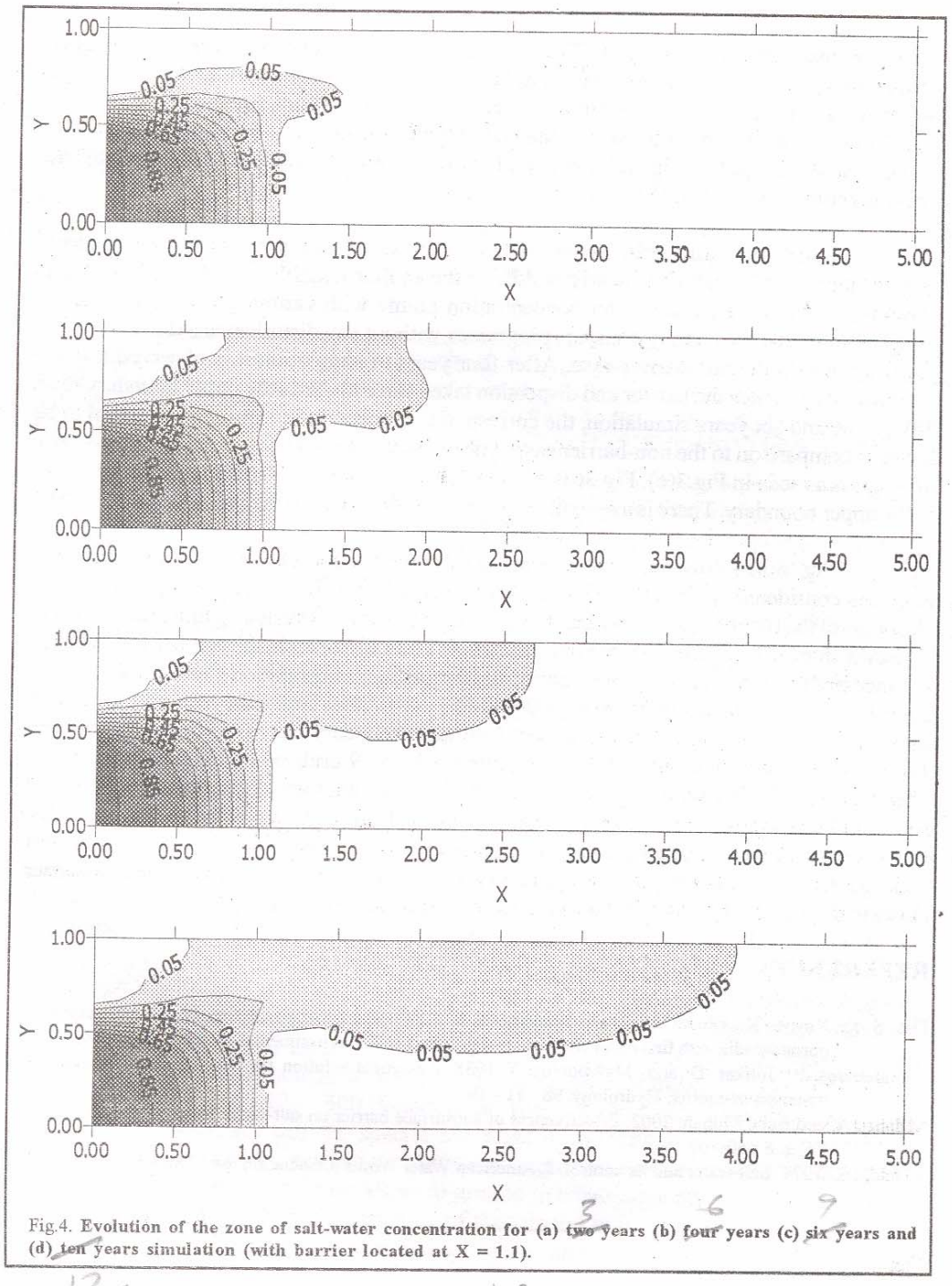

Fig.4. Evolution of the zone of salt-water concentration for (a) two years (b) four years (c) six years and (d) ten years simulation (with barrier located at X = 1.1).

Figure 4. Evolution of the zone of salt-water concentration for (a) 3 years (b) 6 years (c) 9 years and (d) 12 years simulation (without barrier located at X = 1.1)

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A contribution on *myxosoma* Infection in Cultured *Oreochromis niloticus* in Lower Egypt

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ABSTRACT: Heavy infection with myxosoma spp. has been recorded among cultured ***Oreochromis niloticus*** from Sharkiya governorate at Lower Egypt. Prevalence of the infection exceeded 80 % of all examined fish. Head cysts and hole in the head like lesions have been recorded among all examined fish. The causative agent "*myxosoma*" has been identified using classical taxonomical and histopathological techniques. The genetic non-relatedness of the salmonid ***Myxosoma cerebralis*** to the African cichlid *myxosoma* spp has been confirmed using a polymerase chain reaction (PCR) involving a specific primer of the gene encoding 18-S ribosomal RNA of ***M. cerebralis***. [Nature and Science. 2006;4(4):40-46].

KEYWORDS: *Oreochromis niloticus*, *Myxosoma*, Lower Egypt, Molecular Identification

INTRODUCTION

Myxosoma spp. is the best known of 1300 parasites grouped in the phylum Myxozoa and the first shown to possess a 2 host life cycle including fish and an aquatic oligochaete, ***Tubifex tubifex*** (Markiw & Wolf 1983, Wolf & Markiw 1984, Wolf et al. 1986). Myxosporean research in Africa dates back to the late 19th century with Gurley (1893) as one of the earliest authors referring to the continent. The African continent boasts over a 100 myxosporean species from freshwater, brackish and marine fishes of which 84 infect primarily freshwater fishes (Fomena and Bouix 1997) and this number is continuously growing. When comparing the known African myxosporeans to the more than 1,300 species described worldwide, it is evident that for a huge continent with such high fish diversity, a large gap exists in the knowledge on the occurrence and distribution of these parasites.

El-Mansy (2005) revised the taxonomy of myxosporean species, using specimens isolated from plasmodia situated in the infected cornea of ***Oreochromis aureus***, ***O. niloticus*** or ***Tilapia zillii*** inhabiting the River Nile, Egypt. In addition, he described the histological effects of the parasite on the infected tissues. He also indicated that the spores of ***Myxobolus heterosporus*** had a variety of shapes expressing remarkable heteromorphism. He also, reported the presence of five main myxobolus like spore types and tailed-spores. In the same study, El-Mansy (2005) also indicated that light and electron microscopy supported that spores of a myxobolus-like morphology coexisted with so-called tailed-spores in one plasmodium and some transitional stages from myxobolus-like spore types to tailed-spores were observed. Therefore, he concluded that some tailed-spores may be simply heteromorphs of myxobolus.

Numerous species of myxosoma *(*formerly known as myxobolus*)* have been reported among different African tilapia species within the last few decades. Among these myxosporean species , the ***Myxobolus Bütschli***, 1882 species from tilapia of the Okavango River and Delta, ***Myxobolus africanus*** Fomena, Bouix and Birgi, 1985 from the gills and fins of ***Hepsetus odoe*** (Bloch, 1794), ***Myxobolus camerounensis*** Fomena, Marqués and Bouix, 1993 from the gill arch of ***Oreochromis andersonii*** (Castelnau, 1861), ***Myxobolus hydrocyni*** Kostoïngue and Toguebaye, 1994 from the gills of ***Hydrocynus vittatus*** Castelnau, 1861, ***Myxobolus nyongana*** (Fomena, Bouix and Birgi, 1985) from the gills of ***Barbus poechii*** Steindachner, 1911, ***Myxobolus* cf. *tilapiae*** Abolarin, 1974 from the buccal cavity of ***Tilapia rendalli rendalli*** (Boulenger, 1896), ***Myxobolus etsatsaensis*** sp. n. from the gills of ***Barbus thamalakanensis*** Fowler, 1935, ***Myxobolus paludinosus*** sp. n. from the gills of ***Barbus paludinosus*** Peters, 1852. There are currently 11 myxobolus species parasitizing cichlids in Africa (Baker 1963, Abolarin 1974, Landsberg 1985, Faisal and Shalaby 1987, Sakiti et al. 1991, Fomena et al. 1993).

Despite the presence of numerous reports on the myxosporean affecting African cichlids as well as non cichlid fishes such as salmonid fishes worldwide, none of this reports described the pathological lesions as well as molecular work reported in the current study. The current study also presents a unique variety of diagnostic approaches used for the diagnosis of myxosoma infection in ***O. niloticus***.

MATERIALS AND METHODS

Fish

In the midsummer of 2006, 100 ***Oreochromis niloticus*** (average size 50 gm) were collected from an earthen pond at Abbasa Fish Farm, Sharkiya Governorate, Lower Egypt and brought alive to the Fish Diseases and Management Laboratory (FDML) at Faculty of Veterinary Medicine, Cairo University. Tilapias were kept in well-aerated, temperature adjusted water aquaria until examined.

Sampling and Sample processing.

Tilapias were euthanized with an overdose of MS 222 (Tricaine methane sulfonate, Finquel- Argent Chemical Laboratories, Washington) and visually inspected for any abnormalities before examination is adopted. Lesions were photographed and documented using Sony digital camera (Japan).

Skin, gill scraps were performed on all examined fish and photos for the plasmodial stage of the myxosoma spp. were taken and sketched then used for taxonomical identification of the myxosoma spp. Also, eyes of the fish were examined for the presence of any myxosporean spores within both cornea and lens. Further, the fish were cut open using the standard triangular dissection technique then impression smears from different organs including liver, spleen, kidneys, gall bladder were made. Stomach and intestinal scraps were also made from the examined fish. All samples were freshly examined using regular light microscope as well as dissecting microscope. Some samples required special staining using Giemsa stain.

Sections from the head cysts, head cartilages, eyes, gills, brain, liver, spleen, kidneys, intestine and gonads were stored in 10 % neutral formalin and sent for histopathology. Histopathology was performed on 1-5 µm sections of the above mentioned organs and stained with hematoxylin and eosin (H&E) and or special stain (Giemsa stain). Method and criteria used for histopathological examination was adopted from Baldwin et al. 2000.

Samples from the head cysts, head muscles, kidneys were collected into microfuge tubes and stored in -30 for PCR testing. The following method was adopted for DNA extraction: A total of 500 µl of phenol: chloroform: isoamyl alcohol (25:24:1) was added to each lysed myxospore preparation, and each preparation gently inverted several times to form an emulsion. The organic phase was separated from the aqueous phase by centrifugation at 1700 × *g* for 10 min at room temperature. The upper aqueous phase, containing DNA, was transferred to a new micro-centrifuge tube and an additional 500 µl of phenol: chloroform: isoamyl alcohol added. The solutions were mixed and centrifuged as before. The aqueous phase was again transferred to a new micro-centrifuge tube, 500 µl chloroform added, and the solutions mixed and centrifuged as before. The DNA was precipitated by adding 800 µl of ice-cold ethanol and incubating at –20°C for 1 h, followed by centrifugation at 12 000 × *g* for 10 min at 4°C. The supernatant was discarded and the DNA pellet permitted to air dry. The pellet was re-suspended in 50 µl of TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0) and held overnight at room temperature. Samples were warmed to 65°C for 1 h to ensure solubilization and subsequently allowed to return to room temperature prior to use in the PCR reaction.

Primers used were adopted from that published by Andree et al. (1998) as (5'-GCATTGGTTTACGCTGATGTAGCGA- 3') and (5'-GGCACACTACTCCAACACTGAATTTG- 3'). The standard reaction volume was 50 µl (45 µl of master mix and 5 µl of DNA template). The PCR master mix was comprised of PCR buffer (300 mM Tris, 75 mM ammonium sulfate, pH 9.0), 2.5 mM MgCl, 400 µM dNTPs, 20 pmol of each primer, and 2 U µl–1 taq DNA polymerase (Fisher Scientific). All reagents were stored at –20°C and kept on ice after thawing. Taq polymerase was the last reagent added. Amplifications were performed using a thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). A denaturation step in which samples were held at 95°C for 5 min took place before amplification cycles began. One complete cycle consisted of 1 min at 95°C, followed by 2.5 min at 65°C, followed by 1.5 min at 72°C. This cycle was repeated 35 times, after which an extended elongation step of 10 min at 72°C concluded the program. When necessary, amplified DNA samples were stored at 4°C.

RESULTS & DISCUSSION

Clinical examination of the examined tilapia revealed the presence of number of clinical abnormalities including, frontal head cysts (Figure1& 2) which usually progress to hole in the head in most of cases (Figure 3), skin erosions, fin rot, and corneal opacity. A relatively small % of the examined fish (5 %) revealed the presence of ring of cysts that surrounded the iris of the eye (Figure 4). Wet mount, Methylene blue and Giemsa stained slides examination of the affected eyes revealed the presence of high number of plasmodial stages of myxosoma spp. within each cyst. Such myxosoma spp. was sketched and further identified as ***M. heterosporus*** **(Baker, 1963)** according the recent revision published by El-Mansy (2005). However, there is a very close similarity between the plasmodial stage of the identified ***M. heterosporus*** and that of ***M. tilapiae***. The polar capsules of ***M. heterosporus*** are, however, more pyriform, compared with the more spherical polar capsules of ***M. tilapiae***. Histologically, H& E stained sections made from the corneal tissues of the eye

revealed the presence of large number of the ***M. heterosporus*** plasmodial stages associated with localized inflammatory changes and mononuclear cell infiltration (Figure 5).

Clinical examination also revealed that over 80 % of the examined fish were associated with head cysts that usually proceed to hole in the head like lesion. Giemsa stained as well as non stained scraps made from these lesions together with impression smears made from muscles sections of the head cysts revealed the presence of the same type of myxosoma spp plasmodial spores (Figure 7) that presumptively identified as ***M. tilapiae*** with spore body oblong to oval with anterior and posterior ends bluntly rounded, 14.0-15.5 (15.0 ± 0.39) μm in length. Widest region of spore observed towards centre of spore body, 12.0-12.6 (12.3 ± 0.27) μm in width. Two almost spherical to pyriform polar capsules of equal size situated in anterior part of spore. Polar filaments have four to six coils within polar capsules. Interestingly another spp of myxosoma has been detected in impression smears made from kidneys and intestine of the affected fish (Figure 8). The vegetative sporogenic stage is relatively similar to that of ***M. etsatsaensis*** and ***Myxobolus*** sp. described by Obiekezie and Okaeme (1990) from the kidneys and spleen of various cichlid species.

An alternative to histologic examination a polymerase chain reaction (PCR) amplification of a DNA sequence unique to *Myxobolus cerebralis* (Andree et al. (1998) have been performed on the DNA extracts from the muscle sections from the head cysts, heavily infested eyes, and kidneys. Unfortunately, the characteristic 415 bp amplicon of the gene encoding 18S ribosomal RNA of M. cerebralis was not detected in any of the tested DNA extracts of the above mentioned tissues.

The results came in this study, was in full accordance with that of other myxosporean studies which previously described in details the taxonomy of numerous species of myxosoma (formerly myxobolus) affecting African cichlids. However, the unique aspect of this study is tightly associated to the great linkage between rarely recorded pathological finding associated with myxosporean invasion in *Oreochromis niloticus* as well as the usage of new techniques such as molecular tools utilized to confirm the classical taxonomical methods.

The high prevalence (more than 80 %) of myxosporean infection among the examined fish highly suggests that such infection is endemic in the ponds used for rearing of these fish. This suggests that the tubificid oligocheat worm which act as an IMH for the myxosoma spp is highly distributed in an active manner within the mud of such earthen ponds and that's why more than one spp of myxosoma have been reported in this study. The presence of myxosporean plasmodial stages within external as well as internal organs as kidneys and intestine strengthen the same conclusion.

The myxosporean ***Myxosoma heterosporous*** detected in the cornea of 5 % of the affected fish and its associated histological changes were similar to those of the infected cornea of O. aureaus, O. niloticus and T. zilli described by El-Mansy (2005).

The more vigorous picture was the development of head cysts in the frontal and occipital areas in the affected tilapia. Such cysts were highly populated with large number of myxsporean ***Myxosoma tilapiae*** and or ***M. heterosporus***. These cysts ultimately ruptured leaving a hole in the head like lesion in the frontal aspect of the head. The pathological sequence of such myxosporean invasion highly recommend the assumption that myxosporean parasites secretes certain kinds of proteolytic enzymes that liquefy the infected tissue and enables quick increase of the spores in each affected head cyst . The proteolytic enzymes might include some enzymes that digest the cement material between the connective tissue, skin, and other tissues. Thus a hole in the head like lesion is the ultimate fate of such myxosporean invasion has developed.

The measures of the myxosoma spp. reported in the current study coincides with those of ***M. tilapiae, M. heterosporus*** reported by Reed et al. (2002) and El-Mansy et al. 2005. While those detected in the kidneys, intestine were in accordance with that of ***M. etsatsaensis*** and ***Myxobolus*** sp. described by Obiekezie and Okaeme (1990) from the kidneys and spleen of various cichlid species.

Myxosoma cerebralis specific PCR has been used to detect the gene encoding for *M. cerebralis* 18 S ribosomal RNA within the DNA extracts of affected tilapia tissues. The aim was to confirm the specificity of such test to identify the myxosporean parasite to the species level and confirm close relatedness of ***M. cerebralis*** of salmonids to their closely related cichlid spp as those reported in this study. Unfortunately, the specific 415bp band was not obtained with any of the above tested tissues of ***O. niloticus*** which suggests two main facts. First, the cichlid myxosporean are genetically different from those of salmonids. Second, in vivo expression of the cichlid myxsporean genes are majorly different from that of salmonids.

In conclusion, this study is a unique mixture between classical and recent diagnostic tools through which prevalence and severity of myxosoma infection have been clearly presented and discussed. This study can be the first bead in the chain of new diagnostic approach for the myxosporean parasites affecting African cichlids.

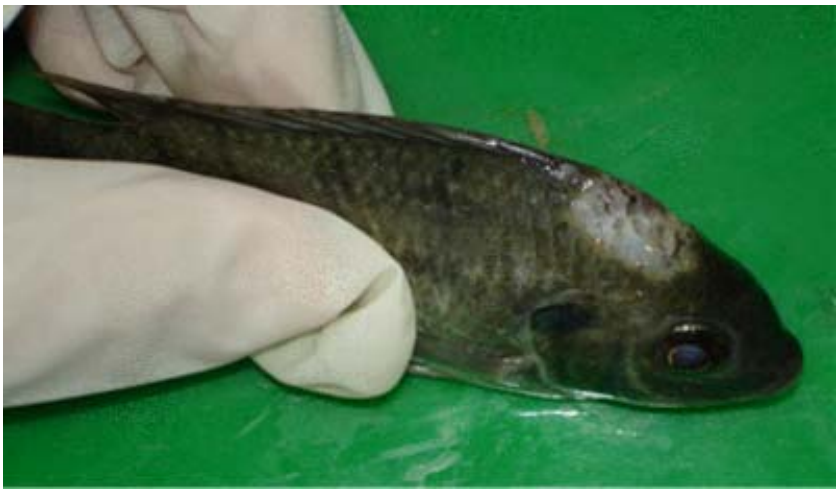

Figure 1. *Oreochromis niloticus* with frontal head cyst (Myxospoearn vegetative plasmodial stages were isolated from the cyst scrap).

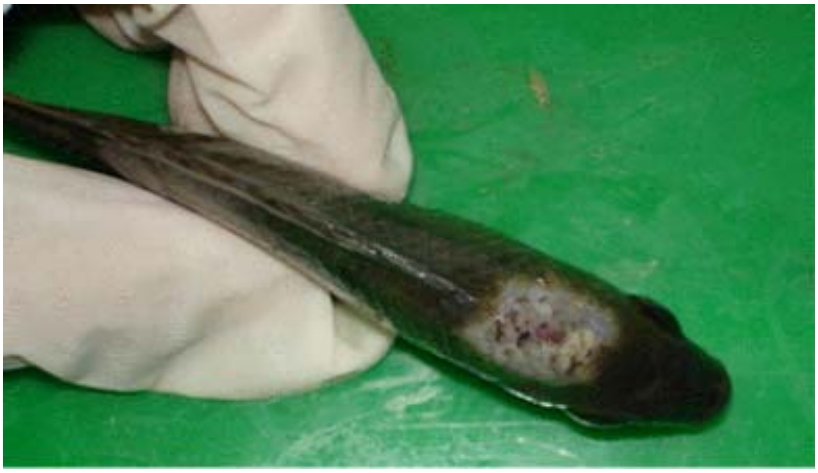

Figure 2. *Oreochromis niloticus* with more severe frontal head cyst (notice the progression of the lesion).

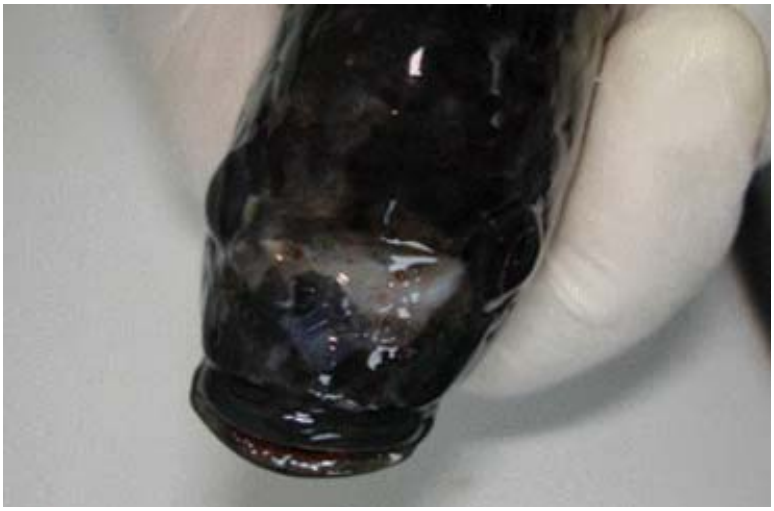

Figure 3. *Oreochromis niloticus* showing a ruptured head cyst leaving a hole in the head like lesion. *M. tilapiae* and *M. heterosporus* were isolated from the infection site.

Figure 4 *Oreochromis niloticus* with a corneal myxosporean cyst ring surrounding the iris.

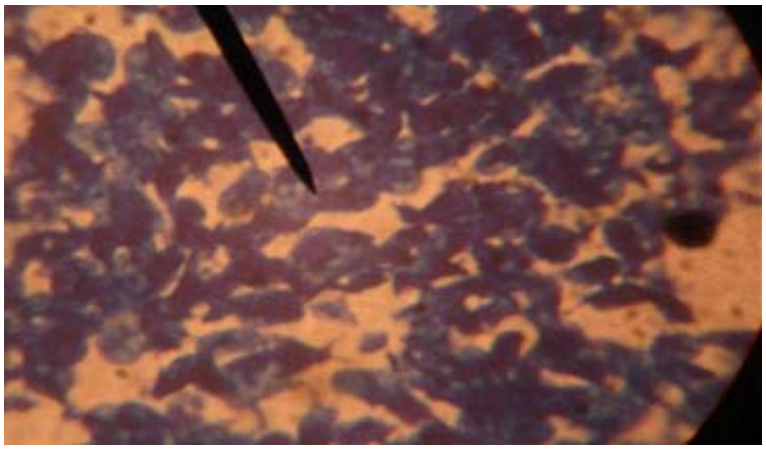

Figure 5. A Giemsa stained histopathological section of the eye (Cornea) showing heavy infiltration of the tissue with deeply stained myxosporean vegetative plasmodial stages.

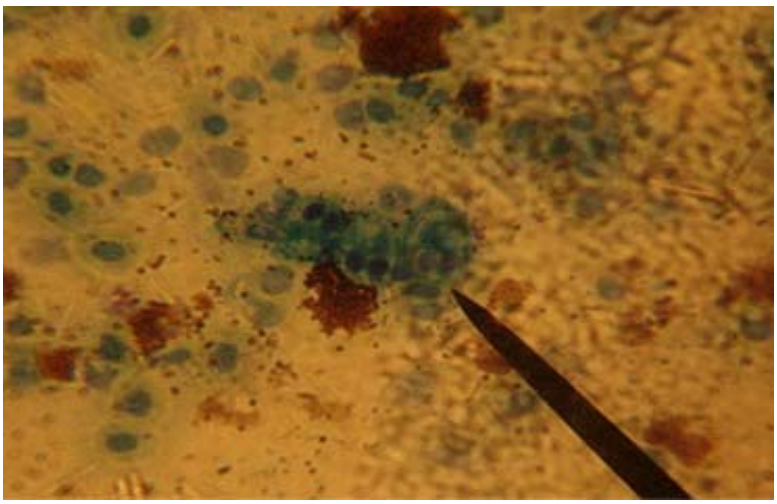

Figure 6. A Giemsa stained wet mount from the corneal cysts showing M. heterosporus plasmodial vegetative spores.

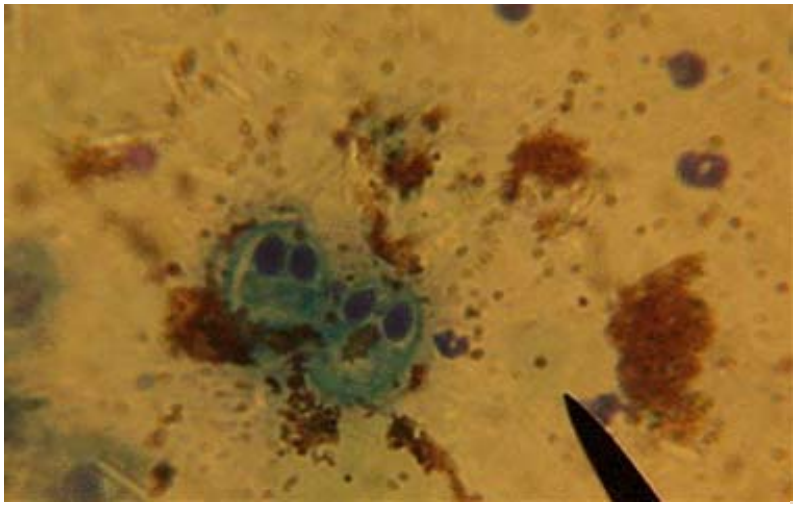

Figure 7. Vegetative plasmodial spore of *M. tilapiae* in Giemsa stained wet mount from the head cyst.

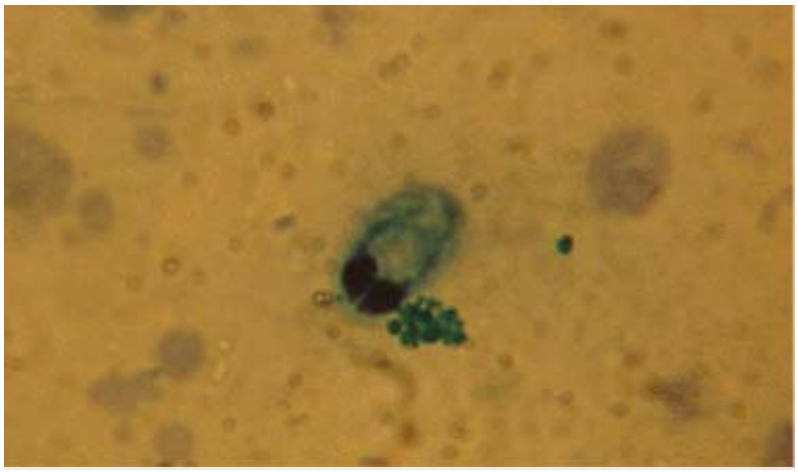

Figure 8. A Giemsa stained wet mount from the intestinal scrap showing deeply stained plasmodial vegetative stage of a myxosoma.

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The Feature of Service Industry Development on the Old Industrial Bases in China's Northeast

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Abstract: Service (tertiary) industry plays an important role in economic growth, creating more jobs, speeding up urbanization, improving people's living standard. Therefore, in the process of adjusting and rebuilding the old industrial bases in China's northeast, we must pay great attention to developing the service sector. First, we analyzed the general situation of the service industry development in China's northeast three provinces. Second, we compared with the internal services and the economic efficiency comparison of service industry in China northeast three provinces. At last, we gave some suggestions for developing service industry development in China's northeast three provinces. [Nature and Science. 2006;4(4):47-52].

Keywords: industry; China; northeast; development; comparison

I. Introduction

China central government implemented to develop the strategy of the old industrial bases in china's northeast (China's northeast refers to Liaoning, Jilin, Heilongjiang province). So in recent year, China's northeast these three provinces have showed a rapid economic development. The service industry in these provinces has developed rapidly and become an important power of economic growth, along with the second industry. It pushed increasing of the three provinces' GDP forward, and improved industrial structure of the three provinces' economy. The total value created by Heilongjiang province's service industry increased year by year, from the year 2000 to 2005, the added value of the service sector rose from RMB 102.75 billion to RMB 186.67 billion, increased by 1.82 times. The proportion of service industry added value in the GDP increased steadily, it kept at 31%to 33%; Jilin service industry added value reached RMB 140.28 billion in 2005, the proportion in the GDP was 38.8%. However since 2001, the added value of Liaoning service industry has exceeded RMB 200 billion, the proportion in the GDP went up to 40%, which was much higher than Heilongjiang and Jilin province, and it developed fastest among the three. The proportion of Heilongjiang service industry added value in the GDP is 33.9%. It is not only lower than Liaoning's 40.5% and Jilin's 38.8%, but also lower than the national average level 40.3% (Table 1).

II. Analysis on the internal services of China northeast three provinces

On the whole, the services sector in Heilongjiang, Jilin, Liaoning province has maintained rapid and favorable development, the internal services also increased steadily. In which, the development of transportation, warehousing and post was well. Growth rate and pulling effect on GDP was enhanced, traditional service industries like wholesale and retail trade, catering service always played an important role in the GDP .We can see from the indices, added value, growth rate and proportion in the GDP, each of them is

far higher than that in other internal services. Insurance, finance, and real estate increased rapidly year by year, it reflected prosperity in both investment and sale.

Table 1. Value added of the tertiary industry and proportion to GDP

RMB billion

Year	China	Proportion to GDP（%）	Heilongjiang	Proportion to Heilongjiang	Jilin	Proportion to Jilin	Liaoning	Proportion to Liaoning
2000	29904.6	33.4	1027.5	31.6	620.0	34.1	1851.0	39.7
2001	33153.0	34.1	1153.0	32.4	741.9	36.5	2043.9	40.6
2002	35132.6	33.5	1266.0	32.6	822.3	36.7	2258.4	41.4
2003	38885.7	33.2	1396.8	31.5	892.2	35.4	2487.9	41.4
2004	43384.0	31.8	1559.9	29.4	1017.9	34.4	2823.9	41.1
2005	73395.6	40.3	1866.7	33.9	1402.8	38.8	3244.6	40.5

Source：China statistical yearbook-2005

Table 2. Added value of internal service industries in 2004

RMB billion

Region / Sector	Heilongjiang			Jilin			Liaoning		
	Added value	Increasing rate	Proportion（%）	Added value	Increasing rate	Proportion（%）	Added value	Increasing rate	Proportion（%）
GDP	5303.0	19.7	100	2958.2	17.3	100	6872.7	14.5	100
Service	1559.9	11.7	29.4	1017.9	14.1	34.4	2823.9	11.7	41.1
Transportation, Warehousing and Post	306.2	10.0	5.8	180.2	18.3	6.1	613.0	23.7	8.9
Wholesale, Retail trade, Catering service	479.7	13.0	9.0	368.3	12.4	12.5	896.0	11.6	13.0
Finance, Insurance	38.96	4.0	0.7	21.5	5.5	0.7	159.7	9.9	2.3
Real estate development	134.6	9.2	2.5	67.3	1.8	2.2	206.1	9.4	3.1
Other service	600.4	12.6	11.4	380.6	16.8	12.9	949.0	11.0	13.8

Source：China statistical yearbook-2005

a：Increasing rate and growth rate of GDP are calculated on the basis of the last year

But we can also see from Table 2, the internal services of Heilongjiang province, especially the development of modern service, knowledge-and-technology-intensive service sector, falls behind the other internal sectors. However the proportion of traditional service is on the high side. Heilongjiang's growth rate of transportation, warehousing, post and telecommunications is 10% in 2004, which is lower than Jilin's 18.3% and Liaoning's 23.7 %; The growth rate of wholesale and retail trade, catering service is 13%, which is equivalent to Jilin's and higher than Liaoning's 11.6%, but the total added value of wholesale and retail trade in Heilongjiang province is RMB 47.97 billion, and RMB 36.83 billion in Jilin, both of them are lower than Liaoning's RMB 89.6 billion. And the indices of finance, insurance, education are all below Liaoning province. Whether in the field of total output, economic growth rate or contribution to GDP, Liaoning province is better than Heilongjiang and Jilin province.

III. The economic efficiency of service industry on the old industrial bases in China northeast

III.1. The employment growth

Petty-Clark's law pointed out that, with the continuously increased in per capita national income, social labor force distributing in tertiary industry shows that labor force of service industry will increase. In other words, the development of service industry can promote employment. In regard to the number of service industry employees in the northeast three provinces, from 1996 to 2004, the number of service employees in the northeast three provinces increased from 3.684 million to 3.896 million, the proportion of employment rose from 29.3% to 34.9%. Although the total number of service industry employees has increased continuously, the number of added quantity is low, promotion effect on employment is not enough. In recent ten years, the proportion of service industry employees to the province employees rose only by 0.7 point, but the proportion of Jilin and Liaoning province rose by 5.6 point and 7.1 point respectively.

In order to measure how much an industry contributes to the region's economy, we regard the number of employment brought by this industry as an important index. Seen from the comparison, Heilongjiang service industry contribution to employment increase is lacking of efficiency.

Table 3. Distribution of employed person in 2004（%）

Industry / Region	Primary industry	Second industry	Tertiary industry
Heilongjiang	49.1	20.4	30.5
Jilin	46.5	18.6	34.9
Liaoning	36.8	24.8	38.4

Source：China statistical yearbook-2005

III.2. The tourism development

In recent years, tourism of Heilongjiang, Liaoning, and Jilin province developed fast. For example in Heilongjiang province, the total income of tourism was RMB 28.03 billion in 2005, made up 5.1% of the GDP. On the other hand, the characteristic tourism formed scale gradually, Ice Snow tourism kept rapid development. It also paid attention to exploit forest tour resources, frontier tour resources and humanity travel resources. The province has successfully hold "Haerbin Ice Snow Festival, Wudalianchi Water-drinking Festival, Jingpo Lake Golden Autumn Festival, Zhalong Crane Festival, Jiamusi Three Rivers Festival, and Yichun Forest Festival".

Heilongjiang foreign exchange earnings from tourism has increased from $1.89 hundred million in 2000 to $3.02 hundred million in 2004, rose by 59.79%, Jilin foreign exchange earnings in tourism has increased from $0.58 hundred million in 2000 to $0.96 hundred million in 2004, rose by 65.52%, Liaoning foreign exchange earnings in tourism has increased from $3.83 hundred million in 2000 to $6.13 hundred million in 2004, rose by 60.05%. Although the increased range of Heilongjiang province was smaller than Jilin province, the foreign exchange earnings in Jilin province hasn't exceed $100 million all along. Tourism of Liaoning province found itself both the foreign exchange earnings and the proportion of the nation tourism earnings in an advantageous position. There are two main reasons result in the tourism differences among the three provinces: The first, whether there are special tour resources in the area or not. In Liaoning province, there are inshore tour resources, so the tourism earnings are big; the second reason, whether or not the government gives enough strong support, the enterprises exploit and create new tour resources, the successful holding of "Haerbin Ice Snow Festival" is just an example. However Jilin province hasn't set up an advantage aspect. So the trend that tourism development in Jilin province went behind the other two provinces.

III.3. The output capacity

In order to compare with the output capacity of the service in the northeastern three province, we computer the labor productivities, take the value of output per unit of labor as the index to consider the labor capacity, this index shows that per unit of labor can create the value in some distinct, and tells the true that the labor productivities. The results show the labor productivity belongs to different levels, the absolute number of each of them differs near RMB 5000 yuan. Considering the output capacity, Heilongjiang is 20.7% higher than Jilin, but it is below the Liaoning, it stands in the middle, Liaoning output capacity is highest, exceed 19.5% than Heilongjiang, and 44.3% than Jilin. It has absolute advantage on the top, but Jilin has absolute disadvantage. Therefore, we should improve the output capacity of the service of Heilongjiang and Jilin.

Table 4. International tourism foreign exchange earnings

USD million

Region / Year	China	Heilongjiang	Heilogjiang/ China	Jilin	Jilin/ China	Liaoning	Liaoning/ China
2000	16624	189	1.14%	58	0.35%	383	2.30%
2001	17792	250	1.41%	76	0.43%	463	2.60%
2002	20385	297	1.46%	86	0.42%	550	2.70%
2003	17406	244	1.40%	66	0.38%	454	2.61%
2004	25739	302	1.17%	96	0.37%	613	2.38%

Source：Calculate on China statistical yearbook

Table 5. 2004 tertiary industry value of output per unit of labor

RMB

Region	Employment (10000persons)	Value added of the tertiary industry (RMB100 millions)	Value of output per unit of labor (RMB/person)
Heilongjiang	494.5	1559.9	31545
Jilin	389.6	1017.9	26127
Liaoning	749.1	2823.9	37697

Source：Calculate on China statistical yearbook-2005

IV. Suggestions for developing service industry on the old industrial bases in China's Northeast

Seen from the development of service industry in China east coastal areas, some important elements have significant effects on the development of service industry. It includes labor force, capital, technology, advantage of region, sufficient degree of elements, perfect infrastructure and market opening level to the outside world. So the government should take proper political measures to speed up the development of service industry in the northeast three provinces, especially promote the development of modern service industry, and make great effort to narrow the gap between prosperous regions and impoverished regions. At the same time, we must seize the opportunity to vigorously developed the northeast old industry bases.

1. Pay attention to the development of service industry, draw up a strategic development planning.

We should change traditional concept that we attach much importance to primary industry and secondary industry more than tertiary industry, thus we should make development of service industry priority. That is the strategic issue to improve international competitive power, increase employment, adjust industrial structure and create new economic growth. It is the important role that modern service industry in the future international competition, we must place industry development on strategic height. It requires us to exert ourselves in developing modern service, lay a strong developing foundation and enhance the actual strength as a whole.

2. Press ahead to optimize and upgrade the industrial structure of service sector

We have the labor resources advantage in china's northeast, therefore we can make great effort to increase export of labor-intensive service in the process of developing service industries. Especially we should pay attention to develop professional services. And we should make use modern technology to transform traditional industries, encourage developing knowledge-and-technology-intensive industries and capital-intensive ones, press ahead to optimize and upgrade the industrial and export structure.

3. Relax restrictions on market permission; speed up the opening of service industry

There are some problems in service industries of the northeast three provinces, monopoly of management, excessive restrictions on entering to the market, low transparency, for instance telecommunications, bank, insurance. This not only undermines the order of fair competition, but also leads to the short of bringing forth new ideas, low efficiency, and competitive power deficiency. These service industries have received the highest voice calling for opening to the outside by the WTO member state. We should reduce market monopoly, and relax the approving standard for civil-funded enterprise, permit some capable enterprises to invest and operate these industries, encourage blazing new trails by full competition, and create favorable conditions for the northeast three provinces opening door.

4. The government should give its coordinative functions to create fine external environment for the development of modern service industry.

The governments should create fine external environment for their own province. Firstly, they should strengthen infrastructure constructions of the service sector and create a fine environment to absorb foreign capital. This will considerably increase the speed and efficiency opening door. Secondly, the governments should accelerate to foster the talent of service industry, establish new majors which are short of in the universities, increase post qualification training and set up qualification standardization system of the service sector. Besides, the governments ought to make the local laws and regulations perfect conform to the WTO rules and international practice, enhance the sense of law and risk defense to help enterprises resist huge attack from excessive competition; avoid undue turbulence in order to maintain the economy increasing.

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Retreat of Himalayan Glaciers – Indicator of Climate Change

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Abstract: Glaciers are the coolers of the planet earth and the lifeline of many of the world's major rivers. They contain about 75% of the Earth's fresh water and are a source of major rivers. The interaction between glaciers and climate represents a particularly sensitive approach. On the global scale, air temperature is considered to be the most important factor reflecting glacier retreat, but this has not been demonstrated for tropical glaciers. Mass balance studies of glaciers indicate that the contributions of all mountain glaciers to rising sea level during the last century to be 0.2 to 0.4 mm/yr. Global mean temperature has risen by just over 0.6^0 C over the last century with accelerated warming in the last 10-15 years. The major impact will be on the world's water resources. Many climatologists believe that the decline in mountain glaciers is one of the first observable signs of the human induced global warming. [Nature and Science. 2006;4(4):53-59]

Keywords: Himalayan; glaciers; climate change; mountain

Mountain regions covering about one-fifth of the Earth's land surface are an important source of water, energy, minerals, forest and agricultural products as well as area of recreation. Geographers have produced numerous definitions aiming to distinguish mountain environments from non-mountains ones; many of them have build on common perceptions of what constitutes a mountain, and none of them is fully quantitative. The global mountain area defined is almost 40 million km^2, or some 27 per cent of the total Earth's surface area. Mountain ecosystems support vibrant livelihoods, and include significant watershed resources, biological diversity of unique flora and fauna. They act as a barometer of global climate change. These fragile ecosystems are vulnerable particularly towards the adverse effects of climate change at global level and need specific protection and conservation strategies against the problem. Many climatologists believe that mountains provide an early glimpse of what may come to pass in lowland environments.

Ice sheets, ice streams, floating ice shelves and mountain glaciers together constitute the cryosphere, an integral and dynamical part of Earth's land-ocean-atmosphere system. The cryosphere is very sensitive to changes in temperature and its various components are sensitive monitors of climate change. Polar ice sheets respond very slowly to climate change with response time of 100-1000yrs whereas mountain glaciers respond rapidly on the order of seasons to decades.

The Himalaya encompasses the world's third largest glacier systems after Antarctica and Greenland occupying about 15% of the mountain terrene,

increasing to about double this size with the winter ice core. The glacier systems are being classified as mountain or ice caps. As the Himalayan glaciers are mountain glaciers, therefore they exhibit a typical differentiation with the Antarctica and Greenland. Mountain glaciers constitute only about 3% of the glacierized area of earth. The importance of these glaciers system is because they may be melting rapidly under present climatic conditions and therefore makes large contribution to rising sea level. They are estimated to store freshwater stocks of approximately, 12 billion m^3, but have been observed to be shrinking rather fast, faster than the average global rate. In India, there are more than 5,000 glaciers on the southern slope of Himalayas covering an area of nearly 38,000 Km^2. The distributions of these glaciers are higher in North-West than in the North-Eastern part of the Indian Himalayas due to the criss-cross mountains, altitude variations and different climatic environment.

We live in a time of significant climate change, with almost all regions of the world experiencing accelerated and ongoing continuous and permanent warming of the environment in the recent decades. Few natural environments are able to testify this long term warming trend as tangibly as the world's mountains glacier systems. In India, the work on the recession of Himalayan glaciers started during the period of 1970 onwards. Studies have revealed that most glaciers in Himalaya and Karakoram region are in receding mode. The ongoing glacier studies have revealed that glaciers are retreating with an average rate of 18m - 20 m $year^{-1}$. Glacier snout is the best indicator of the glaciers advance and retreat over a period of few years and decades (Table 1) (GSI, 1999, Srivastva et al., 2001, Naithani et. al., 2001, D.P. Dobhal, 2004). It has also been observed that the rate of recession for both the small glaciers (<5 km) and large glaciers (>10 km) are more or less the same, which indicates that the future of small glaciers is not very encouraging. This can be alarming as the number of small glaciers far exceeds the number of large glaciers. Alpine glaciers are in retreat in almost all mountain belts of the world. Many of the smallest alpine glacier complexes are likely to disappear in the forthcoming two decades. Large glacier systems particularly those at high altitudes, such as massive tidewater glacier systems in Alaska, are also thinning and retreating. Their future in the current century will depend on the condition, whether climate stabilizes or continues to warm in the near future.

Weather and climate shape the physical environment. As a result, changes in climate should be clearly reflected in the ongoing changes to the seas, lakes, rivers and land of the world. Changes in climate also affect plants and animals. Glaciers are a source of continuous water supply to perennial river systems and two of the world's largest rivers, the Indus and Brahmaputra originate from these glacial lake systems and thereby ensure round the year irrigation facility to agriculture, which is the main string of economy of the developing nations like of India. The average annual run-off of Indus, Ganges and Brahmaputra rivers is 208, 494 and 510 km^3 $year^{-1}$, respectively. Varying estimates of water resources in the Himalayan region have been made. Murthy (1978) estimated Himalayan water resources around 245 km^3 $year^{-1}$, Gupta (1983) and Kawosa (1988) estimated the total amount of water flowing from the Himalayas to the plains to be around 8643 km^3 $year^{-1}$. Bahadur (1998a) re-evaluated his earlier estimates of 200–500 km^3 $year^{-1}$as 400-800 km^3 $year^{-1}$as melt water contributions from the snow and glacier fields in the high mountain region. Despite these widely differing estimates of the water resource of the Himalayan region, the water output could be the highest from any single mountain range in the world (Stone,

1992). They are powerful tourist attractions and bear a great influence on stream flow and the strategic enterprise dependent on it, such as power generation, irrigation, municipal water supplies, fish and other forms of aquatic life and recreation.

Fluctuations in the physical environments of glaciers and ice caps in cold mountain areas have been systematically observed for more than a century in various parts of the world and therefore they are considered to be highly reliable indicators of worldwide warming trends of the environment. The interaction between glaciers and climate represents a particularly sensitive approach (Kaser, 2001; Wagnon et al., 2001). The tropical glaciers provide important proxy data in climate change research (IPCC, 2001; Oerleman, 2001). It is now being an item of great interest. Mountain glaciers and ice caps are, therefore, key variables for early-detection strategies in global climate-related observations (Forel, 1895). The global retreat of mountain glaciers during the 20th century is striking. Trends in long time series of cumulative glacier-length and volume changes represent convincing evidence for fast and sudden climatic change at a global level. Since 1990, the Intergovernmental Panel on Climate Change (IPCC) has documented such changes as an evidence for the existence of global warming, independent of the various surface temperature data sets.

The 20th century was a period of dramatic glacier retreat in almost all alpine regions of the globe, with accelerated glacier and icefield melt in the past two decades. According to the World Resource Institute, the total size of the world's glaciers has declined by about 12% in the twentieth century. The first phase of this glacier retreat was associated with emergence from the Little Ice Age that ended in the 19th century. Twentieth century warming was amplified over the continents, with a temperature rise of close to about 1ºC. Observations from alpine elevations are inadequate to assess whether this surface warming has been amplified at altitude, but the punishing impact on mountain glaciers and icefields is unequivocal. Small glacier systems have rapid response times to climate perturbations, and these systems exhibit the most visible changes. In Montana's Glacier National Park, ice-covered area decreased by 73% (99 km^2 to 27 km^2) from 1850 to 1993. Glacierized area in the Alps has decreased by 40% since 1850, with an estimated volume loss of 50%. Spain has 13 glaciers remaining, a decline from 27 glaciers in 1980. Tropical ice caps in the Andes and Africa are disappearing at a similar rate; Mt. Kilimanjaro's icefields have diminished by 82% by area since 1912, from 12 km^2 to just over 2 km^2 in 2000.Approximately 33% of this retreat has come in the last 20 years. On the global scale, air temperature is considered to be the most important factor reflecting glacier retreat, but this has not been demonstrated for tropical glaciers (IPCC, 2001). However work carried out in Kilimanjaro concludes that increased air temperature governs the glacier retreat in a direct manner (Kaser, 2004).It is more difficult to assess the impacts of climate change on large glacier systems, as their dynamical response time can be many decades, and these systems are found in colder regions (higher latitudes and altitudes), where ice fields are comparatively less sensitive to climate change. The effect of global warming on the cryosphere in mountain areas are most visibly manifested in the shrinkage of mountain glaciers and in reduced snow cover duration (Barry, 2002).

Glaciers are a relative newcomer to the mountain scene, despite their immense impact on the landscape. The first buildup of ice covered approximately 240,000 years ago and ended 128,000 BP. Mountain glaciers are melting at unprecedented rates. Over the last century,

glaciers in the European Alps and Caucasus mountains have shrunk to half their size while in Africa 8% of Mount Kenya's largest glacier remains. If the current trend continues many of the world's mountain glaciers including all those in the Glacier National park will vanish entirely. The major impact will be on the world's water resources. Many climatologists believe that the decline in mountain glaciers is one of the first observable signs of the human induced global warming.

Over the past 30 years majority of the Himalayan glaciers have been retreating and thinning. In Bhutan, glaciers are retreating at an average rate of 30-40 m per year[2]. In Central Asia, glaciers are wasting at exceptionally high rates. In the northern Tien Shan (Kazakhstan), glaciers have been collectively losing 2 sq km of ice (0.7% of their total mass) per year since 1955, and Tuyuksu glacier has receded nearly a kilometer since 1923. Glaciers in the Ak-shirak Range (Kyrgyzstan) have lost 23% of their area since 1977, similar to area losses in the northern Tien Shan (29% from 1955-1990) and the Pamirs (16% from 1957-1980). In the Chinese Tien Shan, Urumqihe Glacier lost the equivalent of 4 m ice thickness from 1979-199524, and the Chinese Meteorological Administration predicts that China's northwestern mountains will lose over a quarter of their current glacier coverage by 2050.

Mountain glaciers are sensitive indicators of climate change, although which parameter is playing an important role and quantitative relationship between climate change and glacier fluctuations is still ambiguous, but it corresponded to a warming of ~0.3ºC in the first half of the 20th century in the northern hemisphere. On the global scale, air temperature is considered to be the most important factor reflecting glacier retreat, but this has not been demonstrated for tropical glaciers (IPCC, 2001). However work carried out in Kilimanjaro concludes that increased air temperature governs the glacier retreat in a direct manner (Kaser, 2004). In the last 25 years a second 0.3ºC warming pulse caused northern hemisphere temperatures to rise to unprecedented levels in the last 1,000 years, with the 1990s representing the warmest decade and 1998 the hottest year of the millennium. Glaciers in the Himalaya are receding faster than in any other part of the world and, if the present rate continues, the likelihood of them disappearing by the year 2035 is very high Thus, climate change is shrinking the mountain glacier and directly affecting the landscape and threatening water supplies all over the globe. The above explanation of facts clearly revealed that the Himalayan glaciers can be considered as a reliable indicator of climate change and are a major cause of concern worldwide.

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Table 1. Snout Recession rates of some glaciers in Himalaya

S. No	Glacier	Period of Measuring	Period (years)	Recession (metres)	Average rate (myr^{-1})
1	Milam glacier	1849- 1957	108	1350	12.50
2	Pindari glacier	1845-1966	121	2840	23.40
3	Gangotri glacier	1935-1996	61	1147	18.80
		1962-1991	29	580	20.0
		1996- 1999	3	76	25.33
4	Tipra bamak glacier	1960-1986	26	325	12.50
5	Dokriani Glacier	1962-1991	29	480	16.5
		1991-2000	09	161.15	18.00
6	Chorabari glacier	1962-2003	41	196	4.8
7	Shankulpa glacier	1881-1957	76	518	6.8
8	Poting glacier	1906-1957	51	262	5.13
9	Bara Shigri glacier	1956-1963	07	219	31.28
		1977- 1995	18	650	36.11
10	Chotta Shigri Glacier	1987-1989	03	54	18.5
		1986-1995	09	60	6.7
11	Sonapani glacier	1909-1961	52	899	17.2
12	Kolai glacier	1912-1961	49	80	16.3
13	Zemu glacier	1977-1984	07	193	27.5
14	Arwa valley	1932-1956	24	198	8.25
15	Trilokinath	1969-1995	26	400	15.4
16	Dunagiri	1992-1997	5	15	3.00
17	Chiba	1961-2000	39	1050	26.9
18	Meola	1961-2000	39	1350	34.6
19	Jhulang	1962-2000	38	400	10.5

Source: GSI (1999), Srivastva et al. (2001), Naithani et. al. (2001), D.P. Dobhal (2004), Oberoi et al. (2001)

Plate 1- Origin of Ganges from Gangotri Glacier

Plate 2-View of Dokriani Glacier

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Study on the Genetic Transformation of Gentian by Gene Recombinant

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Abstract: Transformation recombinant vector pMHL7133-Gus linked with rol gene which be cloned from Agrobacterium Rhizogenes R1000 through Agrobacterium tumefaciens LBA4404 into explant of gentian lamina, inducing rol gene express and producing hair root. Meanwhile, using the Agrobacterium Rhizogenes R1000 infect gentian directly as a comparison, we built two sets of transform system of Agrobacterium for hairy root through researching on all kinds of factors deeply and optimizing transform condition. As a result, the production rate of induced hairy root using Ti plasmid to mediated recombinant vector transforming Gentian is better than the hairy root mediated by Ri plasmid. [Nature and Science. 2006;4(4):60-67].

Keywords: gentian; Agrobacterium Rhizogenes; Agrobacterium tumefaciens; hairy root

1. Material and Method

1.1 Material

The seeds of gentian are processed by 0.25% gibberellin for 24 hours, and then wash them 3-5 times. Washing the seeds with the 70% alcohol and sterile water 3-4 times, and disinfect the seeds with 2% NaCIO for 15-20 mins. Washing them with sterile water 4-5 times, and inoculate on the MS culture medium in 25±2℃, 2000-2500 Lux light intensity, 16 h light period, and 8h dark condition for about 40 days. It can be used to transform when it grows to about 0.5-1 cm^2.

1.2 Activation of Agrobacterium

Pick single colony of Agrobacterium tumefaciens LBA4404 (with PMHL7133 — rol plasmid) and Agrobacterium Rhizogenes R1000, inoculated respectively in a LB liquid medium with rifampicin (rif 50 mg/L) and kanamycin (the km 50 mgs/L) ,and the YEB culture medium with streptomycin (the smr 50 mgs/L) in 28 ℃ cultivations stay overnight.

Take out 1 ml seed liquid inoculated in the 100 ml LB and the YEB liquid medium respectively, the 150-180 rpms/mim amplification cultivation, till the mold liquid logarithmic phase (OD 600=0.4-0.6), centrifuge in 4000rpm/min 10min, leave up pure, and dilute to OD_{600}=0.05～0.2 with the MS liquid medium, used for a plant genetic transformation.

1.3 The induction of gentian hairy root

Shear the gentian euphylla with the ophthalmic scissors under the asepsis condition carefully, cultivated in the culture medium 0 h-48 h, then take out to soak into Agrobacterium tumefaciens liquid with different concentration 3min-6min, use the asepsis filter paper sucks away surplus the mold liquid, place on the co-culture medium 25±2℃ to cultivate for 24 h-60 h darkly. Flush the explants using the asepsis water 3-5 times, then soak them into the MS liquid medium which has 500 mg/L cef but without hormone for 10-15min, fluttering often and lightly. Flush with the asepsis water again after take out, transfer to the solid MS medium contains 300 mg/lcef (LBA4404) and 500 mg/l（R1000）cef without hormone to induce the hairy root

under 25±2℃, 16 h light period, and 8h dark condition. Compare with the uninfected gentian.

In order to optimize the transform condition and exalt the transform efficiency, we made an **L_{16}（4^5）** orthogonal experiment on four cardinal factors of the genetic transformation in two different species of Agrobacterium Rhizogenes. Put the hairy root induction rate that combine three duplication experiments output by each experiment as the evaluate index.

Table 2-5. Factors and levels of orthogonal experiment design L_{16}（4^5）

Level	Factor			
	advance cultivation time（h）	concentrationOD_{600}	induce time（min）	co-culture time（h）
	A	B	C	D
1	0h	0.05	3min	24h
2	24h	0.1	4min	36h
3	36h	0.15	5min	48h
4	48h	0.2	6min	60h

1.4 The amplification of gentian hairy root

Infected euphylla of Agrobacterium can outgrow hairy root at the petiole and the wound edge, and grow fast in the first 6 day, about 1-1.5 cm. When it grows to about 3cm, take the hairy root of the growth haleness, inoculate in the 1/2 MS solid medium, 25℃s cultivated darkly. Every 5-7 days subculture for once. 3 times after subculture, cultivated in the 1/2 MS liquid medium, each one puts one root. Observing growth circumstance after 20 days.

1.5 PCRs examine on transformed plants

Extract plant total DNA by using the method CTAB. Take 1 ul DNA as the PCR template, take the both ends sequence of the rol gene of the induction hairy root as a primer, carry on the PCR amplification. Meanwhile, check against by taking the plasmid DNA as positive compare and take uninfected hairy root DNA as negative compare.

2. Result

2.1 The study on main factors of the transform efficiency

We made an **L_{16}（4^5）** orthogonal experiment on four cardinal factors of the genetic transformation. After 20 days, statistics the induction frequency of hairy root. Put the hairy root induction rate that combine three duplication experiments output by each experiment as the evaluate index.

The hairy root induction rate=the explants number of the creation hairy root/total explants number.

Table 2. Factors and levels of orthogonal experiment design L_{16}（4^5）

Number	A	B	C	D	E	Induction rate %
1	A1	B1	C1	D1	E1	34 27
2	A1	B2	C2	D2	E2	28 17
3	A1	B3	C3	D3	E3	8 4

4	A1	B4	C4	D4	E4	0	0
5	A2	B1	C2	D3	E4	44	30
6	A2	B2	C1	D4	E3	26	15
7	A2	B3	C4	D1	E2	12	7
8	A2	B4	C3	D2	E1	4	0
9	A3	B1	C3	D4	E2	22	12
10	A3	B2	C4	D3	E1	38	21
11	A3	B3	C1	D2	E4	17	11
12	A3	B4	C2	D1	E3	7	4
13	A4	B1	C4	D2	E3	60	35
14	A4	B2	C3	D1	E4	51	38
15	A4	B3	C2	D4	E1	10	6
16	A4	B4	C1	D3	E2	6	0
（Ti）							
T1	70	160	83	104	0		
T2	86	143	89	109	0		
T3	84	47	85	96	0		
T4	127	17	110	58	0		
X1	17.5	40	20.75	26	0		
X2	21.5	35.75	22.25	27.25	0		
X3	21	11.75	21.25	24	0		
X4	31.75	4.25	27.5	14.5	0		
R	14.25	35.75	6.75	12.75	0		
（Ri）							
T1	48	104	53	76	0		
T2	52	91	57	63	0		
T3	48	28	54	55	0		
T4	79	4	63	33	0		
X1	12	26	13.25	19	0		
X2	13	22.75	14.25	15.75	0		
X3	12	7	13.5	13.75	0		
X4	19.75	1	15.75	8.25	0		
R	7.75	25	2.5	10.75	0		

T: Sum of different factors at any level（n=1,2…n）

X: Mean of different factors at any level（n=1,2…n）

R: R=max $\{x_1,x_2\ldots x_n\}$ -min $\{x_1,x_2\ldots x_n\}$

From Table 2 it is clear that in the experiment that mediated transform gentian by Agrobacterium tumefaciens, the average induction rate of the number 13 is the tallest, 60%. Number 4 is the lowest, 0%. The difference of each inheritance affects is $R_B>R_A>R_D>R_C$, the mold liquid concentration> the prepared cultivation

time>the co-culture time>induce time. The influence of the mold liquid concentration is the biggest. The prepared cultivation time is 48h, the mold liquid concentration 0.05, induce time 6min, the co-culture 36h for the most suitable match.

2.2 The influence of getting the explants on the induction rate

The induction rate is closely related to the age of upgrowth time and the physiology state of the explants. The more tender of the tissue, the more easily infected by Agrobacterium. But the short age of the seedling is sensitive to the Agrobacterium, it is easily to die after infected. As figure 3 shows, the age of 45ds gentian euphylla seedling is the best to be the explant. The induction rate of the hairy root can increase to 60%.

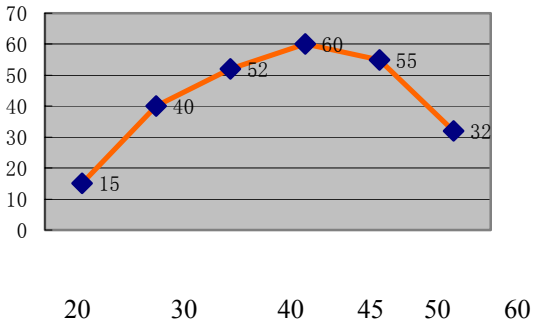


Figure 3. Effect of seedling age on frequency of hairy root formation

2.3 The influence of the append AS concentration on induction rate

The usage of phenol material may raise the expression of the Vir region and the transform rate of the explants for medicine. But AS has the dissimilarities to induction rate of different kinds of hairy root. The most suitable AS concentration to different plant is different. As figure 4 shows, the AS usage promotes to the induction of the hairy roots, the best concentrate is 20 μm, but the rate only raised 10% compared with the one without AS. The effect wasn't obvious.

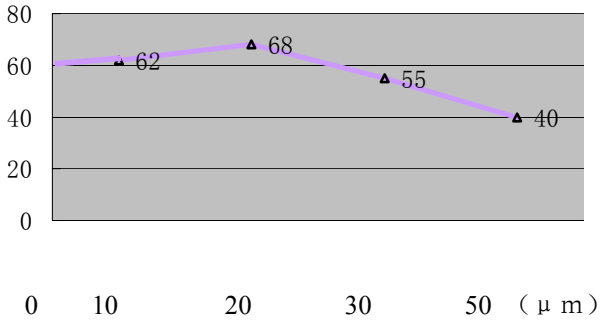


Figure 4. Effect of AS on frequency of hairy root formation

2.4 The extraction of the gentian hairy root DNA

We use CTAB method to extract hairy roots DNA which inducted by Agrobacterium Rhizogenes and Agrobacterium tumefaciens (Figure 5).

Figure 5. DNA amplification of regenerated

1: DL 1,5000 marker

2: Hairy root DNA of gentian induced by Ti Plasmid

3: Hairy root DNA of gentian induced by Ri Plasmid

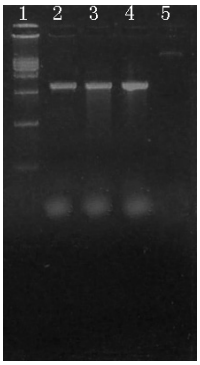


Figure 6. Extraction of plant total DNA after transformation test by PCR

1: DL 1,5000 marker

2-3: PCR of Hairy root DNA of gentian induced by Ti Plasmid

4: PCR of Hairy root DNA of gentian induced by Ri Plasmid

2.5 PCR test on transformed gentian hairy root

We make PCR on the hairy roots DNA inducted by two kinds of Agrobacterium (use the system on 2.4.1), the amplified DNA has the same size with rol gene. We identify it is a transgene plant initially.

3. The influence of the factors in orthogonal experiment on gentian transformation

3.1 The influence of different kinds of Agrobacterium

We found in the experiment that the rate of hairy root inducted by the gentian mediated transform by LBA4404 Agrobacterium is reach to 60%, but the rate of hairy root inducted by the gentian mediated transform by R1000 is only 38%. It has two following reason: 1. Different Agrobacterium Rhizogenes has different transformation ability because of different plasmid feature. 2. LBA4404 can not induce to hairy root as Agroitbacterium tumefaciens by itself. The hairy root is formatted by the expression of rol gene controlled by plant expression vector promote regulation. So the high frequency of the transform rate is because of the plant expression vector that make the rol gene high express.

3.2 The usage of the hydroxybenzene

Some plant semaphore molecules urge the activation and efficiently express of Vir region,and it is necessary to the transform of T—DNA. It also can improve the transform rate and sensitive. Currently AS has already widely used in the genetic transformation system of the plant, but be

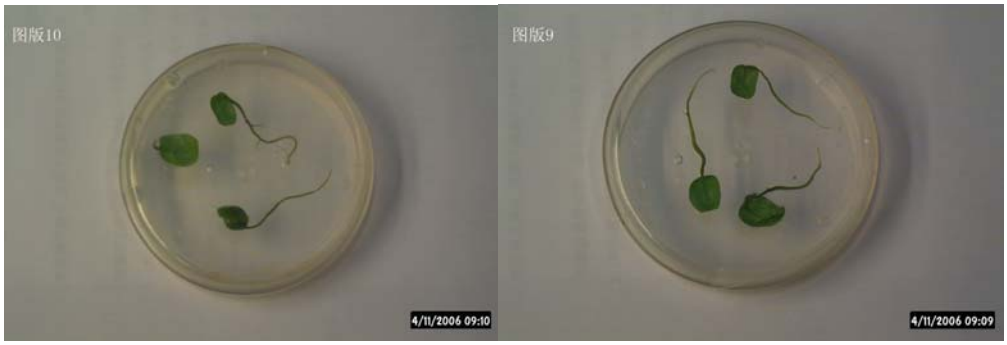
the plant also exists some phenol materials that has the same function. So the addition of AS is not for all the specieses. The addition of AS or high concentration of AS may cause the low transform rate. The reason of it is the over dose will generate poison to harm to the explants. In this experiment, a small quantity of AS can increase the induction frequency. The most suitable concentration is 20 ums. But the induction frequency only raised to 68% from original 60%. So we can conclude that the hydroxybenzene produced by explants itself make an important role in the process of vir

region activation but not the environment influence. So we don't have to append AS considering from the simplification of the test and the financial issue.

4. The morphological observation of hairy root

After the co-cultured explants switch to the culture medium which containing the bacteriostat, culturing in the scattering light for 8-16hours. It outgrows hairy root after infected 8-10 days. The hairy roots grow on the vein nerve of the lamina incision, minority of them grow on the incision boundary. One lamina can grow 1-3 roots generally. The velocity of the roots is fast in the first 4days. The average increase is about 0.4 cm everyday, and after 4 days it becomes 0.2cm per day. The outgrowth velocity of the hairy roots mediated by Agrobacterium tumefaciens is better than the roots mediated by Agrobacterium Rhizogenes. The roots lost the geotropism. They grow along the culture medium surface or grow stick the wall of the bottle. It appears lateral roots after 3 weeks. It performed a feature of lateral roots and much more root hairs. The explants haven't infected can't grow hairy root, and died gratully. So we can observe the transgene explants initial.

ly.



The hairy roots of transgene gentian mediated by Ti

The hairy root transformed by R1000

Figure 7. The hairy roots of transgene gentian mediated by Ti and the hairy root transformed by R1000

5. Study on induced hairy root mediated — transformed by Agrobacterium tumefaciens

Since the first time the scientists inserted the exogenous gene to Ti plasmid of Agrobacterium tumefaciens, and put them into plant cell to regenerate new plant in 1998, the plant genetic engineering technique got to a very fast speed development. During several decades, it has got a very successful achievement on the aspects of antivirus, antiherbicide, antiworm, antidrought, and anticold etc. It generated grate influence on agriculture production, medical hygiene, environment hygiene and food etc. At the same, the vector system mediated by Ti plasmid became mature. It became the most useful transform technology.

After 80's, the Ti plasmid of Agrobacterium Rhizogenes became more and more valuable in the aspects of hairy roots induction and secondary metabolism production. People became to focus the research on getting the hairy roots transformed by Agrobacterium Rhizogenes and regenerating plants in hairy roots, and the aspect of getting plant transgene mediated-transformed by Ri plasmid vector. It was reported many times. In recent years, people began to make a deep research on four gene rolA-D related with the form of hairy root in Ri plasmid. They have already transformed rolA gene into tobacco mediated by Agrobacterium tumefaciens, and found some features like lower plant, lamina crimple, shorten anthotaxy, and lack of roots etc. The roots mediated-transformed by

rolB can form into a great deal of hairy roots. The roots grow very fast, highly bifurcate, and inclining growth. After transformed the rolC gene into tobacco, potato, we found that the transformed plants had some features like lower, apical dominance and male sterility.

Meantime, there is few reports on transforming four induced hairy root genes at the same time into plants. But we were the first one used the medical plant gentian as vector, and induced the hairy roots taking Ti plasmid as plant vector to transform the rol gene successfully. We established the genetic transformation system of rol gene, and used Ri plasmid directly induced hairy roots as the compare. The experiment achieved the aim. We got the high transform rate and growth speed of hairy roots mediated by Ti plasmid. So we can conclude that the rolA-D genes controlled by Agrobacterium Rhizogenes could clone alone, and through the establish of the high efficiency plant expression vectors. We can get the more valuable and the higher produce hairy roots mediated-transformed by Agrobacterium tumefaciens. So this experiment has a practice value that has the theory innovation. Not only for the further researching, but also gave us a new way of thinking and work experience on getting a great deal of hairy roots.

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Cardiovascular Diseases, Protection and Treatment

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Abstract: Heart is the flowing center of the blood. As the number one killer in the world, cardiovascular diseases cause 12 million deaths in the world each year. Male mortality fell by more than 60 percent in Japan, and by 50 percent or so in Australia, Canada, France and the United States. The average rates of hospital discharges in the European Union were 2,190,000 for cardiovascular disease; 629,000 for coronary heart disease; and 356,000 for stroke. These data are for the latest year available. In Africa, Western Asia and Southeast Asia, 15-20 percent of the estimated 20 million annual deaths are due to cardiovascular diseases. This translates to 3 to 4 million deaths, bringing the total for developing countries to 8 or 9 million, or about 70 percent more than that for developed countries. This article is giving a brief description of the cardiovascular diseases as the references to the scientists and the normal people. [Nature and Science. 2006;4(4):68-78].

Keywords: artery; blood; cardiovascular diseases; heart; vein

1. Cardiovascular Disease in the World

Heart is the flowing center of the blood that offers the whole animal body for the nutrition and oxygen needed. The vascular system including arteries and veins take the task to flow the blood through the animal body and heart is the pump as the blood flowing energy resource. The health condition is significant important for any person.

More than 50 million Americans alone display blood pressures considered outside the safe physiological range (Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure.1997. *Arch. Intern. Med.* 157(21):2413–46).

Cardiovascular diseases cause 12 million deaths in the world each year, according to the third monitoring report of the World Health Organization, 1991--93. They cause half of all deaths in several developed countries, and are one of the main causes of death in many developing countries - and the major cause of death in adults. Many cardiovascular events aren't fatal but may be sufficiently debilitating to seriously affect functional ability. This is hard to assess without reliable morbidity data, but it may well be that 25 - 30 percent of the cardiovascular disease burden arises from disabling sequelae of stroke or other forms of heart disease.

Significant declines are recorded for total cardiovascular disease mortality as well as specific cardiovascular diseases over the last few decades. Male mortality fell by more than 60 percent in Japan, and by 50 percent or so in Australia, Canada, France and the United States. A similar pattern is seen for females.

Less impressive declines (20--25 percent) in cardiovascular disease mortality have been recorded for the Scandinavian countries, as well as Ireland, Portugal and Spain.

Cardiovascular disease death rates have risen by 40 percent in Hungary and the former Czechoslovakia, by almost 60 percent in Poland and by almost 80 percent in Bulgaria. These rates reflect massive increases in adult male mortality.

In both developed and developing countries, deaths from cardiovascular disease still account for almost 50 percent of all deaths. In the third monitoring report of the World Health Organization, 1991--93, it was stated that cardiovascular diseases are the principal cause of mortality in Europe, accounting for more than 50 percent of all deaths in those older than age 65. Premature death rates from cardiovascular disease range from 40.5 per 100,000 in France to 248 per 100,000 in Latvia, a ratio of 1 to 6. Globally, premature mortality due to cardiovascular disease is 2.5 times higher in men than in women.

The average rates of hospital discharges in the European Union were 2,190,000 for cardiovascular disease; 629,000 for coronary heart disease; and 356,000 for stroke. These data are for the latest year available. (3)

In Africa, Western Asia and Southeast Asia, 15--20 percent of the estimated 20 million annual deaths are due to cardiovascular diseases. This translates to 3 to 4 million deaths, bringing the total for developing countries to 8 or 9 million, or about 70 percent more than that for developed countries.

An annual survey on cardiac interventions in Europe is performed by the working group on Coronary

Circulation of the European Society of Cardiology. Results from 25 countries with an overall population of 525 million showed that in 1993 a total of 756,822 coronary angiograms were reported. This represents a 12 percent increase from 1992. Germany (37 percent), France (21 percent), UK (10 percent), Italy (6 percent) and Spain (5 percent) registered 79 percent of all the coronary angiograms performed.

A total of 183,728 PTCAs were reported in 1993, 24 percent more than 1992. Germany ranked first in per capita procedures, followed by France, Holland, Belgium and Switzerland. In 1993 there were 6,444 stents implanted in patients in 14 reporting countries. This represented a 53 percent mean increase over 1992.

In both developed and developing countries, deaths from cardiovascular disease still account for almost 50% of all deaths. In the third monitoring report of the World Health Organization, 1991--93, it was stated that cardiovascular diseases are the principal cause of mortality in Europe, accounting for more than 50% of all deaths in those older than age 65, and they cause 12 million deaths in the world each year. Many cardiovascular events aren't fatal but may be sufficiently debilitating to seriously affect functional ability. This is hard to assess without reliable morbidity data, but it may well be that 25--30% of the cardiovascular disease burden arises from disabling sequelae of stroke or other forms of heart disease.

2. Heart Anatomy

The heart is the center of the cardiovascular system that flows the blood to the body. It is a hollow, muscular organ that weighs 250-350 grams, about 12 cm long and 9 cm wide at its broadest point, and 6 cm thick. It beats over 100,000 times a day to pump 7,000 liters of blood per day through over 40,000 kilometers of blood vessels. The blood vessels form a network of tubes that carry blood from the heart to the tissues of the body and the return it to the heart.

The heart is situated between the lungs and is a component of the mediastinum, the mass of tissue between the lungs that extends from the sternum to the vertebral column. About 2/3 of the heart mass lies to the left of the body's midline. The heart is enclosed and held in place by the pericardium. The wall of the heart is divided into 3 layers: epicardium (external layer), myocardium (middle layer), and endocardium (inner layer). The interior of the heart is divided into 4 cavities called chambers that receive the circulating blood. The 2 superior chambers area called the right and left atria. Each atrium has an appendage called auricle that increases the atrium's surface area. The atria are separated by a partition called the interatrial septum. The 2 inferior chambers are the right and left ventricles that are separated from each other by an interventricular septum. The muscle tissue of the atria and ventricles is separated by connective tissue that also forms the valves. The coronary sulcus separates the atria from the ventricles that encircles the heart and houses the coronary sinus and circumflex branch of the left coronary artery. The anterior interventricular sulcus and posterior interventricular culsus separate the right and left ventricles externally. The sulci contain coronary blood vessels and a variable amount of fat.

The right atrium receives blood from all parts of the body except lungs through three veins (superior vena cava, inferior vena cava, and coronary sinus), and then delivers the blood into the right ventricle, which pumps the blood into pulmonary trunk. The pulmonary trunk divides into a right and left pulmonary artery, each of which carries blood to the lungs. In the lungs, the blood releases its carbon dioxide and takes on oxygen. Blood returns to the heart via four pulmonary veins that empty intro the left atrium. The blood then passes into the left ventricle, which pumps the blood into the ascending aorta. From here the blood is passed into the coronary arteries, arch of the aorta thoracis aorta and abdominal aorta. These blood vessels and their branches transport the blood to the heart and all other body parts, except the lungs.

The thickness of the four chambers varies according to function. The atria are thin-walled because they need only enough cardiac muscle tissue to deliver the blood into the ventricles with the aid of gravity and a reduced pressure created by the expanding ventricles. The right ventricle has a thicker layer of myocardium than the atria, since it must send blood to the lungs and back around to the left atrium. The left ventricle has the thickest wall, since it must pump blood at high pressure through literally thousands of kilometers of vessels in the head, trunk, and extremities. As each chamber of the heart contracts, it pushes a portion of blood into a ventricle or out of the heart through an artery. On order to keep the blood from flowing backward, the heart has structures composed of dense connective tissue covered by endothelium called valves. Atrioventricular valves lie between the atria and ventricles. The wall of the heart, like any other tissue, has its own blood vessels. The flow of blood through the numerous vessels that pierce the myocardium is called coronary (cardiac) circulation (Heartonline, 2005).

Heart is the flowing center of the blood, and it supplies the whole body of an animal for the requirement of the oxygen and nutrients that are delivered by blood to everywhere of the body. Figure 1 shows the flowing of the blood in a human body (Figure 1).

The flowing of the blood:

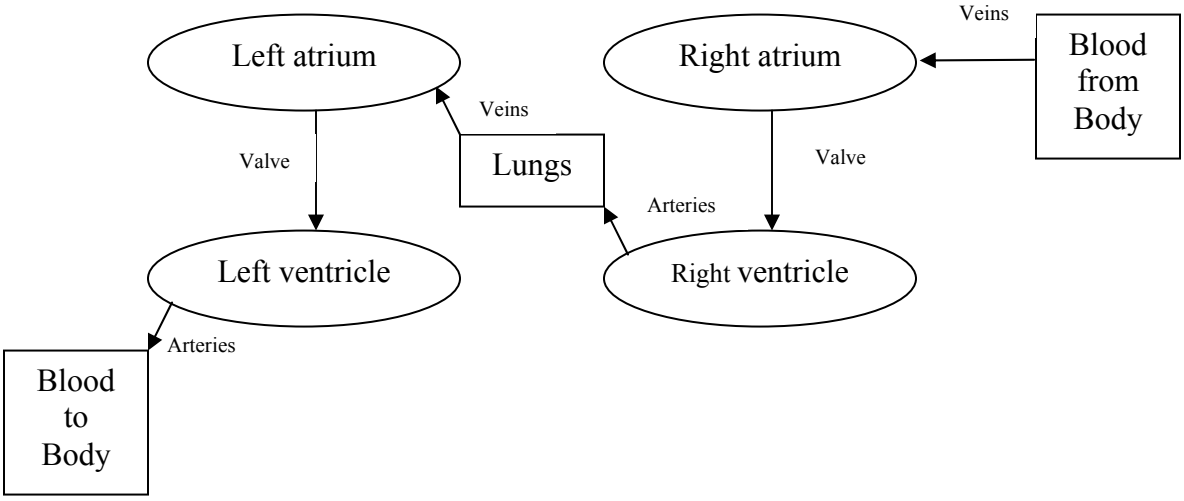

Figure 1. Flowing of the blood

3. Anemia

Definition: Anemia is a lack of red blood cells and/or hemoglobin. There are over 30 different types of anemia, including sickle cell anemia. Although most forms of anemia can be successfully treated, there are some forms that are chronic, damaging or even life-threatening. Severe cases of anemia have been associated with an enlarged heart, cardiomyopathy and other heart-related conditions. Anyone experiencing symptoms such as chronic fatigue, weakness, paleness and shortness of breath (especially while physically active) is urged to get a check-up with their physician.

Prevention: Eating a balanced, heart-healthy diet can help prevent the most common forms of anemia. Other types of anemia are present since birth, or develop for unknown reasons, and have no known prevention strategies.

Treatment: A popular misconception is that people who have anemia should simply take iron supplements. Iron supplements should be avoided unless prescribed by a physician, because an excess level of iron could damage the heart or other vital organs. Most forms of anemia can be successfully treated with supplements, injections of specific vitamins and/or increased intake of nutrient-rich foods (Heartonline, 2005).

4. Angina

Definition: Angina (angina pectoris) is a type of temporary chest pain, pressure or discomfort. It is a symptom of cardiac ischemia — a condition in which the heart is not getting enough oxygen-rich blood to feed itself and the rest of the body. In one type of angina, called stable angina, the attacks happen only when the heart needs extra oxygen, like when a person climbs a long flight of stairs or jogs across the street. In other types of angina, such as unstable angina, angina attacks may occur even when a person is at rest.

Prevention: It is important to have a heart-healthy diet that is low in cholesterol and fats and oils, and make a good control of diabetes and high blood pressure.

Regular exercise: Quitting Smoking and staying away from second-hand tobacco smoke.

Treatment: Medications used to treat angina either increase the supply of oxygen to the heart muscle, or reduce the heart's need for oxygen. These medications include the following: Beta-blockers; nitrates; calcium channel blockers; antiplatelet; anticoagulant; angioplasty; atenting; airectional atherectomy; aoronary artery bypass grafting; ainimally invasive bypass surgery; aransmyocardial revascularization; axternal counterpulsation (Heartonline, 2005).

5. Arrhythmia

Definition: An arrhythmia is an irregular heartbeat resulting from any change, deviation or malfunction in the heart's conduction system — the system through which normal electrical impulses travel through the heart. An arrhythmia may be unusually fast (tachycardia) or unusually slow (bradycardia). Some

arrhythmias are signs of more serious heart problems, and others are not. An arrhythmia may be brief and unnoticeable, or it may be startling, obvious or even fatal (Table 1, Table 2)

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Table 1. Arrhythmia Prevention

Quitting or reducing reducing alcohol and caffeine intake
Quitting smoking
Avoiding certain medications (e.g., decongestants)
Using stress management techniques

Table 2. Arrhythmia Treatment

Beta-blockers
Calcium channel blockers
Digoxin
Surgery to implant an artificial pacemaker
Surgery to implant an implantable cardioverter defibrillator (ICD)
Electrophysiological surgery with catheter ablation.

6. Atherosclerosis

Definition: Also known as "hardening of the arteries," atherosclerosis is a disease in which the arteries are hardened and narrowed as a result of plaque, which has built up along the inside of the artery walls. The disease is a chief contributor to cardiovascular disease. Atherosclerosis may begin as early as childhood, but it is the advanced stages of this condition that are the most dangerous later in life. These advanced stages can cause a narrowing (stenosis) of the artery and speed the rate at which the artery is blocked or closed altogether (occlusion). If the affected artery is one of the coronary arteries (located on the surface of the heart), then a lack of oxygen-rich blood to the heart (cardiac ischemia) could cause coronary artery disease (CAD) and, consequently, increase an individual's risk of the following: Angina, Heart attack, Cardiac arrest, Sudden cardiac death (Table 3, Table 4).

Table 3. Atherosclerosis Prevention

Quitting smoking
Controlling diabetes
Controlling high blood pressure (hypertension)
Reducing the amount of fats and oils and cholesterol
Achieving and maintaining a healthy weight (avoid Obesity)
Keeping a routine of moderate to vigorous exercise
Learn your family medical history
Getting regular physical examinations
Stress management
Controlling chronic depression

Table 4. Atherosclerosis Treatment

Antiplatelets (e.g., aspirin) - inhibit the formation of blood clots.
Beta-blockers - reduce the workload of the heart by blocking the effect of adrenaline on the heart.
Nitrates - work directly on the muscles of the heart and blood vessels, causing them to relax and allowing oxygen-rich blood to reach the heart.
Cholesterol reducing drugs - lowers the patient's level of fats (lipids) in the blood, such as cholesterol and triglycerides.
ACE inhibitors - block the production of a substance that causes blood vessels to tighten, allowing more oxygen-rich blood to flow into the heart.
Vitamins - may also be helpful, especially folic acid, which counteracts the dangerous effects of an amino acid called homocysteine.
Balloon angioplasty - A balloon-tipped catheter presses plaque back against the artery walls, increasing the amount of room through which blood can pass through the vessel.
Stenting - A wire mesh metal tube called a stent can be inserted into the area of a damaged artery.
The stent acts as a scaffold, stretching and supporting the artery walls, and permitting blood to flow freely through the previously blocked vessel.
Atherectomy - uses one of three catheters, all of which destroy plaque by cutting it away.
Depending on the technique used, the residue is pulverized and 1) allowed to flow harmlessly through the bloodstream; 2) removed as the catheter is withdrawn; or 3) vacuumed through the catheter and out of the body during the procedure.

7. Diabetes

Definition: Diabetes is a disorder in the body's ability to use blood sugar (glucose). Glucose is the main source of energy for the human body, which is taken from the starches and sugars that people eat and travels through the bloodstream, circulating throughout the body. Normally, the body's tissues can absorb the glucose and use it for energy with the help of hormone insulin that is produced in the pancreas (an organ next to the stomach). Unless the body has enough insulin and the ability to use insulin properly, glucose will simply build up in the bloodstream and then get flushed from the body in the urine, rather than go into the cells to feed them. Therefore, people with untreated diabetes may have dangerously high blood sugar levels. These high blood sugar levels can lead to a variety of symptoms (e.g., weakness) in the short-term, and serious consequences such as heart attack, stroke or other consequences of diabetes in the long-term.

There are two types of diabetes: Type I and Type II. Type I is thought to be caused by a combination of genetic factors and environmental factors that result in a lack, or complete absence, of insulin. For example, a viral infection can cause the immune system to attack itself. As a result, the body may destroy over 90% of its own insulin-producing (beta) cells in the pancreas. Much more common, Type II diabetes has been linked to obesity (weighing more than 20% of one's ideal weight), inactivity and being over 40 years old. Diabetes can also be caused by pregnancy (a pregnancy complication known as gestational diabetes), drug use or the use of certain steroids.

Patients with Type II diabetes do manufacture insulin, sometimes even more so than necessary, but for some reason their bodies reject and/or do not detect it, resulting in what the body perceives as a deficiency. This insulin blockage is due to cell abnormalities of unknown cause in the liver and muscles. The onset of this type of diabetes, also called adult-onset diabetes, usually occurs after age 30 (Table 5, Table 6).

Table 5. Diabetes Prevention

Maintaining a balanced diet low in fats and oils, low in sweets, and high in fiber.
Eating regular meals and light snacks
Lowering cholesterol levels
Maintaining proper weight to avoid obesity — a major risk factor for Type II diabetes
Engaging in regular exercise, which lowers blood sugar levels and helps the body to use insulin

Table 6. Diabetes Treatment

Insulin injections
Diet treatment e.g., sugar control
Rest
Exercise
Nutrition

Diabetes Treatment: Treatment for Type I currently requires insulin injections, although other strategies for taking insulin are currently being researched (e.g., nasal spray or skin patch). Type II requires medication and sometimes insulin injections. Both types require lifestyle changes that include diet. People with Type II diabetes are often treated with medication. Depending on the severity of the condition, they may be able to take oral medications instead of injections. However, some insulin injection may still be used. Insulin is generally injected by the patient (or a child patient's parent) under the fat layer of the arm, leg or stomach.

8. Hypertension

Definition: Hypertension is the medical term for high blood pressure. Blood pressure is the measure of the force of the blood pushing against the walls of the arteries — the blood vessels that carry blood from the heart to the rest of the body. When the heart contracts to pump out blood, pressure is highest. This measurement is called the systolic pressure. After pumping, the heart relaxes and pressure drops to its lowest point just before a new beat. This measurement is called the diastolic pressure. The measurement of an individual's blood pressure is always expressed as systolic pressure over diastolic pressure. For example, normal blood pressure for adults is considered to be in the range of 120/80 millimeters of mercury. Generally, blood pressure above 140/90 is considered to be high for adults, and blood pressure under 90/60 is considered to be low for adults. Hypertension, or high blood pressure, is a condition commonly associated with narrowing of the arteries. This causes blood to be pumped with excessive force against the artery walls. It is a sign that the heart and blood vessels are being overworked. Untreated, hypertension will cause the heart to eventually overwork itself to the point where serious damage can occur. For instance, the heart muscle can thicken (hypertrophy) and function abnormally, or dilate and constrict less forcefully (dilated cardiomyopathy). There may also be injury to the brain, the eyes (retinopathy) and/or the kidneys (nephropathy). Hypertensive patients are also at increased risk of heart disease and stroke. Most cases of high blood pressure have no cure, but the overwhelming majority can be managed and controlled with diet and medication (Table 7, Table 8).

Table 7. Hypertension Prevention

Quitting smoking.
Losing weight.
Controlling diet.
Getting adequate amounts of vitamins and minerals.
Engaging in regular aerobic exercise.
Limiting alcohol
Limiting salt intake to 2,000 milligrams (2 grams) of sodium per day.

Table 8. Hypertension Treatment

Monitor their blood pressure at home, under the guidance of their physician.
Diuretics - Medications that promote the formation of urine in the kidneys, causing the body to flush out excess fluids and minerals, especially sodium.
Alpha-blockers and beta-blockers - Medications that inhibit alpha and beta receptors in various parts of the nervous system, which slows the heart rate. This helps arteries to relax, decreases the force of the heartbeat and reduces blood pressure. Beta blockers are especially useful in patients with heart disease.
ACE inhibitors - These medications are types of vasodilators that help to reduce blood pressure by inhibiting the substances in the blood that cause blood vessels to constrict. Recent studies suggest that this class of drugs may be superior to others in preventing stroke, heart disease and kidney disease in patients (especially diabetics) with risk factors for vascular disease. They are also very useful in patients with established heart disease.
Angiotensin II receptor blockers (ARBs) - This new class of drugs is showing good results and great promise in reducing hypertension-related complications. Although beta blockers, ACE inhibitors and diuretics are currently used most often in the treatment of hypertension, ARBs may be prescribed more often in the future.
Calcium channel blockers - These are types of vasodilators that inhibit the flow of calcium into heart and blood vessel tissues, which reduces tension in the heart, relaxes blood vessels and lowers blood pressure. Unfortunately, most studies have not shown that these agents reduce the risk of death from hypertension, and some of these medications may increase the risk of death from hypertension.
Using stress management techniques.

9. Obesity

Definition: Obesity is a condition in which people are more than 20 percent over their ideal weight. Currently, more than one-third of Americans are obese (Table 9, Table 10).

Table 9. Obesity Prevention

Reducing fat intake - In the last few years, research has suggested that cutting one's fat intake is a more effective weight loss strategy than counting calories. A number of nationally recognized weight loss programs ask people to keep track of the types of foods they eat (such as fats, meats or dairy) rather than the number of calories included in those different types of food.
Choosing a more active lifestyle - Moderate and sustained physical activity is essential to achieving and maintaining a healthy weight. Aerobic exercise, such as walking, swimming or stair climbing, can help to keep weight off while at the same time providing many other benefits to an individual's overall health and well being.
Avoiding smoking and alcohol use - These strategies for relieving stress do not solve whatever problem is at hand, and often make situations worse (e.g., by causing new health problems). By using healthy "stress-burning" techniques instead of excessive alcohol use or smoking, individuals can reach weight loss goals more quickly.
Counseling on diet - Some people have trouble controlling their food intake not because of hunger, but because of emotional needs. People who use food to try to fill a feeling of emptiness, to comfort themselves or to gain a sense of control may benefit from speaking to a qualified counselor about the situation. Inpatient programs are available around the country, in which people stay overnight as they relearn to eat only when hungry and to satisfy emotional needs through other means.

Table 10. Obesity Treatment

Drugs used to treat obesity usually affect levels of certain hormones (e.g., serotonin and noradrenaline) in areas of the brain associated with food intake and satisfaction. This temporarily decreases the urge to eat and, with appropriate diet and exercise, supports weight loss efforts. There are some drawbacks, however. These drugs can produce serious side effects, such as high blood pressure in the blood vessels of the lungs (pulmonary hypertension) and valvular heart disease. These medications should only be used by patients whose treatments are closely monitored by a qualified physician.
Cutting calories
Starting an exercise program
Using healthy stress management techniques
Surgical intervention - In rare cases, surgery may be the treatment of choice for people with clinically severe obesity who have not been helped by other medical therapies. One common surgical technique is gastric stapling (the "tummy tuck"), which uses bands or staples to decrease the amount of space in the stomach available for food. Another type of surgery is the gastric bypass, in which part of the small intestine is bypassed as food passes out of the stomach. The gastric bypass involves significant changes in how food and drink can be taken in, and people are encouraged to learn all about the surgery before having it done. For all surgical candidates, an integrated medical program must be in place to provide guidance on diet, physical activity and support before and after the surgery.

10. Plaque Rupture

Definition: LDL ("bad") cholesterol produces poisons (toxins) that damage the lining (endothelial cells) of the inside wall of an artery. This damage contributes to the formation of tiny wounds or lesions on that inside wall. Other fatty materials in the bloodstream (e.g., triglycerides) are attracted to those lesions and begin to build up there. White blood cells rush to the site of the irritation to devour harmful substances, but only cause the lining of the artery to become sticky, attracting even more LDL molecules. Clot-producing platelets begin to collect over the site, releasing still more irritating substances and trapping more fatty particles and white blood cells. This gradual build-up of fatty materials and toxins is known as plaque. As the plaque continues to build up, some of the plaque formations develop a relatively thick covering (due to calcification). These types of plaque are considered to be stable plaques and are a primary cause of hardened and narrowed arteries (atherosclerosis). Other types of plaque are known as unstable plaques, which (in comparison to stable plaques) have the following: A larger fatty core; More white blood cells encased within; A thinner, softer, more unpredictable coating that might be stripped off at any time without warning. If the coating of an unstable plaque is stripped off, this is known as a plaque rupture (Table 11, Table 12).

Table 11. Plaque Rupture Prevention

Reducing the amount of fats and oils and cholesterol in diet
A routine regimen of moderate to vigorous exercise
Quitting smoking
Controlling diabetes.
Controlling high blood pressure.
Getting regular physical examinations.

Table 12. Plaque Rupture Treatment

Antiplatelets (e.g., aspirin or clopidogrel) - inhibit the formation of blood clots by decreasing the ability of platelets (the body's natural blood-clotters) to bind together. In the case of atherosclerosis, antiplatelets prevent a damaged vessel from becoming blocked due to excessive concentration of platelets.
Anticoagulants - also help to minimize the formation of blood clots.
Lifestyle changes
Cardiac catheterization - During this procedure, a thin tube (catheter) is inserted through a blood vessel in the body (usually the groin) and fed all the way to the heart. Once in place, the physician may choose to do a balloon angioplasty, which uses a balloon-tipped catheter to press plaque back against the artery walls, increasing the amount of room through which blood can pass through the vessel. A variant of the balloon angioplasty is the laser angioplasty.

11. Stroke

Definition: Also known as a cerebrovascular accident (CVA), a stroke is a life-threatening event in which part of the brain is not getting enough oxygen. A stroke can cause oxygen-starved brain cells to die within minutes. Rapid response to stroke can result in little apparent damage, but a stroke left untreated for too long can result in neurological and tissue damage (e.g., permanent loss of speech or paralysis) or death. There are two different types of strokes: Ischemic and Hemorrhagic (Table 13, Table 14).

Table 13. Stroke Prevention

Controlling hypertension (high blood pressure) - Blood pressure abnormalities must be continually monitored and controlled because they are a chief contributor to strokes.
Getting treatment for atrial fibrillation or atrial flutter.
Getting treatment for sleep apnea.
Learning stress management techniques and seeking help for depression or drug abuse.
Reducing cholesterol levels, perhaps by taking cholesterol-reducing drugs.
Increasing one's level of exercise.
Maintaining an ideal weight.
Refraining from or quitting smoking.
Limit use of alcohol to about one glass of wine or one beer per day, which may help in the prevention of strokes.
Controlling diabetes.
Eating a heart-healthy diet.

Table 14. Stroke Treatment

Drugs
Rest
Nutrition
Exercise

People having symptoms of a stroke should call 911 immediately. Maintaining breathing in patients who may be losing consciousness. This is done through the use of breathing equipment and/or supplemental oxygen. People may be advised by their physician to take aspirin or other antiplatelet agents to help prevent the formation of blood clots.

For people who have obstructed or partially obstructed carotid arteries, and have already had a stroke or TIA related to that condition, a surgery known as a carotid endarterectomy may be an option to prevent another life-threatening event. This surgery may also be performed if the person has not had a stroke or TIA but has greater than 80 percent blockage in the carotid arteries. A carotid endarterectomy involves the removal of fatty build-up from the carotid arteries supplying blood to the brain. While the person is under general anesthesia, the plaque from the artery is removed along with the entire inner lining of the artery. Carotid stent placement within the carotid arteries is a less invasive catheter-based procedure currently being studied as an alternative to surgery.

A carotid aneurysm that has not yet ruptured may be diagnosed early, particularly if it was causing warning signs that led the patient to seek treatment. Surgery may be necessary to repair the aneurysm, preventing a hemorrhagic stroke.

Performing a computed tomography (CT) scan to determine whether someone is suffering from an ischemic stroke or a hemorrhagic stroke. If it is an ischemic stroke, then thrombolytic medications may be given intravenously to dissolve the obstructing blood clots. However, giving thrombolytic medications to a patient having a hemorrhagic stroke would worsen the existing bleeding in the brain and should be avoided. Special attention may be given to maintaining nutritional needs intravenously or through the mouth and preventing pneumonia, a common complication after a stroke.

People who survive a stroke will often need to undergo treatment (i.e., stroke rehabilitation) to deal with some of the long-term effects of the event. The goal of the treatment is to minimize as much neurological damage as possible, such as impaired movement or speech. The sooner that treatment is begun, the more likely it is that patients will regain significant functions. Individuals may also experience depression, which may be related to the temporary or permanent loss of basic functions. If this should occur, patients are urged to seek the help of a qualified counselor for support and treatment.

Glossaries and special notes:

Plasma fibronectin. A protein that is believed to protect brain cells from damage from an ischemic stroke. A recent study published in Nature Medicine (2001) found that mice lacking the ability to produce this protein suffered 40 to 50 percent more damage from strokes than mice with normal levels of this protein.

ORP150 (oxygen-regulated protein 150 kD). A chemical that is created in the body as a result of oxygen starvation. Another study published in Nature Medicine (2001) reported that brain cells treated with ORP150 were only 40 to 50 percent as likely to die from oxygen starvation as untreated cells.

Stem cell transplants. Stem cells are basic cells that have the ability to develop into many different types of cells. They start out very similar to each other, but depending on where they develop, the cells become highly specialized to their individual functions. Researchers are investigating a variety of methods in which stem cell transplants could be used as a treatment for stroke damage and other conditions involving damaged brain cells.

Hypothermia. Researchers at the Cleveland Clinic Foundation in Ohio are currently studying whether lowering a patient's body temperature can decrease the amount of damage that occurs during a severe stroke.

Endovascular Photo Acoustic Recanalization (EPAR). A laser that is fed through a catheter into the brain to destroy blood clots that could potentially cause a stroke. Clot busting drugs are currently the only method available to break up an existing blood clot in the brain. However, they can take up to an hour to be effective. EPAR can take less than a minute, restoring blood flow and oxygen to the brain and drastically reducing the amount of stroke damage.

PPI. A drug that blocks the production of a protein called Src. After a stroke, Src causes blood vessels to leak, which lead to brain swelling and damage. A study published in Nature Medicine (February 2001) found that when PPI was given to mice within 15 minutes of an arterial blockage, the resulting stroke produced 70 percent less damage.

Cholesterol-lowering drugs. A study published in Circulation (January 2000) indicated that one type of statin may decrease a patient's risk of stroke and diabetes. It is not known whether this effect is common to all cholesterol-reducing drugs or just the specific drug studied in the trial (pravastatin).

Atherosclerosis. A disease in which the arteries are hardened and narrowed, dual to the gradual build-up of plaque on their inner walls.

Hypertension, or high blood pressure, is a condition commonly associated with narrowing of the arteries. This causes blood to be pumped with excessive force against the artery walls. It is a sign that the heart and blood vessels are being overworked. Untreated, hypertension will cause the heart to eventually overwork itself to the point where serious damage can occur. For instance, the heart muscle can thicken (hypertrophy) and function abnormally, or dilate and constrict less forcefully (dilated cardiomyopathy). There may also be injury to the brain, the eyes (retinopathy) and/or the kidneys (nephropathy). Hypertensive patients are also at increased risk of heart disease and stroke. Most cases of high blood pressure have no cure, but the overwhelming majority can be managed and controlled with diet and medication.

Hypertension is a major health problem in the United States, where more than 50 million people (1 in 4 adults) have the condition, according to the American Heart Association. It is especially common among African-Americans, who are one of the most likely ethnic groups in the world to be diagnosed with high blood pressure. It has also been diagnosed in two-thirds of Americans over 65 and a growing number of children.

According to the American Heart Association's *2001 Heart and Stroke Statistical Update*, more than 30 percent of Americans with high blood pressure are unaware that they have this potentially life threatening

condition; another 26 percent are on medication, but do not have their blood pressure under control.

Diabetes is a disorder in the body's ability to use blood sugar (*glucose*). Glucose is the main source of energy for the human body. It is taken from the starches and sugars that people eat. It travels through the bloodstream, circulating throughout the body.

Normally, the body's tissues can absorb the glucose and use it for energy with the help of *insulin*. Insulin is a hormone produced in the pancreas (an organ next to the stomach) that is normally secreted when glucose levels are high. Unless the body has enough insulin and the ability to use insulin properly, glucose will simply build up in the bloodstream and then get flushed from the body in the urine, rather than go into the cells to feed them. Therefore, people with untreated diabetes may have dangerously high blood sugar levels. These high blood sugar levels can lead to a variety of symptoms (e.g., weakness) in the short-term, and serious consequences such as heart attack, stroke or other consequences of diabetes in the long-term.

Diabetes affects about 10 to 20 million Americans, which is about 6 percent of the United States population. Because diabetes often runs in families, those with one or more diabetic relatives are advised to be especially vigilant in maintaining a low-fat, low-to-moderate sugar diet and exercising regularly. African-American, Hispanic, Asian and Native American individuals are at a higher risk of developing the condition. A quick, simple blood test or urinalysis can check for diabetes.

There are two types of diabetes: Type I and Type II. Type I is thought to be caused by a combination of genetic factors and environmental factors that result in a lack, or complete absence, of insulin. For example, a viral infection can cause the immune system to attack itself. As a result, the body may destroy over 90 percent of its own insulin-producing (*beta*) cells in the pancreas. Much more common, Type II diabetes has been linked to obesity (weighing more than 20 percent of one's ideal weight), inactivity and being over 40 years old. Diabetes can also be caused by pregnancy (a pregnancy complication known as *gestational diabetes*), drug use or the use of certain steroids.

Before the discovery of insulin in 1921, the long-term prognosis for diabetics was not good. Today, most diabetes can be managed and controlled with a combination of insulin treatment (either medications or injections) and lifestyle modifications. Other strategies for taking insulin, such as nasal sprays and skin patches, are currently being researched.

Obesity is a condition in which a person is more than 20 percent over his or her ideal weight. It is the second leading cause of preventable death, contributing to serious health problems such as cancer, heart disease and stroke. There are a number of treatments available for obesity, including cutting calories, starting an exercise program, using healthy stress management techniques and getting supportive counseling. Medications are also available, if necessary. In severe cases, surgery (e.g., gastric stapling or gastric bypass) may be necessary.

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Role of Gap Junction in Atherosclerosis and Thrombosis

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Abstract: The scientific objective of this study is to test the hypothesis that the antioxidant β-carotene will prevent or reduce plaque disruption and thrombosis in atherosclerotic rabbits. This protective effect on plaque disruption will be mediated by enhanced gap junctional intercellular communication (GJIC). This article is giving a brief description of the tactics to study the role of gap junction in atherosclerosis and thrombosis. [Nature and Science. 2006;4(4):79-89].

Keywords: artery; blood; cardiovascular diseases; heart; vein

1. Introduction

The scientific objective of this study is to test the hypothesis that the antioxidant β-carotene will prevent or reduce plaque disruption and thrombosis in atherosclerotic rabbits. This protective effect on plaque disruption will be mediated by enhanced gap junctional intercellular communication (GJIC). To test this hypothesis, plaque disruption will be triggered in atherosclerotic rabbits as we described (Abela, et al. 1995; Hage-Korban, et al. 1999; Ma, et al. 2002, 2003) and GJIC will be measured with this rabbit model.

1) Plaques will be induced by the combination of a high cholesterol diet and balloon injury. Plaque disruption and thrombosis will be less in atherosclerotic arteries pretreated with β-carotene compared to non-treated control arteries. Despite a normal appearing endothelium after eight months following balloon injury, balloon de-endothelialized arteries are still vulnerable to disruption. However, our preliminary data suggest that β-carotene may protect the atherosclerotic arteries from plaque disruption and thrombosis.
2) Gap junction function in endothelial cells will be enhanced in arteries pretreated with the antioxidant β-carotene. Since gap junction function can be altered by oxidized LDL cholesterol and antioxidant treatment can modify oxidized LDL in atherosclerotic plaque, it is expected that the GJIC will be greater in β-carotene treated rabbit arteries.
3) Antioxidants have been shown to preserve endothelium-derived relaxing factor (Mugge, et al. 1991; Drexler, et al. 1999). Atherosclerotic rabbit arteries pretreated with β-carotene will preserve vasodilatation to acetylcholine challenge in a physiologic chamber when compared to control (non β-carotene treated rabbit arteries).
4) GJIC will be inhibited in β-carotene treated rabbits to evaluate if the GJIC is the key way for the β-carotene protection on atherosclerotic arteries from plaque disruption and arterial vasodilatation.

The proposed studies address an area of the critical importance to unstable cardiovascular syndromes. The applicant is a potential independent research scientist who has successfully accomplished many related projects and trained many students and medical research-fellows under the supervision of Dr. George S. Abela.

2. Background and Significance

Plaque disruption and thrombosis are the major events leading to unstable cardiovascular syndromes. Studies of pathologic specimens have shown convincingly that plaque fissuring is an important cause of acute myocardial infarction, crescendo angina and sudden ischemic death (Davies and Thomas, 1984). Early studies have demonstrated that plaque disruption frequently occurred in patients with fatal coronary thrombosis. Studies using coronary angiography and angioscopy have shown that plaque fissuring and disruption are common findings in patients with unstable angina and unstable events and that ulcerated plaque is much more commonly associated with occlusive thrombus that are smooth and non ulcerated plaque (Mizuno, et al. 1991).

Clinical trial have demonstrated that individuals receiving β-carotene had 49% fewer total vascular events and 44% fewer major coronary events including myocardial infarction and sudden death than did control subjects receiving placebo (Gaziano, et al. 1990). Also, vitamin E has been shown to reduce coronary events in both men and women on long-term supplementation (Rim, et al. 1993).

Evidence suggests that oxidation of LDL is important in the development of atherosclerosis (Steinberg, et al. 1989). Oxidized LDL is more readily taken up by macrophages than the native LDL. Also, oxidized LDL is chemotactic for circulating monocytes and may be cytotoxic to endothelial cells. The presence in plasma of an electronegative LDL subfraction cytotoxic for endothelial cells has been reported and the LDL shows proinflammatory activity on endothelial cells and may contribute to early atherosclerotic events (De-Castellarnau, et al. 2000). Antioxidants such as vitamin E have been shown to increase resistance to LDL oxidation

(Esterbauer, et al. 1991) and reduce platelet adhesion (Jandak, et al. 1988).

LDL cholesterol alters endothelial function resulting in abnormal vasoreactivity of human coronary arteries (Vita, et al. 1990). This is related to reduced endothelial mediated endothelium-derived relaxing factor (EDRF) production result in regional vasospasm at sites of dysfunction appears to persist up to 4 weeks following balloon injury. Duration of the endothelial dysfunction is related to severity of the initial vascular injury (Weidinger, et al. 1990). The aorta of the rabbits with balloon injury is highly vulnerable to disruption and thrombosis for up to 8 months following injury (Abela, et al. 1995). This is despite the presence of a normally appearing endothelium by scanning electron microscopy.

The presence of a thin collagenous cap overlying a lipid rich pool is associated with the morphology often seen in disrupted plaque with overlying platelet thrombus (Falk, 1989). In the proposed study, we hypothesize that antioxidants will prevent or reduce plaque disruption and thrombosis by preserving EDRF mediated vasodilator response and enhanced gap junction protein activity. This is expected to provide stabilization of vulnerable atherosclerotic plaques.

Both behavior and growth of individual cells depend on the cells with which they are in contact. Cell-to-cell communication is important for the maintenance of tissue homeostasis and control of growth and differentiated function. Since the gap junction is the only known structure by which the interior of adjacent cells are connected, it has been postulated that factors important for control of intercell communication is exchanged through these protein channels (Lowenstein, et al. 1979; Trosko and Chang, 2000).

Structural and functional studies have identified communicating gap junctions in endothelial cells and smooth muscle cells in vivo and in vitro (Little, et al. 1995). The gap junctional channel has been shown to admit low molecular weight molecules including anions, cations, camp, IP3 and calcium, but to exclude proteins and nucleic acids. For example, elevation of intracellular calcium concentrations has been associated with decreased GJIC and increased levels of camp with increased GJIC (Hossain, et al. 1989). These channels have been implicated in the tumor-promotion phase of carcinogenesis and more recently in the atherosclerotic process. Inhibition of GJIC has been demonstrated in cultured smooth muscle cells by the cytokine TNF-α and upregulation of GJIC by β-carotene in cultured human fibroblasts (Mensink, et al. 1995; Zhang, et al. 1995). Currently, little is known of the effects of there agents on vascular endothelium. The present proposal will investigate the effect of oxidized LDL on GJIC in atherosclerotic arteries and the effect on physiologic function of the vessels.

Unsaturated fatty acids inhibit GJIC in a reversible fashion and at doses above particular chemical thresholds (Trosko and Chang, 2001). In cultures of normal aortic cells the number of coupled cells is significantly higher than in cultures of atherosclerotic cells and gap junctional communication between cells loaded with lipid inclusions is lower than that between cells free of excess of intracellular lipids. In cultures of human skin fibroblasts the rate of intercellular communication is comparable to that in cultures of atherosclerotic cells. It is hypothesized that the reduced gap junctional communication in atherosclerotic human aorta is associated with alterations in the degree of smooth muscle cell differentiation (Andreeva, et al. 1996).

But, other reports show that in atherosclerosis and hypertension, vascular smooth muscle cells are stimulated to proliferate and exhibit enhanced gap junction protein expression (Kurjiaka, et al. 1998). Immunohistochemical staining followed by *in situ* hybridization on sections of human atherosclerotic carotid arteries revealed strong expression of gap junction connexin43 messenger RNA by macrophage foam cells. These results suggest that tissue-specific conditions present in atherosclerotic arteries induce expression of connexin43 messenger RNA in monocyte/macrophages (Polacek, et al. 1993).

It is reported that β-carotene (1-10 μM, 1-5 days treatment durations) did not affect GJIC, gap junction protein (connexin43, Cx43) expression, or growth in vitro of non-transformed (C10) or neoplastic (E9 and 82-132) murine lung epithelial cells, but β-carotene enhanced GJIC and Cx43 expression and reduced the growth of C3H10T1/2 murine fibroblasts (Banoub, et al. 1996). These indicate that the effects of β-carotene on GJIC and growth are cell-specific. Although the antioxidant properties of lycopene are thought to be primarily responsible for its beneficial properties, evidence is accumulating to suggest other mechanisms such as modulation of intercellular gap junction communication, hormonal and immune system and metabolic pathways may also be involved (Rao and Agarwal, 2000). Studies on vascular reactivity in atherosclerotic rabbits have demonstrated that vitamin E at low doses improves the EDRF mediated vasodilatory response (Keaney, et al. 1994). The organ chamber system that we have used is designed to study vasoreactivity of whole artery preparation under systemic perfusion pressures. We will use the same system to evaluate vasoreactivity of rabbit arteries in this proposal.

Atherosclerosis is associated with inflammation and acute coronary events (Li, 2004). CRP, IL-6, PAI-1, tissue factors may provide links between systemic inflammation and the outcomes at a localized cardiovascular event. Studies have demonstrated that several arteries may be involved in the acute event other than the one that has occluded (Buffon, 2002; Mukherjee, 2002). In this study we will also determine if there is an association among the expression of inflammation factors, acute coronary events and gap junction.

This proposed project would reveal the GJIC in the atherosclerosis, plaque disruption and thrombosis procedures and the link of the β-carotene and GJIC in these procedures.

3. Preliminary Studies

Working with George S. Abela, the applicant has done many experiments related this proposed project. All the techniques required by atherosclerotic rabbit model and thrombosis triggering have been done by the applicant (Abela, et al. 1995; Hage-Korban, et al. 1999; Ma, et al. 2002, 2003, 2004) and GJIC measurements by the project consultants (Trosko, et al. 2000).

1) Rabbit Atherosclerosis, Thrombosis and Vasoactivity

Twenty-four New Zealand white (NZW) rabbits were divided into 4 groups: normal rabbits as control-control (Group I, n=4)**;** atherosclerosis (induced by diet of 1% cholesterol plus arterial wall balloon injury), non-thrombus triggered and non-β-carotene treatment as control (Group II, n=4); atherosclerosis, thrombus triggered by Russell's viper venom (RVV, 0.15 mg/kg, IP) and histamine (0.02 mg/kg, IV) but non-β-carotene treatment (Group III, n=8); atherosclerosis, thrombus triggering and β-carotene (30 mg/kg, i.v.) treatment (Group IV, n=8). After rabbits were killed isolated carotid arteries were placed in a dual perfusion chamber and tissues were kept in liquid nitrogen for biochemical measurements. Both carotid arteries from each rabbit were perfused with oxygenated physiologic buffered solution at 37^{o}C and 60 mmHg. Baseline vasodilation was determined using norepinephrine (NE, 1×10^{-6} M) preconstriction, and pharmacological challenge was performed with acetylcholine (Ach, 1×10^{-5} M) and sodium nitroprusside (SN, 1×10^{-5} M). Vessel diameter was measured by a computer planimetry system.

The experimental results showed that all the normal rabbits had no atherosclerosis but all the rabbits in other three groups had atherosclerosis. The average weight of aorta for Group I was 1.62±0.55 g. The average weight of aorta for Groups II, III and IV was 3.65±0.37 g and there was no significant difference within the 3 groups. The average weight of aorta arteries for Groups II, III and IV was about 2.25 times heavier than that of Group I ($p<0.01$). Half of the triggered rabbits developed thrombosis. The ratio of the thrombus surface area on the aorta in Group III rabbits to that on the aorta in Group IV rabbits was 1.56. Group II rabbits got atherosclerosis, but no thrombus because rabbits of this group had no thrombus triggering by RVV and histamine. Group I rabbits got neither atherosclerosis nor thrombus. Thrombus surface area on aorta of Group III rabbits was higher than that of Group II rabbits and Group IV rabbits. The number of thrombi in Group III rabbits was two times as that in β-carotene treatment rabbits. The average of total cholesterol in aorta was 2729±334 μg/g for rabbit Groups II, III and IV, and 458±83 μg/g for Group I. There was no significant difference for aorta cholesterol among Group II, III and IV but was significant difference between the average of the above 3 groups and Group I ($p<0.01$). The vasodilation activity of artery in response to norepinephrine, acetylcholine and sodium nitroprusside was Group I>Group II>Group IV>Group III (inter-group ratio of the arterial vasodilation after pharmacological challenge was 1.2-3.5, $p<0.01$-0.05). There was no significant difference of glucose and protein content in aorta arteries among 4 groups. β-Carotene has potential pharmacological effects on atherosclerotic rabbits. β-Carotene reduced thrombosis triggering but not cholesterol content in the artery. This gives the possibly that β-carotene was involved other ways such as enhancing GJIC between the atherosclerostic artery cells.

This results in a platelet rich thrombus on a disrupted plaque in over 70% of treated rabbits (Figure 1) (Ma, et al. 2003).

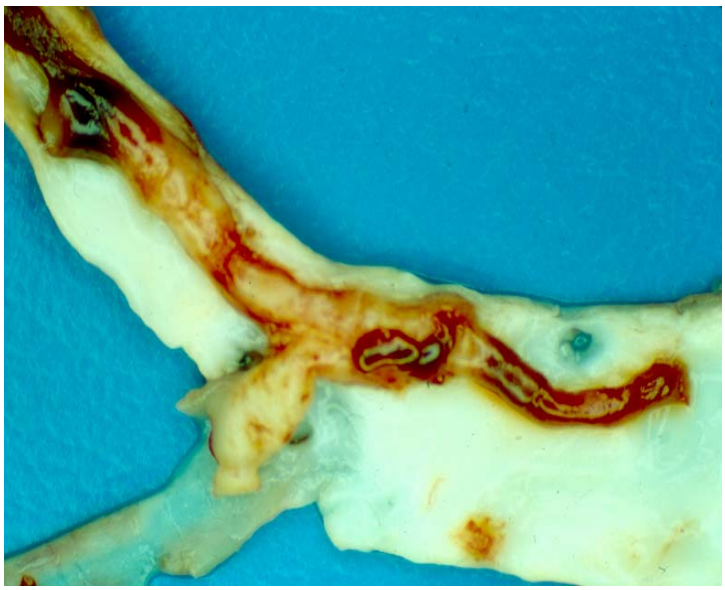

Figure 1. Gross examination: White thrombi with attached fibrin rich thrombi can be seen on the intimal surface of the aorta in more than half the triggered rabbits.

Consultants have measured the effect of LDL and GJIC in endothelial cells. These data have demonstrated that LDL inhibits GJIC in cultured porcine endothelial cells (Scaffidi, 1992).

2) C-reactive Protein Measurement

Atherosclerosis was induced in 17 NZW rabbits using balloon deendothelialization and feeding a high cholesterol alternating with normal chow for 9 months. Triggering was induced by Russell viper venom (0.15 mg/kg; i.p.) and histamine (0.02 mg/kg; i.v.) given at 48 and 24 hr prior to sacrifice.

Materials and Methods

Serum CRP levels were evaluated under three conditions:

1. **Normal rabbits**
2. **After induction of atherosclerosis**
3. **Following pharmacological triggering**

Model of Plaque Disruption and Thrombosis:

Atherosclerosis was induced in 17 NZW rabbits using balloon deendothelialization and feeding a high cholesterol alternating with normal chow for 9 months.

Triggering was induced by Russell viper venom (0.15 mg/kg; i.p.) and histamine (0.02 mg/kg; i.v.) given at 48 and 24 hr prior to sacrifice. This results in a platelet rich thrombus on a disrupted plaque in over 70% of treated rabbits (Figure 1) (Abela, 1995).

CRP Levels:

Rabbit specific high sensitivity ELISA was developed to detect the levels of serum CRP concentrations.

Serum samples were obtained by venous puncture of the ear veins in control rabbits (n=3), and atherosclerotic rabbits, before (n=6) and after triggering (n=8).

4. Results

Serum CRP levels increased significantly following each intervention. Levels on normal chow were very low and more than quadrupled after cholesterol feeding and were more than ten fold after thrombus triggering (Figure 2). Furthermore, CRP was significantly higher in rabbits that had thrombi than those that did not develop thrombi after triggering (Figure 3).

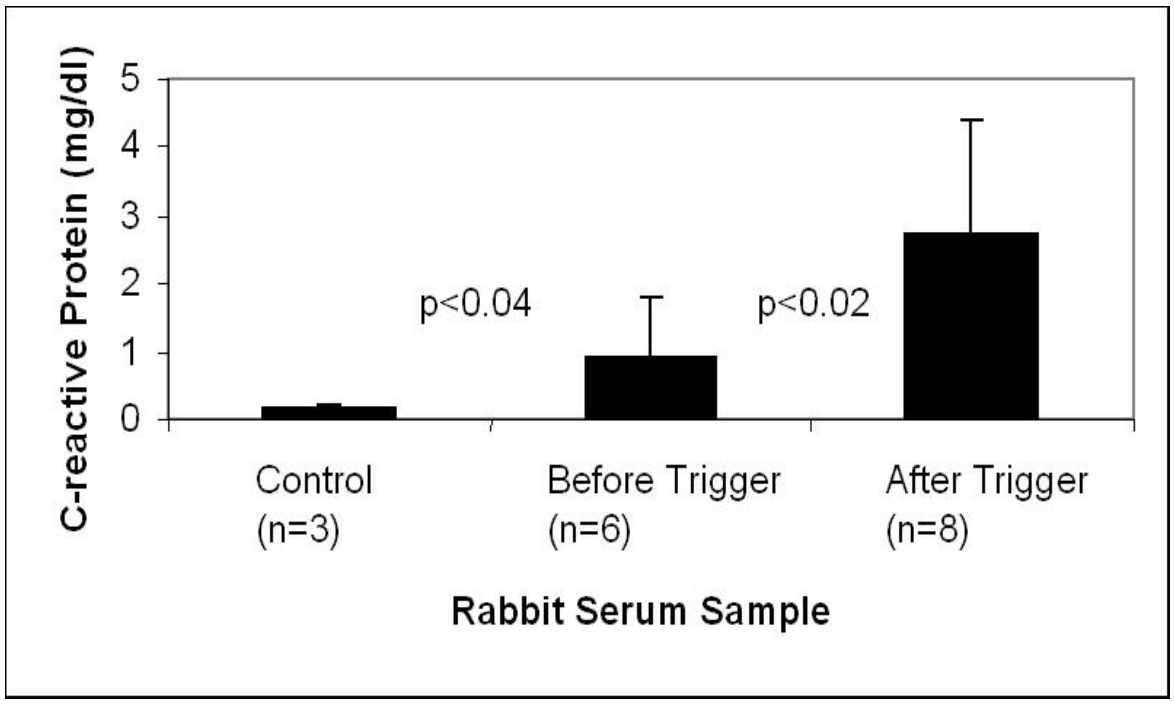

Figure 2. CRP levels were lowest at baseline (Control) and rose following feeding a high cholesterol diet (Before Trigger) and jumped further after triggering (After Trigger).

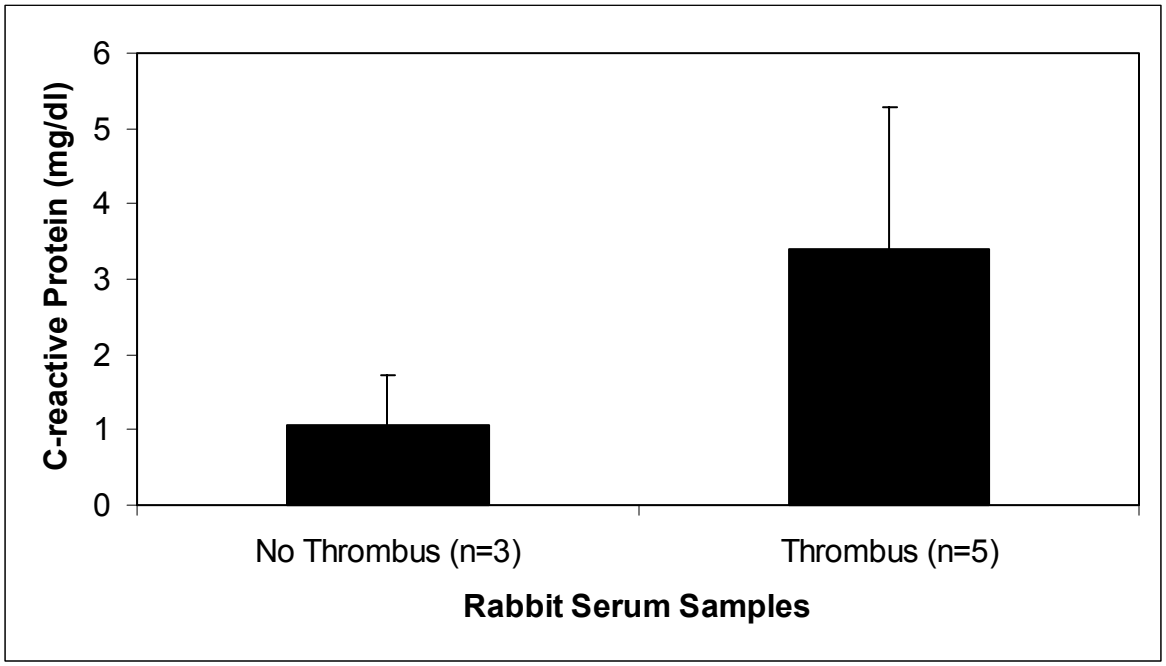

Figure 3. CRP levels in rabbits that developed thrombus were significantly greater than those without a thrombus ($p<0.01$).

Table 1. Preparation of the four rabbit groups

Groups	Treatments	n	Normal Diet	Cholesterol Diet (1%)	Balloon Trauma	RVV and Histamine	β-carotene
I	Control-control	40	Yes	No	No	No	No
II	Control	40	Yes	Yes	Yes	No	No
III	Non-β-carotene	40	Yes	Yes	Yes	Yes	No
IV	β-carotene	40	Yes	Yes	Yes	Yes	Yes

5. Research Design and Methods

1) Atherosclerosis and Thrombosis

A. Atherosclerotic Rabbit Model:

To study the function of gap junction in the protection of β-carotene on atherosclerosis and plaque disruption, the first step is to induce atherosclerosis in rabbit model.

One hundred and sixty, male, New Zealand White rabbits weighing between 2.5 and 3.2 kg (Harlan-Sprague Dawley, Inc., Indianapolis, Indiana) are divided into 4 groups as shown in the Table. The method of establishing atherosclerotic rabbit model and thrombus triggering is done as described previously (Abela, et al. 1995; Hage-Korban, et al. 1999).

The control-control group (Group I) will be fed a regular diet (Harlan-Sprague Dawley, Inc., Indianapolis, Indiana) for 6 months and without pharmacological thrombus triggering. Rabbits in Groups II, III and IV will have balloon-induced arterial injury, then will be maintained on a 1% diet (Harlan-Sprague Dawley, Inc., Indianapolis, Indiana) for 1 month followed by an alternated regular diet for another 1 month for total of 6 months up to pharmacological thrombus triggering.

The catheter will be advanced in a retrograde fashion to the aortic valve and then withdraw 3 cm. The balloon is inflated with 1.5 cm^3 of air, and the catheter is retracted down to the iliofemoral artery. This will be repeated three times in each rabbit. Rabbits are anesthetized with ketamine (50 mg/kg, IM, Fort Dodge Animal Health, Fort Dodge, Iowa) and xylazine (20 mg/kg, IM, The Butler Company, Columbus, Ohio) in this surgery process.

Briefly, one week after initiating the atherogenic diet, balloon-induced arterial wall injury of the aorta (endothelial debridement) will be performed with a 4F Fogarty Arterial Embolectomy catheter (0.9×40 cm, Baxter Healthcare Corporation, Irvine, California) introduced through the right femoral artery cutdown. Atherosclerosis will be achieved by feeding a 1% cholesterol diet for 1 month, followed by a one-month period on a normal diet. This cycle will be repeated for a total of 6 months during the three years period of the proposal. Serum cholesterol will be checked prior to killing.

B. Thrombosis of Pharmacological Triggering:

Only the rabbits that are underwent balloon-induced arterial injury and are maintained on an alternative 1% cholesterol diet (Groups II, III and IV) had atherosclerosis then could be triggered thrombus. For thrombus triggering in atherosclerotic rabbits (Groups III and IV), Russell's viper venom (RVV, 0.15 mg/kg, Sigma Chemical Co., St. Louis, Missouri) is given by intraperitoneal injection at 48 and 24 hours before the rabbits are sacrificed. Thirty minutes after each RVV injection, histamine (0.02 mg/kg, Sigma Chemical Co., St. Louis, Missouri) is administered intravenously on an ear. For rabbits of Group IV, β-carotene (30 mg/kg, BASF Corporation, Mount Olive, New Jersey) is injected intravenously 8 days before sacrificed. After intravenous administration of heparin sulfate (1000 U/rabbit, IV, Elkins-Sinn, Inc., Cherry Hill, New Jersey) to prevent postmortem clotting, rabbits are anesthetized by injecting Nembutal sodium solution (pentobarbital 50 mg/ml, 1 ml/kg rabbit, Abbot Laboratories, North Chicago, Illinois) through a marginal ear vein. Procedures are performed according to Michigan State University's Animal Care and Use Committee approved protocol.

C. Aorta artery and Thrombus Evaluation and Quantitation:

Aorta, left femoral arteries and both carotid arteries are removed immediately after the rabbits are sacrificed. The artery diameter vasodilation, thrombus, plaque and total surface area of aorta are measured. The tissues of heart, liver and kidney are stored immediately in liquid nitrogen until biochemical measurements.

The total surface area of aorta, the surface of aorta covered with atherosclerotic plaques, the surface area of aorta covered with *ante mortem* thrombus, the number and weight of thrombi on the aorta from the aortic arch to the distal common iliac branches are evaluated. The surface area is evaluated by a color charge-coupled device camera (TM 54, Pulnix, Sunnyvale, California) and digitized by an IBM PC/AT computer with a color image processing subsystem. The digitized images are calibrated by use of a graticule. Surface area is measured by use of a customized quantitative image analysis package.

Measurements will be made of the total surface area of the aorta and the iliofemoral branches, the surface area covered with atherosclerotic plaque, and the surface area covered with thrombus. Images of the arterial surface will be collected with a Pulnix TMC-7 color video camera and digitized by an Apple Macintosh Quadra 950 computer equipped with a NuVista 2M color graphics card and color image processing cubsystem. The digitized images will be calibrated using a graticule, and surface areas measured using a quantitative image analysis package. Tissue samples will be taken from the ascending, upper and lower abdominal aorta. Tissue preparation will be performed as indicated for the tests described below. Arterial tissue samples will be obtained after sacrifice and snap frozen for immunoperoxidase studies.

Arterial samples will then be processed for light microscopy using hematoxulin and eosin, Masson's trichrome, and Verhoffe's elastic stain. It is expected that two tissue blocks will be obtained per rabbit. This will result in an estimate of 180 samples. Our laboratory has the expertise to process these tissues as demonstrated by the preliminary data.

D. Artery diameter responding evaluation:

After rabbits are killed the both isolated carotid arteries from each rabbit are placed in a dual perfusion organ chamber and perfused with oxygenated physiologic buffered solution (PBS) (NaCl 119 mM, KCl 4.7 mM, $CaCl_2$ 2 mM, NaH_2PO_4 1.2 mM, $MgSO_4$ 1.2 mM, $NaHCO_3$ 22.6 mM, glucose 11.1 mM and Na_2EDTA 0.03 mM) under 60 mmHg flow pressure and 2.5 ml/min flow rate at 37°C. Baseline vasodilation is determined using norepinephrine (1×10^{-6} M) preconstriction, and pharmacological challenge is performed with acetylcholine (1×10^{-5} M) and sodium nitroprusside (1×10^{-5} M) successively. Vessel diameter is measured by a computer planimetry system. The data are calculated according to the formulas: Ach-NE (%)=(Ach-NE)/NE×100 and SN-NE (%)=(SN-NE)/NE×100 separately, where Ach, NE and SN represented the diameter (mm) of the arteries that are perfused by the PBS containing a corresponding chemical.

E. Tissue Culture:

In this project, primary cells will be cultured as the bypass observations. Endothelial cells and smooth muscle cells isolated from aorta and femoral arteries of all four rabbit groups will be cultured under the standard tissue culture technique (Davies, 1990). The GJIC and biochemical examinations will be made for the cultured cells.

2) Gap Junction Intercellular Communication

Gap junctional intercellular communication (GJIC) plays an important role in the regulation of cell growth, migration, and differentiation. How β-carotene influences the gap junction intercellular communication (GJIC) in atherosclerotic rabbit arteries and plaque disruption condition will be the key question in this project. GJIC in all the rabbit groups and cultured cells will be measured. The technique of GJIC measurement will be supported in Drs. Chang and Upham's lab (Trosko, et al. 2000).

GJIC will be assessed in β-carotene treated and non-treated rabbit aorta using the scrape-loading dye transfer method. The test demonstrates the intact function of the gap junction protein by visualizing the crossing of a dye (Licifer Yellow) from cells injured by a cutting blade to adjacent intact cells. Although the test has been used primarily in co-cultures of various cell types, it has also been used in intact specimen (El-Foulty, 1987; Christ, et al. 1995). Gap junction will be measure in cultured cells and isolated tissues (Emdad, et al.2001).

Immediately after sacrificing the rabbits with an overdose of pentobarbital, the aorta will be excised, a longitudinal incision will be made and the specimen will be

pinned flat on a cork board. The sample will be washed with phosphate buffered solution (PBS: 137 mM NaCl, 2.68 mM KCl, 8.10 mM Na_2HPO_4, 1.47 nM KH_2PO_4, 0.68 mM $CaCl_2$, 0.49 mM $MgCl_2$) and incubated in Dulbeccos modified Eagle's Medium (DMEM) containing 10% fetal calf serum at 37°C and 5% CO_2 atmosphere prior to the experiments. Each sample will be inspected under a dissection microscope for morphological uniformity of the endothelium prior to dye studies, and atherosclerotic-appearing, normal-appearing and thrombus-involved areas of each animal will be identified and marked. Cells will be rinsed with PBS and covered with PBS containing 0.05% Lucifer Yellow and tetramethyl rhodamine dextran (Molecular Probes, Inc., Eugen, Ore). The dye will be scraped-loaded by scalpel incision into cells in the previously marked areas and incubated for 3 minutes at room temperature. Because of its low molecular weight, Lucifer Yellow can be transmitted between adjacent cells via gap junctions, but does not diffuse through intact plasma membranes. The high molecular weight rhodamine Dextran (10,000 daltons) cannot cross the gap junction channels or diffuse through intact plasma membranes, and therefore, serves to identify the primary loaded cells.

Following the scrape-loading procedure, samples will be rinsed several times in PBS to remove excess dye, fixed in formalin to trap the dye in place, and examined under a Nikon epifluorescence microscope for evidence of dye transfer from scrape-loaded cells to contiguous cells. Using the ACAS 570 laser cytometer, the data will be digitized and expressed as the average (percent) fluorescence SEM and normalized using an arcsin square root transformation procedure.

3) Biochemical and Histological Examinations

A. G-6-P, G-6-Pase, ATP/ADP/AMP, cAmp, cGMP, Adenylyl Cyclase, Guanylyl Cyclase, GTPase, and G-proteins:

There are often cell-cell communications through gap junction. Besides gap junction-mediated intercellular communication through GJIC, other signal pathways such as cAMP pathway, also play important roles in the intercellular signal transduction mechanism (Romanello, et al. 2001). In order to determine if β-carotene treatment alters the signal pathways in the rabbit with atherosclerosis, thrombosis and plaque disruption condition, and in order to find if there is relationship between GJIC and the other signal pathways in atherosclerotic rabbits under the antioxidant β-carotene treatment, the following measurements will be performed on homogenates of the arteries from all the rabbit groups and cultured cells.

Glucose-6-Phosphate (G-6-P) (Beutler, 1984): 0.5 ml of Tris-HCl (1 M, with 5 mM EDTA, pH 9.0) added to 1 ml of NADP (2 nM), 0.01 ml of β-mercaptoethanol, 1.2 ml of homogenized tissue extract, and 1.3 ml of H_2O. Read baseline absorbance at 340 nm then add 5 l of G-6-PD (150 U/ml). Record fluorescence till stable reading is obtained. Add glucose phosphate isomerase 10 l (1500 U/ml) and repeat record fluorescence. Add 10 l of G-6-P standard (50 M) and record fluorescence, and then add 20 l of G-6-P standard (50 M) and read fluorescence.

Glucose-6-phosphatase (G-6-Pase): Glucose-6-phosphatase measurement is followed Harper method (Harper, 1965). 0.1 ml of tissue homogenate (100 mg tissue/ml) in citrate buffer (0.1 M, pH 6.5) is added into a test tube and incubated at 37°C for 5 minutes. 0.1 ml of glucose-6-phosphate (0.08 M) is added and the sample is incubated at 37°C for 5 minutes, then 5 ml of trichloroacetic acid (10%, w/v) is added and centrifuged at 9,000×g at 4°C for 5 minutes. 1 ml of the supernatant is taken into a test tube and 5 ml of ammonium molybdate solution (2 mM) then 1 ml of reducing solution (42 mM 1-amino-2-naphthol-4-sulphonic acid, 560 mM SO_3) is added. The sample is incubated at room temperature for 30 minutes then absorption is measured at 660 nm.

AMP and ADP (Beutler, 1984): 50 µl of Tris-HCl (1 M, with 5 mM EDTA, pH8.0) added to 20 µl of $MgCl_2$ (0.1 M), 200 µl of NADP (2 mM), 700 µl of homogenized tissue extract, 5 µl of H_2O, 50 µl of phosphoenolpyruvate (15 mM), 100 µl of NADH (2 mM), 50 µl of lactate dehydrogenase (240 U/ml) and 5 µl of ATP (20 mM). Read baseline absorbance at 340 nm at 37°C. Then add 10 µl of pyruvate kinase (Type II, 140 U/ml) and read absorbance at 340 nm for AMP content. Then add 10 µl of adenylate kinase (myokinase, 725 U/ml) and read 340 nm for ADP content.

ATP (Beutler, 1984): 100 µl of Tris-HCl (1 M, with 5 mM EDTA, pH8.0) added to 20 µl of $MgCl_2$ (0.1 M), 200 µl of NADP (2 mM), 50 µl of glucose (20 mM), 200 µl of homogenized tissue extract, 400 µl of H_2O, and 5 µl of G-6-PD (60 U/ml diluted in β-mercaptoethanol-EDTA stabilizing solution). Read baseline absorbance at 340 nm at 37°C. Then add 10 µl of hexokinase (400 U/ml) and read absorbance at 340 nm until constant value is obtained.

cAMP and cGMP (Armbruster, 1990; Sambrook, 1989): cAMP and cGMP will be measured by standard ELISA and Western Blotting method. The cAMP and cGMP antibodies will be bought from Sigma (Product No. A0670 and G4899).

Adenylyl cyclase catalytic activity (Johnson and Salomon, 1991): 250 l of the reaction solution is incubated at 30°C for 10 min, which contains 20 mM Tris-HCl, pH7.5, 1 mM [α-^{32}P]ATP (10 cpm/pmol), 2 mM cAMP, 5 mM MgCl2, 1 mM EDTA, 1 mM beta-mercaptoethanol and 0.1% (w/v) BSA. After the incubation 600 µl of 120 mM zinc acetate is added to stop the reaction. cAMP is isolated with Dowex AG-50W-X8 column (BioRad) and the radio labeled product [α-^{32}P]cAMP is measured by a scintillation counter.

Guanylyl cyclase catalytic activity (Domino, et al. 1991): The assay buffer (3 ml) is prepared as the following: 600 µl of 200 mM 2-(N-morpholino)ethanesulfonic acid (MES), 180 µl of 100 mM $MnCl_2$ or $MgCl_2$, 600 µl of 10 mM 1-methyl-3-isobutylxanthine (MIX), 600 l of 10 mM cGMP, 300 l of 10 mg/ml bovine serum albumin and 720

μl of H_2O, 1.5 mg of creatine kinase and 33.1 mg of creatine phosphate (disodium salt). Take 50 μl of the buffer and add 25 μl of 2 mM GTP (50 nmol) containing 500,000 cpm [α-^{32}P]GTP, incubate at 37^{o}C for 5 min. Start the assay with the addition of 25 μl of the enzyme source (tissue extract solution). Controls will not be incubated and the reaction stopped by adding 500 μl of 120 mM zinc acetate prior to the adding the enzyme. The control is used to subtract background radioactivity when calculating the amount of cGMP formed. Non-enzymatic formation of cGMP will be tested by adding 25 μl of the buffer from the enzyme source or 25 μl of enzyme boiled in a 100^{o}C water bath for 5 min in separate reaction mixtures. The assay reaction will be stopped by adding 500 μl of 120 mM zinc acetate. Samples are then placed in an ice bath. Once all of the assay reactions have been stopped, 600 μl of 144 mM sodium carbonate is added to precipitate 5'-nucleotides, including unreacted [α-^{32}P]GTP. Centrifuge at 2000×g for 10 minutes. Samples could be frozen and thawed prior to centrifugation. Pour the sample over a neutral alumina column (0.7×15 cm, Econo-Column from BioRad). Elute [α-^{32}P]GTP with 5 ml of 100 mM Tris-HCl, pH7.5, into scintillation vials. Add 10 ml of scintillation fluid or H_2O and determine the radioactivity of the samples in a scintillation counter. Recoveries will be determined by measuring the absorbance at 252 nm of aliquots from each sample before and after column separation or with a tracer amount of cGMP. Recovery is usually between 60%-70%.

GTPase (Kikuchi, 1988): GTPase is the enzyme for the degradation of GTP. GTPase can be measured by the detection of ^{32}P from [^{32}P]GTP. The GTPase activity is determined by a modification of the method of Kikuchi (1988). Briefly, 20 μl of the sample is incubated for 30 min at 30^{o}C in 80 l of the reaction mixture containing 20 mM Tris-HCl at pH7.5, 1 mM EDTA, 1 mM DTT, 5 mM $MgCl_2$, 0.8 M NaCl, 3 mM L-α-dimyristoylphosphatidylcholine, and 1 μM [^{35}P]GTP (2000-3000 cpm/pmol). After the incubation, 50 μl of aliquots is added to 0.75 ml of ice-cold 5% (w/v) charcoal in 50 mM NaH_2PO_4. The mixture are centrifuged at 1000×g for 10 min at room temperature, The amount of ^{32}Pi released from [γ-^{32}P]GTP is then estimated by counting the radioactivity of 0.5 ml of the clear supernatant.

G Proteins (Ohmori, et al. 1989): G proteins are GTP-binding proteins. GTP combines with G proteins specifically. Incubate sample with radioactive-labeled GTP and isolate free GTP then measure radioactivity in the sample to measure G protein amount. Samples are incubated for 2 hours at 30^{o}C in 40 μl of the reaction mixture containing 20 mM HEPES at pH8.0, 1 mM EDTA, 1 mM DTT, 0.8 mM NaCl, 6 mM $MgCl_2$, 3 mM L-α-dimyristoylphosphatidylcholine, and 1 μM [^{35}S]GTPγS (1000-2000 cpm/pmol, Boehringer Mannheim). The reaction will be stopped by the addition of about 2 ml of the ice-cold 20 mM Tris-HCl at pH8.0 containing 100 mM NaCl and 25 mM $MgCl_2$ followed by rapid filtration on nitrocellulose filters. Filters are washed five times with the same ice-cold buffer. After the filter are dissolved in 8 ml of scintillation mixture, the radioactivity will be counted, G-proteins will be measured in endothelial cell suspension in normoglycemic and hyperglycemic culture medium.

Atherosclerosis is associated with inflammation and acute coronary events (Li, 2004). C-reactive protein (CRP), a non-specific inflammatory marker may provide a link between systemic inflammation and the outcomes at a localized cardiovascular event. Studies have demonstrated that several arteries may be involved in the acute event other than the one that has occluded (Buffon, 2002; Mukherjee, 2002). In this study we try to determine if there is an association between the systemic inflammatory response and an acute event. This was performed using an atherosclerotic model of plaque disruption and thrombosis that has been previously reported (Abela, 1995).

Elevated levels of C-reactive protein (CRP) have been associated with increased risk for development of cardiovascular events. In order to follow the trend of CRP over the course leading to an acute event, we evaluated CRP levels under three conditions: normal rabbits, atherosclerotic rabbits before and after pharmacological triggering of plaque rupture and thrombosis. As previously reported, plaque rupture and thrombosis is induced using Russell viper venom (RVV) and histamine in an atherosclerotic rabbit model. Methods: Atherosclerosis was induced with balloon deendothelialization and feeding a high cholesterol diet for 9 months. Serum samples were obtained from control rabbits (n=3), and atherosclerotic rabbits, before (n=6) and 48 hours after RVV and histamine-induced thrombosis (n=8). Rabbit specific high sensitivity ELISA was developed to detect the levels of serum CRP concentrations. Results: CRP levels were significantly lower in control normal rabbits compared to rabbits with atherosclerotic plaques. Our results further demonstrate that rabbits with RVV and histamine-triggered thrombosis had significantly higher levels of serum CRP than non-triggered atherosclerotic rabbits. Conclusion: The rise of serum CRP levels both after cholesterol feeding and the sudden rise after pharmacological triggering of thrombus may help using of CRP to evaluate not only the long-term risk but also a more short-term risk of events if CRP levels increase acutely.

B. Total Tissue Cholesterol and Serum Cholesterol:

Cholesterol plays a key role in the atheroclerosis. To distinguish the rabbit atherosclerotic situation, total tissue cholesterol and serum cholesterol will be measured.

Midthoractic and midabdomical aorta tissues are sampled. Total cholesterol (free and individual ester) in the tissue is measured by high-performance liquid chromatography (HPLC) (Kim and Chung, 1984). Each sample of aorta is ground to a fine powder with anhydrous sodium sulfate and extracted twice with 5 ml of chloroform:methanol (2:1). The extract is dried under nitrogen and re-dissolved in 5 ml of isopropanol. A portion of isopropanol extract is filtered, dried and re-dissolved in the mobile phase. Sample (0.1 ml) is injected into the HPLC

column and separated by using a Waters Radial-Pack C18 column eluted isocratically with acetonitrile:isopropanol (45:55 by volume) at 2 ml/min. The absorbance of elute is measured at 210 nm with a UV detector. Total cholesterol concentration is calculated by comparing the peak areas of samples with those obtained from the standard (Sigma Chemical Co., St. Louis, Missouri) (Witztum, et al. 1985).

Total serum cholesterol is obtained by enzymatic assays of blood samples collected from the rabbits before they are killed. This is done with a Sigma Diagnostics Kit for cholesterol (Sigma Chemical Co., St. Louis, Missouri).

C. Glucose Concentration:

Sigma Glucose Diagnostic Kit (Sigma Chemical Co., St. Louis, Missouri) is used. The method of the instruction by Sigma is followed for this evaluation.

D. Total Protein Concentration:

Bio-Rad Protein Assay Dye Reagent Kit (Bio-Rad Laboratories, Hercules, California) is used and the kit instruction is followed.

E. Inflammation Factors Detection:

Inflammation factors CRP, PAI-1, tissue factor, and IL are measured by ELISA.

F. Electron Microscopy:

The tissue samples are fixed overnight in 4% glutaraldehyde (Fisher Scientific, Pittsburgh, Pennsylvania) with 0.1 M phosphate buffer (pH 7.4). Artery segments of 5 mm each are subjected to critical point drying in liquid CO_2, mounted on tubs, and gold-coated in a sputter coater. The intimal surface is examined in a scanning electron microscope (SEM) (JEOL JSM-6400V, Tokyo, Japan). Tissue sections are obtained and processed routinely for ultrastructural examination for transmission electron microscope (TEM). Thin sections are stained with uranyl acetate and lead citrate and then examined with a TEM microscope (BEI preamplifier, Au Evirotech Company, Germany).

4) Statistical Analysis

With Jandel Scientific program, SigmaStat (Sigma Chemical Co., St. Louis, Missouri) will be used for data statistical analysis. Data will be presented as mean ± Std.Dev. $P<0.05$ is considered statistically significant difference. Intergroup comparisons will be conducted using multivaritate analysis ANOVA. It is expected that atherosclerosis and plaque will reduce the GJIC function and β-carotene treated arteries will relatively enhance GJIC function.

6. Potential Difficulties and Limitations and the Alternative Approaches to Achieve the Aims

A. Platelet adhesion maybe altered by antioxidants. Studies have demonstrated that platelet adhesion is lowered in patients on vitamin E (Jandak, et al. 1988). It maybe that this mechanism is an important contributor to the early observation of a reduced thrombus size in rabbits with disrupted plaques in β-carotene. In order to address this potential confounding issue, we will perform a bleeding time on the rabbits as described by Blajachman et al. (1979). In brief, a standard incision through the ear of the rabbit is done after warming for 5 minutes to 37°C by immersion into a bath that contains 0.9% NaCl. A 6 mm full thickness incision is made through the ear with a scalpel blade. The selected site avoided areas with visible vessels. The incised ear is then immediately reimmersed in the saline bath and the time for the bleeding to cease is recorded.

B. Attenuation of fluorescence has been shown to occur with β-carotene (Ye, et al. 1993). However, at the concentrations of Lucifer Yellow present in the cells, this has not been a limiting factor during scrape loading/dye transfer experiments (Hossain, et al. 1989). Also, the quenching effect by carotinoid has been shown to be negligible in experiments conducted in Drs. Chang and Upham's lab. Nevertheless, if we find this to be a problem, then an alternative would be to substitute α-tocopherol for β-carotene as the antioxidant agent.

7. **Conclusion and Discussions**

The elevation of CRP levels following cholesterol feeding and pharmacological triggering may provide a means to help monitor progression of acute events. The fact that presence of thrombosis is associated with higher levels of CRP may provide a means to help predict severity of events or even possibly recurrence of events as well.

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