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Role of Nanoparticles to Improve Agricultural Practices and Plant Production

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ABSTRACT: *Nanotechnology is the outmost emerging technology of 21st century. It is an excellent approach to renovate the food and agricultural industry with the help of different modern strategies for the rapid disease detection, molecular management of diseases, improving the aptitude of plants to take up more nutrients. Nanotechnology has the ability to enhance yield of nutrient values and also plays an important role in improving developed systems to monitor ecological conditions. Moreover a number of nano-materials are well known for their potential plant growth promoting effects. This present review article highlights the potential effects of Nanoparticles on flowering, growth, and seed production in plants.*

Key words: Nanoparticles, plant growth, seed production, flowering

INTRODUCTION

Nanoparticles are microscopic particle possessing at least one dimension about less than 100 nm. They are different from bulk materials because of having their characteristic optical, electrical, thermal, physical and chemical properties (Herlekar et al., 2014). They also have a range of applications in the fields of chemistry, medicine, environment, energy, agriculture, information, communication and heavy industry (Phogat et al., 2016). In the 21st century, nanotechnology is appeared with the great impact on public lives, global economy and industries (Mukhopadhyay, 2014). As if we took a glance at the historical part of agricultural implementations of nanotechnology, it emerged only in recent years but the roots of research in this area start developing nearly half a century ago (Dhewa, 2015). Nanotechnology is an effective strategy in the area of productive science used in the consumption of agricultural food waste into bio energy, chemical biosensors development, water purification and increase of plant growth (Abbas et al., 2016).

Nanotechnology appears to have an encouraging potential to make more supportable future perspectives of agriculture. Use of Nanoparticles as fertilizer has countless opportunities to increase crop yield and minimize environmental hazards (Huang et al., 2015). Nanotechnology shows potential in the field of inter-disciplinary research. Application of nanotechnology include improvement of agricultural production using bio conjugated Nanoparticles, animal breeding, transfer of DNA in plants for the growth of insect resistant varieties, nano formulations of agrochemicals for the application of pesticides and fertilizers for crop improvement, nanosensors/ nanobiosensors in crop protection for the identification of diseases and residues of agrochemicals, plant disease diagnostics and postharvest management. The effect of NPs on plants varies from species to species and plant to plant (Farooqui et al., 2016).

Types of Nanoparticles (Tables 1-2)

Gold Nanoparticles

Gold Nanoparticles are outstanding materials in the growth enhancement of various plants such as stem diameter, plant height and number of branches per plant (Arora et al., 2012). It is also reported that gold Nanoparticles (10 ppm) can improve the growth of *Brassica juncea* in terms of increased productivity by giving better seed yield (Gunjan et al., 2014).

Silver Nanoparticles

Silver Nanoparticles (SNPs) attributes as antibacterial, antifungal agents enhanced its use in agriculture and food such as food security, food packing and pathogen detection. It also showed great impact on plant growth and development like germination, root-shoot ratio, seedling growth, root growth and root elongation (Jhanzab et al., 2015)

Titanium nanoparticle

Titanium is the 9th most abundant element and the 2nd most abundant transition metal in the earth's crust. Titanium compounds are very important in the improvement of plants yield of various crops. They act as essential element contents in plant tissues and are very helpful to enhance the catalase, peroxidase and nitrate reductase activities in plant tissues. Titanium oxide nanoparticles can improve nitrogen metabolism, photosynthesis, seed germination and plant growth of spinach (*Spinacia oleracea*) (Khater, 2015).

Titanium oxide Nanoparticles can also act as antioxidants by quenching oxygen free radicals formed during the photosynthetic activity, thereby increasing the photosynthetic rate. They can also improve the ability of absorbing water and inorganic nutrients and breaking down of organic substances during the photosynthetic progression, reducing the oxygen free radicals formed during the photosynthetic process, resulting in the improvement of photosynthetic rate (Abbas et al., 2016).

Zinc oxide Nanoparticle

Zinc oxide Nanoparticles are important in the field of agriculture. The zinc oxide Nanoparticles in the form of colloidal solution can be used as fertilizer. They can boost up the growth and yield of food crops.

Zinc oxide Nanoparticles (1000ppm) are very effective in seedling vigor, seed germination and plant growth and these Nanoparticles are also found to be helpful in increasing root and stem growth in peanuts (Sabir et al., 2014).

Carbon Nanotubes

The transfer of molecules into plant cells is more complex because of the chemistry of plant cell wall and an extra barrier is required in addition to the cell membrane (Khodakovskaya et al., 2009). As carbon nanotubes have ability to penetrate compact plant cell walls and cell membranes to transport molecules. By comparing to the present transportation strategies for plant cell walls like gene gun, microinjection, electroporation, Nanoparticles based methods are found to be advantageous in high efficiency, easy operation, and the capacity to transport molecules other than nucleic acids (e.g. regulatory-active molecules and imaging agents). The development of carbon nanotubes acting as nano-transporters for compact plant cells have fundamental and practical significance for plant intracellular imaging and labeling, genetic transformation and for improving of our knowledge of plant cell biology (Liu et al., 2009).

Copper Nanoparticles

Copper is an essential nutrient used in the development of plants and plays a vital role in the photosynthetic reactions. It activates different enzymes, enhances photosystem performance and contributes in the synthesis of RNA (Adhikari et al., 2012). Copper Nanoparticles are found to be effective during crop growth (Hafeez et al., 2015).

Iron Nanoparticles

Iron Nanoparticles are very effective in seed germination activity, particularly the growth of seedlings. Iron Nanoparticles can penetrate the peanut seed coats to stimulate seed germination by enhancing uptake of water. As iron deficiency occurs in different soils and it causes serious problems to various types of crops such as peanuts. So iron Nanoparticles can be used as fertilizer to enhance the crop yield (Farooqui et al., 2016).

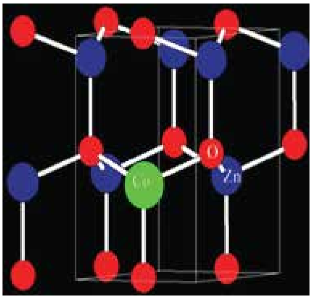
Silicon Nanoparticles

Silicon Nanoparticles showed beneficial effects in a wide range of plants species for their yield, growth, abiotic and biotic resistance. It is very effective as a physico mechanical barrier. It can be deposited on the walls of vascular and epidermal tissues of the stem, leaf and hull in most of the plants particularly monocots and also regulates physiological behavior in plants. Moreover, regulatory effect of the silicon element on plant development and growth under stress conditions is well known (Siddiqui and Al-Whaibi, 2014). Sillicon particles also showed antimicrobial potential by suppressing crop diseases (Servin et al., 2014).

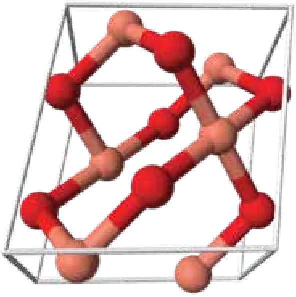
Manganese Nanoparticles

Manganese Nanoparticles have an outmost importance in improving the photosynthetic capability of the plants, thus producing more sugar and can be effective in agricultural sector for the development of plant productivity. Manganese Nanoparticles are found to be more effective in nitrogen metabolism and assimilation as proved by both molecular and biochemical techniques (Pradhan et al., 2014).

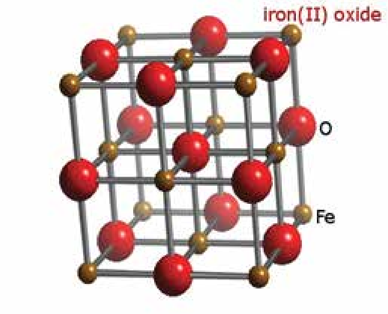
Table 1 : Structures of Nanoparticles



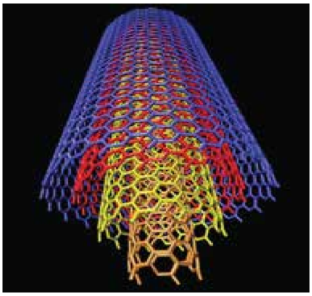
Zinc oxide



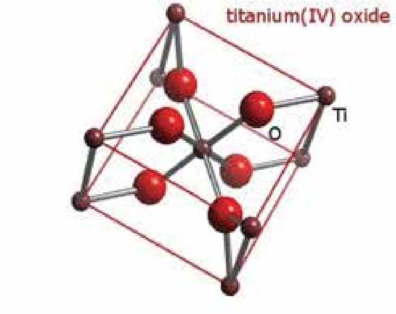
Copper oxide



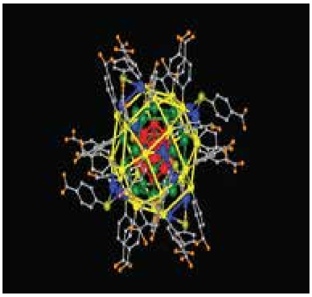
Iron oxide



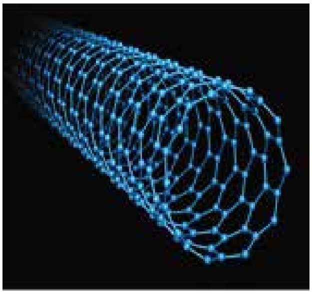
Multibond carbon nanotubes



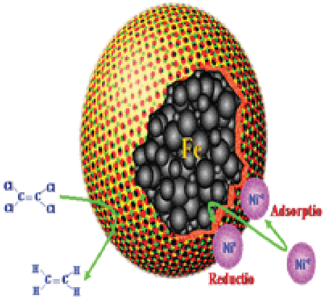
Titanium dioxide



Silver Nanoparticles



Single bond carbon nanotubes (Farooqui et al.,2016)



Zero valent iron

Applications of Nanoherbicides and Nanopesticides

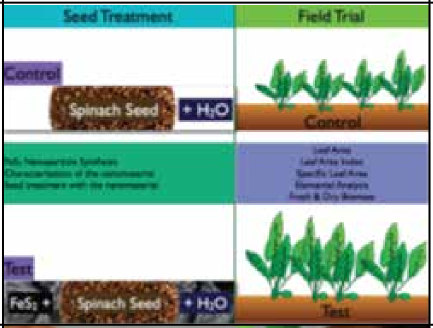
Pesticides and herbicides are usually used in agriculture to get better crop yield and efficiency. The major disadvantages of the use of pesticides are production of pathogen and pest resistance reduces soil biodiversity, decreases nitrogen fixation, pollinator decrease, causes bioaccumulation of pesticides and damages habitat for birds. Nowadays, use of Nanoparticles solve this issue upto much extent and their applications along with herbicides minimized the herbicides dose requirement for weed eradication. By having active constituents and smart delivery system, herbicides are given to the soil according to the soil condition. Silver Nanoparticles also showed pesticidal potential against pathogenic fungi as by inhibiting the conidial germination of genus Raffaelea which causes mortality of oak trees (Singha et al., 2015).

Nanofertilizers

Fertilizers have a vital role in improving food production. Furthermore, excessive applications of phosphorus and nitrogen fertilizers influence the groundwater and also cause eutrophication in aquatic ecosystems. Recently, nanotechnology has progressively moved from the experimental to practical areas. Now nanotechnology has become significantly very important for endorsing the development of environmental friendly and sustainable agriculture like, the development of controlled and slow release of fertilizers. Moreover, encapsulation of fertilizers within a nanoparticle is one of these new facilities which are done in three ways a) coated with thin polymer film, b) the nutrient can be encapsulated inside nanoporous materials, c) delivered as emulsions or particle of nanoscales dimensions. Furthermore, nanofertilizers in combination with nanodevices are used in order to coordinate the release of fertilizer-P and -N with their uptake by crops, thereby reducing undesirable nutrient losses to soil, air and water by direct internalization by crops and avoiding the contact of nutrients with soil, microorganisms, air and water (Veronica et al., 2015)

The application of nitrogen nanofertilizer and potassium nanofertilizer demonstrated significant increase in the absorption of calcium, nitrogen, potassium, phosphorus and magnesium by plants.

Table 2: Effects of various Nanoparticles on the growth and development of plants

Sr. No	Figur	Nanoparticles and plants	End points	Reference
1		iron pyrite (FeS_2) on Spinach	Increase production of spinach	(Srivastava et al., 2014)
2		Iron oxide on Spinach	Enhancement in growth rate and productivity	Jeyasubramanian et al., 2016
3		Silicon dioxide on lentil	Favorable effect on seed germination	Farooqui et al., 2016
4		Sillicon oxide on tomato	Improvement in seedling growth	Siddiqui and Al-Whaibi, 2014
5		Zero valent iron on peanut	seed germination and growth increased	Li et al., 2015

Potassium nanofertilizer also showed most promising effects on plant growth like shoot and root growth (Ajirloo et al., 2015).

CONCLUSION

Nanotechnology is an advance technique effective in all fields of science. Extensive research work for commercializing nano products is going on throughout the world. The purpose of Nanoparticles in agriculture is to reduce nutrient losses and improved yields. Nanoparticles act as one of the most flexible materials, due to their functionalities, miscellaneous properties and applications. Nanoparticles have outstanding optical, physical and antimicrobial properties. As far as their usage is concerned Nanoparticles play an important role in agriculture, where colloidal solution of Zinc oxide Nanoparticles is used in nanofertilizers. Metal Nanoparticles when applied as foliar spray, increases crop production. As the food demand is increasing everyday and the yield of staple food crops is much low, so it is required to commercialize metal Nanoparticles for sustainable agriculture. The point that has been focused on the size of Nanoparticles which plays an important role in the reactivity of the behavior of Nanoparticles. A food demand is increasing day by day and the yield of staple food crops is much low. Moreover, today it is important to improve the productivity of crops to feed the growing world population. So it is required to commercialize metal Nanoparticles for sustainable agriculture.

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Microbial Quality of Fresh and Frozen Fish from Markets of Lahore

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ABSTRACT: *The present study aims at the microbiological analysis of market oriented fresh and frozen fish. Displayed portions of raw fish and frozen carried bacteria that can cause foodborne diseases clearly indicates the risk posed by poor sanitation and unhygienic handling, storage and transportation conditions of fish sold in the markets. Research was designed to compare the presence of indigenous and nonindigenous foodborne bacterial pathogens in displayed, prepacked and packed portions of fish in different markets of Lahore, Pakistan. A total 40 samples of fish were purchased from Super Store (Hyper star), Local Market (local carry shop Mughalpura), Retail shop (Data Darbar), and Fresh water (River Ravi) in Lahore city and analyzed for their microbial quality. All the samples were positive for Total plate count, coliform count, Staphylococcal count, Vibrio count. Among 10 fresh water fish samples mean total Plate Count was 3.8 x 103 CFU/g (log 3.58 ± 0.03526 CFU/g). Among 10 super store fish samples mean total plate count was 4.9 x 104 CFU/g (log 4.05 ± 0.22815CFU/g). Among 10 retail store fish samples mean total plate count was 5.02 x 104 CFU/g (4.19 ± 0.0343 CFU/g) and among 10 local market fish samples mean total plate count was 5.14 x 104 CFU/g (4.212 ± 0.03793 CFU/g). In total, the plate counts range from minimum count log 3.16 to maximum count of 5.30 CFU/g. This study revealed that fish sold in Pakistan could be a source of foodborne bacterial pathogens. Improvements in handling and processing are needed to minimize the prevalence of pathogenic bacteria.*

Key Words: Fish, microbiology, pathogenic bacteria, aquatic microbiology

INTRODUCTION

Pakistan has a domestic and an international market for fish, shrimps and fish products. At the domestic level the catch from marine fisheries or from local sources (river, canal) has been supplied to the local fish markets. Frozen or processed fish is marketed in few large departmental stores in some cities. *Per capita* consumption is only 1.6 kg/annum. On the international level Pakistan has a market for fish and fish products. About 30% of the total fish catch has been exported to 30 countries of the world. Pakistan earns more than 6% of its foreign exchange by exporting fish and fish products. Pakistan's seafood exports have actually decreased during the past decade, with 1992 showing highest figures, i.e., US$ 181 million.

Pakistan's domestic consumption is termed as one of the lowest in the world, at 1.6 kg per person per year (compared to world average of 16.2 kg per person per year. FBS, 2010). Microbes play an important role in the degradation of fish products, thus better knowledge of the microbiological conditions throughout the fish production chain may help to optimize product quality and resource utilization. This study presents the results of research project of the commercially most important fish species harvested and marketed in Pakistan. Fish samples from different markets of Lahore were collected. Totally 40 samples were assessed with respect to microbiological quality, hygiene and food safety. We introduce a quality and safety assessment scheme for Different fish samples originated from various markets depicting their storage, handling and quality characteristics . According to the scheme, in 25 of 40 samplings, sub-optimal conditions were found with respect to quality, whereas in 21 and 9 samplings, samples were not in compliance concerning hygiene and food safety, respectively. The present study revealed that the quality of marketed fish can be optimized by improving the hygiene conditions at some critical points at an early phase of the production chain. Thus, the proposed assessment scheme may provide a useful tool for the industry to optimize quality and maintain consumer safety of fish and fishery products.

Fish was a vertebrate which can be finfish, shell fish (molluscs and crustecean) and any other type of fresh water and marine water which can be used for domestic and animal consumption (Begum et al., 2010). Fish an extra relishing and nutritive source than the plant food. Fish meat was easily digested, generally it was preferred over meat and egg (Shingadia, 2011). Fish an important food item in the developing countries due to its high protein content and nutritional value (Sultana et al.). More than 30,000 species of fish are known, fish from the biggest group in animal kingdom that was used for the production of animal based foods. Nearly 700 of these species are commercially cultured and used for the food production (Olgunoğlu, 2012). Unhygienic food causes many acute and chronic diseases that range from gastrointestinal diseases to various forms of cancer. The spoilage of fish was a complicated process which involves chemical and physical activity of microorganism. Fish products are very perishable, lack of appropriate facilities like exposure of the fish with high temperature and unhygienic condition during handling from the time of catching until it was processed into finished products contributes in major loss of fish quality.

Consumption of Seafood was becoming increasingly acceptable due to the ratio of high proteins, vitamins and poly-unsaturated fatty acids (Ge et al., 2012). Demand of fish and fish products are increasing rapidly around the world (Basti et al., 2006). Worldwide production of fish and fishery products has been doubled since 1970's (Chakraborty et al., 2008). Fish was a very important sources of minerals, proteins, vitamins and omega 3 fatty acids (Goja, 2013). Present research aimed to highlight the risks posed by poor sanitation and unhygienic handling, storage and transportation conditions of fish sold in the market

MATERIALS AND METHODS

Sample Collection

In this investigation the species of fish (*Labeo rohia*) was used, which were collected and randomly selected from the Fresh water, local market, retail market and super store of Lahore. Samples were labeled properly and shifted to Microbiology Laboratory, University of Veterinary and Animal Sciences Lahore.

Preparation of Samples and Enumeration of Microorganisms

Total bacterial count was calculated on the, MacConkey agar. For this purpose, MacConkey agar was weighed and dissolved in the distilled water. *Vibrio* count was calculated on the Thiosulfate Citrate Bile Salt Sucrose Agar (TCBS). For this purpose, Thiosulfate Citrate Bile Salt Sucrose Agar (TCBS) was weighed and dissolved in the distilled water according to the manufacturer instructions. Then it was autoclaved (120°C, 15lbs pressure for 15 minutes). Media was allowed to cool and poured in the sterilized Petri plates under sterilized conditions. These Plates were tested for sterility. 10 fold dilutions were made using Phosphate Buffered saline (PBS). Approximately 100ml Phosphate Buffered saline (PBS) was prepared and then added 9ml in each test tube and then it was autoclaved (120°C, 15lbs pressure for 15 minutes) and cooled down. One gram homogenized fish sample was taken in the test tube, containing 9ml of phosphate buffer saline solution and tenfold serially dilutions were made (Holt,1994).

RESULTS AND DISCUSSION

A total 40 sample of fish samples were purchased from Super Store, Local Market, Retail shop, and Fresh water in Lahore city and analyzed for their microbial Quality. All the samples were positive for Total plate count, coliform count, *Slomonella* count, *Vibrio* count. Among 10 fresh water fish samples mean total Plate Count was 3.8 x 10^3 CFU/g (log 3.58 ± 0.03526 CFU/g). The Counts range from minimum count log 3.48 to maximum count of 3.71 CFU/g. Among 10 super store fish samples means total plate count was 1.13 x 10^4 CFU/g (log 4.05 ± 0.22815CFU/g). The counts range from minimum count log 4.00 to maximum count of log 4.10 CFU/g. Among 10 retail store fish samples mean total plate count was 1.57 x 10^4 CFU/g (log 4.19 ± 0.0343 CFU/g). The counts range from minimum count log 4.15 to maximum count of 4.23 CFU/g. Among 10 local market fish samples mean total plate count was 1.63 x 10^4 CFU/g (4.212 ± 0.03793 CFU/g). The counts range from minimum count log 3.16 to maximum count of 4.30 CFU/g. Among 10 fresh water fish samples mean coliform Count was 1.6 x 10^5 (4.20 ± 0.06822 CFU/g).The counts range from minimum count log 4.14 to maximum count of 4.25 CFU/g. Among 10 super store fish samples mean coliform Count was 9.4 x 10^5 (4.97 ± 0.03108 CFU/g). The counts range from minimum count log 4.30 to maximum count of 5.07 CFU/g. Among 10 retail store fish samples mean coliform Count was 1.06 x 10^5 (5.28 ± 0.0275 CFU/g). Among 10 local market fish samples mean coliform Count was 1.40 x 10^5 (5.14 ± 0.04165CFU/g). Among 10 fresh water fish samples *salmonella* was detected in 10 percent. Among 10 super store fish samples *Salmonella* detected 60%. Among 10 retail market fish samples *salmonella* detected 80 %. Among 10 local market fish samples *Salmonella* was detected in 90% samples.

In current study total plate count was 3.8 x 10^3 CFU/g which was closely resembles to the values reported by (Ali et al., 1991). Another study in which reported results for TPC 4.5 x 10^5 – 9.5 x 10^2CFU/g were different from my research due to conditional differences (Mailoa et al., 2013). Another study in which reported results for total plate count (8.81 ± 0.45 x 10^5CFU / g) reassembled with the findings of (Mandal et al., 2009).

In present study the *Staphylococcal* count was 4.1 x 10^4 CFU/g which was higher than the values 2.50x 10^2 CFU/g as compared to previous report earlier (Ayuba et al., 2013). Similar study revealed the values 1 x 10^4 CFU/g of *Staphylococcal* count, the variation might be due to the environmental conditions.

In present research the *Vibrio* Count was 3.12 x 10^2 which resembled with the values of 4 x 10^4 as reported by Enger et al. 1991. In another study the values of vibrio count was 7.76 x 10^4 CFU/g were high with my values reported by (Hasegawa et al., 1999).

According to my research finding the results were significant which depicts that the storage and handling conditions of some markets of Lahore. The results clearly showed that the microbial quality of fish sold in all the markets was contaminated with pathogens with non significant differences among different markets. Observational data showed pathetic hygienic conditions of the fish sold in local and retail markets while high bacterial load in superstore fish was alarming too. The results also showed that the bacterial load was comparatively lower in superstore fish than other markets. Lowest values of pathogens found in fresh fish indicated the poor handling, transportation and storage conditions. The results also directed that the hygienic conditions of different fish markets were also not appropriate and good. However the microbial quality of fresh water fish was comparatively good (Table 1; Fig. 1).

Table 1 : Microbial Count in samples of fish from different sources

	TPC(CFU/g)	TCC(CFU/g)	SC(CFU/g)	TVC(CFU/g)
Fresh Water	3.8 x 10^3	3.5 x 10^5	4.61 x 10^4	2.06 x 10^2
Super Store	4.9 x 10^4	4.05 x 10^5	4.76 x 10^4	2.62 x 10^3
Local Market	5.14 x 10^4	21 x 10^5	5.02 x 10^4	3.73 x 10^3
Retail Shop	5.02 x 10^4	4.19 x 10^5	4.12 x 10^4	3.59 x 10^3

** Total Plate Counts (TPC), Total Coliform Counts (TCC), Salmonella Counts (SC), Total VibrioCounts (TVC).

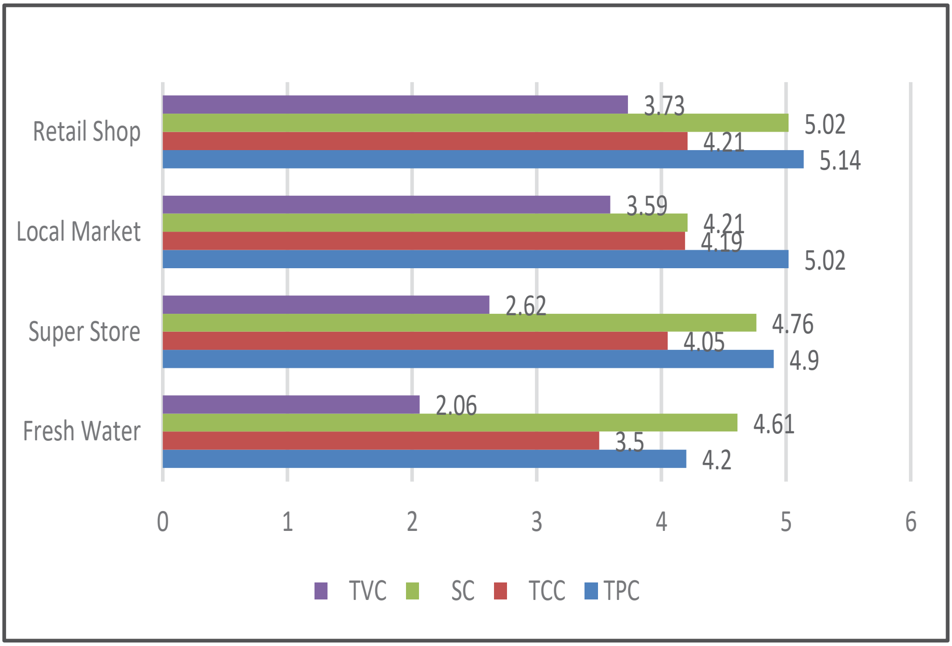

Fig. 1 : Graphical representation of bacterial count in fish samples from different locations in Lahore

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Crimean-Congo Hemorrhagic Fever–Distribution, Diagnosis, Treatment and Control Measures

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ABSTRACT: *The Crimean Congo Hemorrhagic Fever (CCHF), is a highly pathogenic disease that contributes to high morbidity and mortality. It is due to a tick-borne virus, capable of causing hemorrhagic fever that leads to death, specially affecting the human beings, therefore is of outmost importance in the current decade. The disease is widely distributed covering many continents with 40-60% of mortality. Although it is lethal, but still can be avoided; at both community as well as nosocomial level, by adopting proper strategy for management of the disease. This review is a comprehensive discussion about the Crimean Congo Hemorrhagic fever and covers the preventive and curative measures that can be adopted to prevent the outbreak of CCHF.*

Keywords: Crimean Congo Hemorrhagic Fever, Tick borne infection, epidemiology, diagnosis, prevention

INTRODUCTION

Crimean-Congo hemorrhagic fever (CCHF) is a very serious and fatal infection, caused by Crimean-Congo Hemorrhagic Fever virus. The virus belongs to the genus *Nairovirus,* which is one of the five genera of the family *Bunyaviridae,* having approximately 350 species (JCTV, 2014). It causes severe infectious disease in humans (Ergonul, 2006), and the virus has been classified into risk group 4, hence should be handled carefully in Biosafety level 4 (BSL-4) laboratory. The viral hemorrhagic fever associated viral families have resemblance with one another (Geisbert and Jahrling, 2004 but of all hemorrhagic fever associated viruses, Crimean Congo Hemorrhagic virus is of importance due to its wide distribution and extensive epidemics worldwide in the recent decades. Crimean-Congo haemorrhagic fever is characterized by haemorrhagic manifestations with fatality rate ranging from 5% to 30% (Gonzalez et al., 1991). Crimean-Congo haemorrhagic fever is one of the major emerging infectious disease (Lablebicioglu et al., 2015), and It is most widely distributed tick-borne viral infection, geographically widespread with cases reported from different parts of Asia, Africa, the Middle East, Eastern Europe and Greece (Ergonul, 2006). It is the most widely spread tick-borne viral disease with a geographical distribution following that of Hyalomma spp. of ticks, the main vectors of CCHF virus (Drosten *et al.,* 2003).

Structure, Nature and Life cycle of virus:

The CCHF virus (CCHFV) has the evolutionary history of 3100-3500 years (Carroll et al., 2010). It is spherical, uniform sized particle, with diameter of about 100nm (Donets et al., 1977; Zhou et al., 2011; Zivec et al., 2015). The genome consists of single-stranded Negative-sense RNA that is divided into 3 different segments: Small (S), Medium (M) and Large (L). The L, M and S are 11-14.4 kilo bases, 4.4-6.3 and 1.7-2.1 kilo bases in length (Carter et al., 2012). The S segment encodes the nucleo-capsid protein (NP) and a nonstructural protein (NS_s), the M segment encodes precursor of the two envelope glycoproteins G_n and G_c while L segment encodes the RNA-dependent RNA polymerase (Mardani and Keshtar-Johromi, 2007). The non-coding complementary regions called as NCRs give the genome its circular appearance, these regions are usually present at 5' and 3' terminal of all the three segments of the virus genome (Hewlet et al., 1977). The genomes were thought to encode a single protein but it was later revealed that, the S segment encodes a non-structural protein (NS_s) in the opposite orientation to that of NP protein, making it to be considered as ambi-sense virus (Zivec et al., 2015). The virus is enveloped having bi-lipid layer (Acha and Snzyfres, 2003; Whitehouse, 2004). The proteins on the envelope form protrusions of approximately 5-10 nm long. The membrane receptors on the cell surface for the virus has been identified to be nucleolin and G_c as reported by Xiao et al., (2011). It is classified into the following 7 genotypes: Asia-1, Asia-2, Africa-1, Africa-2, Africa-3, Euro-1 and Euro-2 (Alam et al., 2013). The structure of Crimean Congo Virus has been shown in Fig.1.

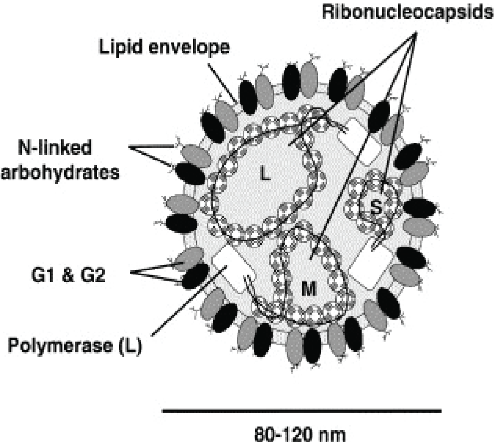

Fig. 1: The internal structure of the Crimean Congo Hemorrhagic Fever with 3 genome segments and lipid envelope (Samreen et al., 2012).

The virus can survive under several conditions and depends on conditions like temperature, pH, humidity and the habitat. CCHFV gets inactivated within 30 minutes at 56°C while gets inactivated for 15 minutes at 60°C. Using chemicals/ disinfectants it can be inactivated usually by the use of 1% hypochlorite and 2% glutaraldehyde. pH also has effect on the virus and can be inactivated at a pH less than 6. The virus has tremendous survival abilities as it can survive in moist conditions for 7 hours at 37°C, for 11 days at 20°C and for 15 days at 4°C. Its survival in dry conditions is reduced for 90 minutes to 24 hours (Public Health Agency of Canada, 2011; OIE, 2014)

The CCHFV binds initially to the cell

surface via receptors that are glycoproteins G_n or G_c. The actual details about the attachment, entry and fusion of the virus remain unidentified till date (Zivec et al., 2016). It is suggested that G_c is responsible for the binding as monoclonal antibodies can neutralize CCHFV infection by targeting G_c as reported by Bertolotti-ciarlet et al. (2005). The cell protein nucleolin has also been suspected to contribute in the entry as its functional interaction has been suggested with the protein G_c (Xiao et al., 2011). But no specific receptors have yet been identified that are primarily involved in the CCHFV entry (Zivec et al., 2016). The entry when accomplished is followed by endocytosis (Clathrin-mediated). CCHFV is also dependent on low pH and presence of cholesterol for its entry (Simon et al., 2009; Garrison et al., 2013; Shtanko et al., 2014) Fig.2.

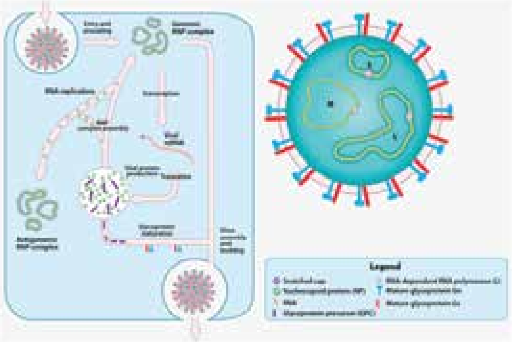

Fig. 2: The replication cycle of Crimean congo virus (Zivec et al., 2016).

Host

Both wild and domestic animals are susceptible including vertebrates and birds. The preference of tick (Hyalomma spp. majorly), carrying CCHFV varies by its stage of development. Larva and nymphs usually prefer ground birds and small mammals. While adults are found more likely to infect the livestock (WHO, 2013; CABI, 2015). Various animals can serve as host because of wide distribution of *Hyalomma* vector. The incidence rate is influenced by climatic changes and ecological conditions in several European Countries. *Hyalomma* life cycle is more adapted to dry climate and arid type of vegetation (Seif et al., 2017). Environment as well as human activities contribute in the occurrence of endemic outbreak in an area, However, this risk can be minimized by changes in use of land, movement and trading of livestock that is infected (Leblebicioglu, 2010).

Disease Distribution and Epidemiology:

The first medical incidence of CCHF was reported in 1944, during investigation on epidemic affecting Soviet troops. It revealed that the 'Crimean hemorrhagic fever' virus causing this outbreak was exactly similar to the 'Congo hemorrhagic fever' virus identified in Africa. These findings gave it the name of 'Crimean-Congo hemorrhagic fever virus' (CCHFV) (Talgat *et al.,* 2013). Since its discovery, more than 140 outbreaks in 52 countries has been reported from all over the world (Appannanavar and Mishra, 2011). Outbreaks in the regions of former Soviet Union (Hoogstraal, 1979; Watts et al., 1988), Africa (Saluzzo et al., 1985; Swanepoel et al., 1987), Middle East countries (El-Azazy and Scrimegour, 1997; Schwarz et al., 1997; Al-Tikriti et al., 1981), Asia including countries like India (Yadav et al., 2014) , Afghanistan (Mustafa et al., 2009), Iran (Sharifi et al., 2009; Keshtkar-Jahromi et al., 2013), Pakistan (Sheikh *et al.,* 2005) have been evinced. In the last two decades, random outbreaks have been reported in Bulgaria, India, Pakistan, Turkey, Iran, Sudan, Greece (Ergonul et al., 2004;

Karti et al., 2004; Papa et al., 2004; Bakir et al., 2005; Aradaib et al., 2010; Papa et al., 2011). CCHFV strain (strain AP92) was isolated in 1975 from *Rhipichepahlus bursa* ticks that were collected from goats in Vergina village, northern Greece (Papadopolous *et al.,* 1980). Antibodies against CCHFV were detected in 4 of 64 residents where strain AP92 was isolated but none of them showed any symptoms that resembled CCHF (Antoniadis and Casals, 1982). A sero-survey was conducted that revealed that 1% of a human population had antibodies to CCHFV (Antoniadis *et al.,* 1990).

In Pakistan, CCHF was first reported in Rawalpindi in 1976 and since then number of CCHF cases have been reported in the country (Sheikh et al., 2005). Pakistan is an endemic country and has the fourth highest number of CCHFV infection cases in Asia after Turkey, Russia, and Iran (Ince et al., 2014). A rapid increase in CCHFV positive cases has been observed in Pakistan since 2009 (Leblebiciglu et al., 2015). Cases usually appear between March and May and between August and October. Several outbreaks of the disease have been reported in Pakistan, that spread over a wide geographic area including Baluchistan, Karachi and Rawalpindi that are the most affected regions (Altaf *et al.,* 1998).

There is a need for global burden of diseases to be described but due to limited resources there is difficulty in collecting data regarding the disease. ProMED (The Program for Monitoring Emerging Diseases), an internet-based reporting system, designed for emerging infectious diseases, threatening human beings (Madoff, 2004; Madoff and Woodall, 2005; Ince et al., 2014) helps to prevent people from infections through spread of information including early preventive measures, prevention from epidemiologic outbreaks and provide more accurate disease control. The source of information are media reports, official reports, online summaries and local observers (Madoff, 2004). The CCHFV infections were reviewed and reported by ProMED to assess and check the reliability of the data obtained by comparing with other published reports. Table.1 indicates case fatality rate of the disease using ProMed as source with publications from 1988 to 2013 (Ince *et al. (* 2014).

Table.1: The case fatality rate in different countries from 1998 to 2013 (Ince et al., 2014).

S.No	Country	Infected patients, n	Fatal cases, n	CFRC (%)
1	Turkey	1406	140	10
2	Russia	891	33	4
3	Iran	323	38	12
4	Pakistan	230	92	40
5	Afghanistan	61	16	26
6	Kazakhstan	54	21	39
7	Kosovo	47	11	23
8	India	42	25	60
9	Mauritania	35	6	77
10	Tajikistan	34	28	82
11	South Africa	17	5	29
12	Bulgaria	6	1	17
13	Greece	5	2	40
14	Iraq	2	2	100
15	Namibia	2	0	0
16	United Arab Emirates	5	2	40
17	Uganda	5	5	100
18	Georgia	1	0	0
19	Oman	1	1	100
20	Senegal	1	0	0
21	Zimbabwe	1	1	100
	Total	3426	451	13

Reservoirs

Reservoirs include animals showing no clinical symptoms. These include cattle, livestock, hares, hedgehogs and vertebrates (CFSPH, 2007; Appannanavar and Mishra, 2011).

Vector

There are both biological and mechanical vectors involved in the transmission of CCHFV.

1. Biological Vector

The virus has been found in the tick of *Culicoides spp*., *Argasidae* (also called as soft ticks), *Ixodidae* (hard ticks), >30 species of ticks has been reported to have CCHFV of which *Hyalomma spp* are considered as the major vector involved in human infection (Fig.3), but it also varies depending on the region it is existing in, for example; in Kazakhstan *Dermatocentor nivens* ticks are considered as vectors (Onishchenko et al., 2005). The ticks other than genus *Hyalomma* include a limited number of species of genus *Amblyomma, Rhicpicephalus* and *Dermacentor* as reported by CABI in 2015.

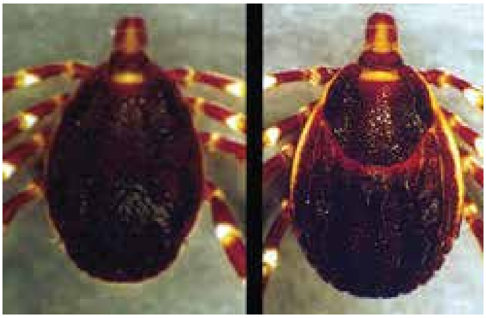
(a) (b)

Fig..3: (a) Male tick (b) female tick. The tick from the genus Hyalomma are the principle vectors involved in the transmission of Crimean-congo virus (WHO, 2013)

2. Mechanical Vectors

Migratory birds, Ungulates and livestock act as mechanical vectors. The ticks attached to the body of the birds are introduced into new areas of unaffected populations (CFSPH, 2015). The Livestock has large number of ticks and so has more potential of spreading the virus as a mechanical vector. The presence of ticks in livestock, is not considered unusual, as a single animal can have 100 ticks e.g., *Hayalomma marginatum*, and thus playing potential role in the transmission of the virus (Estrada-Pena et al., 2012).

Signs and symptoms

CCHFV is characterized by fever and hemorrhage. The infection is difficult to differentiate clinically from other viral hemorrhagic fevers (VHFs) (Leblebicioglu *et al.,* 2016). The major 4 stages of the infection include incubation, pre-hemorrhagic, hemorrhagic and recovery period (Keshtar-Jahromi, 2011). The incubation period is of 3-7 days. It depends on the route of infection, the viral load that has entered into the body and the source of the infection. The blood is the most infectious source of the virus (Appannanavar and Mishra, 2011). 1- 10 organisms are considered as the minimal viral load for the transmission of the disease (Franz et al., 1997). The overall symptoms of the disease include: High fever, myalgia, pain in the abdominal area, headache followed by nausea, diahorrea (without blood). Symptoms such as cutaneous rash, bradycardia and Hypotension are experienced in the pre hemorrhagic phase (Hoogstraal, 1979; Appannanavar and Mishra, 2011).

The hemorrhagic phase is the most severe stage of the disease, it is usually short but rapid and has visibly complicated symptoms like petechiae, epistaxis, hematemesis, melena, hepato- spleeno-megaly (Ergonul et al., 2004; Bakir et al., 2005; Ozkurt et al., 2006). It usually results in the death of the patient due to multiple organ failure and circulatory shock (Sannikova et al., 2007; Doganei et al., 2008). Death occurs in 40-60% of cases.

Transmission

Humans are infected through the bites of ticks, through direct contact with blood or tissues of CCHF patients or viremic livestock (Papa, 2010). The infection can be acquired through the bite of infected tick, by percutaneous and per-mucosal routes, through contact with animal blood or tissues. The aerosol transmission possibility has been under investigation in Russia but up till now, no definite evidence exists (Pshenichnaya and Nanadskaya, 2015). Transmission in workers of agriculture, people working in slaughter houses can easily acquire the infection (Ahtar et al., 2013).

Transmission *via* horizontal and vertical ways can occur in case of CCHFV (Masayuki et al., 2004). Person-to-person transmission and nosocomial transmission of CCHFV was confirmed in 2010 and 2011 when such cases were reported in Ahmedabad, India (Yadav et al., 2014). Human cases of CCHFV most frequently occur among agricultural workers or rural inhabitants after bites from infected ticks (Pshcnichnaya and Nanadskaya, 2015).

Community level transmission in the form of outbreaks has frequently been reported but its nosocomial infection spread through hospital is also life threatening and has been reported in several countries such as Pakistan, India, South Africa, UAE (van Eden et al.,

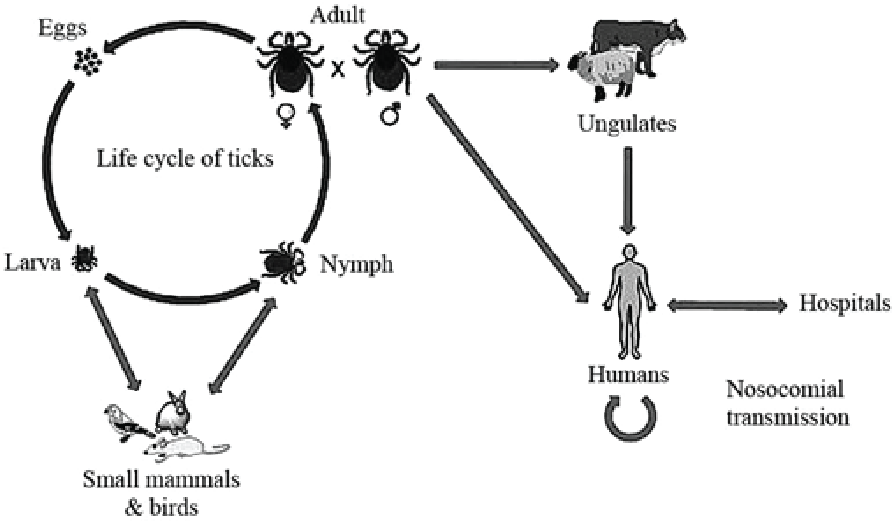

Fig. 4: Transmission and life cycle of tick, in which the larvae after hatching engorge on the small mammals for its nutritional needs. Following molting and converting from nymphs to adults it leaves its primary host and find a large animal to feed on and for reproduction as well. The transmission via ticks involves ticks to animals and humans, nosocomial transmission (from human to other healthy human) (Aslam et al., 2016)

1985; Fisher-Hoch et al., 1995; Mardani, 2001). The CCHF in pregnant patients usually results in abortion or can even cause severe neonatal complications and death. The severity depends on the degree of illness of the mother. The routes of transmission of the virus can be perinatal or intrauterine (Ergonul et al., 2010). Only one study suggests that CCHFV can be transmitted sexually but there is a need for further research on the study of persistence of CCHFV in the body fluids of survivors (Leblebicioglu *et al.,* 2015). Surgical treatment and gastrointestinal bleeding of asymptomatic patients are the most dangerous setups for acquiring CCHFV infection (Shepherd et al., 1985). Fig.4 shows transmission of CCHFV.

Risk Groups

The lack of early diagnosis is a threat for the society and there is a high risk of nosocomial transmission to health care personnel due to splash and needle-stick injuries that may occurred in the absence of adequate personal protective equipment (Seif *et al.,* 2017). Healthcare employees are at risk from occupational infections during patient care The emerging cases of CCHFV in countries lacking CCHFV experience may have problems like infection control challenges, increased risk to healthcare workers and high mortality rate (Pshcnichnaya and Nanadskaya, 2015).

Risk groups include peoples from various walks of life such as Butchers, shepherds, farmers, soldiers, veterinarians, and healthcare workers. CCHFV is a significant public health threat because of high fatality rate, the absence of an FDA-approved vaccine and the potential for human-to-human transmission and nosocomial outbreaks. (Duh et al., 2007).

Diagnosis

Following are some tests that can be conducted to detect the presence of the virus: Enzyme-Linked Immunosorbent assay, Quantitative polymerase chain reaction, (Casals and Tignor, 1974; Swanepoel et al., 1983), Isolation of virus using cell culture and Immuno-histochemical staining. The tests can be conducted by the end of the first day when IgM becomes detectable but produced in a low concentration followed by IgG (Bente et al., 2013).

Other tests that can be used for the detection includes serology are; Complement fixation test, hemagglutination inhibition, Reverse passive hemagglutination inhibition, Indirect Immmuno-florescence, IgM antibody captured (MAC) ELISA (Saijo et al., 2002; Charell et al., 2004; Saijo et al., 2005), Recombinant nucleoprotein (rNP)-based IgG ELISA and Recombinant nucleoprotein (rNP)-based IgM ELISA (Shepherd et al., 1989; Vanhomwegen et al., 2012; WHO, 2013).

The virus can be isolated using cell cultures such as MK2, Vero, BHK-21, and SW-13.4. There are little to no cytopathic effects produced hence Immuno-fluorescence can be used in order to detect the presence of virus using specified monoclonal antibodies. The culturing can be done only in the early phase of the infection and usually takes 2-5 days to produce its results. Biosafety level 4 facilities should be available in order to use the cell line culturing method (Appannnanavar and Mishra, 2011). RT-PCR can be used and is considered as a method of choice for rapid diagnosis. It is better than cell culturing as it saves time and can diagnose within 8 hours. Real Time PCR has additional advantages as it has low rate of contamination, has high specificity, sensitivity and can provide results within few minutes thus saving a lot of time and effort. One step real time RT-PCR was developed by Drosten et al., (2003) for the detection of CCHFV.

Treatment

There is no drug approved by FDA for CCHFV, hence supportive therapy is recommended. The treatment varies with the level and phase of the infection (Oncu, 2013). However, use of Virazole, a ribavirin has been reported to be effective, but only in a few cases (Buttigieg et al., 2014). WHO approved ribavirin based on its in vitro studies (Soares et al., 2010; Ascioglu et al., 2011; Oncu, 2013; Burt and Goedhals, 2015). It can be administered both orally as well as intravenously. Interferons as supportive therapy along with the antiviral are administered for the best results (Appannnanavar and Mishra, 2011). A new immunoglobulin “Venin” has been prepared from the plasma pool of boosted donors which is specific for CCHFV, but the beneficial effects of immunotherapy are very limited (Whitehouse, 2004; Flick and Whitehouse, 2005).

The diverse genetic variation among the strains of CCHFV contributes to be the main obstacle in development of vaccines. The use of inactivated vaccine from brain of mouse has been reported to be administered in former Soviet Union and Bulgaria, but it remains unavailable in most of the countries (Ahmed et al., 2005; Papa et al., 2011).

Prophylaxis/ Preventive measures

Prevention strategies can help reduce the risk of disease. Some strategies include:

- Prevention should be acquired by everyone at both community as well as nosocomial level.
- Appropriate standard precautions should be used in areas having endemics in order to prevent any sort of secondary transmission (Tarantola et al., 2017).
- Special care must be needed at the time of active season of ticks (Aslam et al., 2016).
- Minimization of vertebrate host infections should be done by proper screening and routine examination for presence of ticks responsible to cause the disease.
- Use of unpasteurized milk and raw or uncooked meat should be avoided (Appannanavar and Mishra et al., 2011).
- Workers in the Lab should follow Strict Biosafety precautions and working with such disease requires Bio Safety level-4 setup. Personal protection should be taken against tick bites. Avoid exposure to infected human or animal/livestock.
- In regions of endemics, people should avoid active tick abundant areas, to avoid any chance of infection. Covering of arms and legs is recommended and advised along with the use of light colored clothing to easily identify the tick.
- Application of tick repellent such as

permethrin and di-ethyltoluamide are also effective (CDC, 1995; WHO, 2001; Zavitsanou et al., 2009).

- Use of chemicals that can kill the ticks in livestock production area to avoid any livestock production infection. Acarides are used for such purpose.
- People working with livestock can take practical measures such as clothing, use of gloves, googles to avoid any exposure to the tissue or blood of the livestock (Chin, 2000; WHO, 2001; Zavitsanou et al., 2009).
- The human individual diagnosed or suspected to have infection of CCHFV should be quarantined, and use of gloves, shoe covers, googles, and other barrier-nursing techniques should be adopted. Only staff specialized for the care and nursing should be allowed to deal with the patient.
- The medical instruments and materials should be autoclaved before incineration (LIoyd and Perry, 1998; NIH, 2002). Liquid bleach should be used to decontaminate the surfaces and the sample collecting jar/ bottles should be made decontaminated by applying liquid bleach on the outer surface and use of triple container packing and the sample should be labelled properly. Prevention of transmission in the nosocomial setting can thus be avoided (CDC, 1990; Zavitsanou et al., 2009).
- In case of the death of the patient used of 1:10 bleach solution is recommended and the body should be covered in a plastic bag with an adhesive taping to avoid its contact with the soil (CDC, 1998).
- Use of ribavirin is recommended incase, a person gets exposed to the blood or secretions including bodily fluids of the infected person. For example, it was reported that a health care worker acquired a needle-stick injury and was provided with prophylactic ribavirin did not develop the disease (Smego et al, 2004; Zavitsanou et al., 2009). All these preventive measures can effectively help in avoiding the disease.

CONCLUSION

Although Crimean Congo Hemorrhagic fever, a zoonotic tick-borne infection that can affect a large number of animals specially causing lethality in human beings, was discovered in 12th Century, yet it is hard to cure because of its high degree of genetic variation that makes it a potentially lethal virus. Proper vaccine is still not available for the virus. The application of supportive treatment and precautionary measures are the essential tools for the control and prevention of the disease. Awareness is being developed all around the world about the disease but a lot of work still needs to be done to completely understand the nature of virus and it ways of infection, so as to alleviate the burden of the disease and for its complete eradication.

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Analysis of Human Beta Globin Gene and its Sequence Variants

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ABSTRACT: *Hemoglobin is a protein commonly found in in red blood cells of all vertebrates and invertebrates. It consists of two chains, mainly alpha and beta chain. The globin genes are used as a model system to study gene expression in various eukaryotic organisms. Moreover, these genes controls the gene regulation process. Mutations in these genes can cause destruction in various regulatory pathways encoded by beta globin genes. The introns in beta globin genes mostly interrupts the sequence between codons 30-31 and between codons 104-105. The study involves a comparative genomics approach for identification and analysis of selected human beta globin gene (HBB) by using various computational tools. The nucleotide sequence of HBB gene was obtained from Genbank-NCBI and analyzed to study the transcripts within the coding region of gene by using ensemble software. These transcript maps will help to understand the regulatory features of genes, splice variants and putative indels in the reading frame. Also, the protein encoded by coding region of HBB gene was further analyzed to study various protein domains, profiles and signatures. This was done by using Interpro and CDD domain analysis software. The results are presented along with the proposed utilization of the studied gene. Comparative approaches are not only useful for evolutionary analysis but also can help to understand the function of various conserved genes.*

Keywords: genomics, domain, transcripts, signatures

INTRODUCTION

Hemoglobin is the important iron containing protein in the red blood cells of all vertebrates and invertebrates. It was initially discovered in red blood cells of mammals. The hemoglobin molecule is a heterotetramer composed of two α-globin and two β-globin polypeptides in adults. It was found that each group is associated with heme group. The globin gene can be used as a model system to study gene expression in eukaryotes. Most of the studies related to thalassemia are associated with these genes. They play an important role in gene regulation and development processes. Up till now, great advances have been made for structural analysis of this gene (Borg et al., 2010; Lodish and Jacobsen, 1972).

Globin genes exists in different forms. Among all, five of them which are located on different chromosomes are well characterized. The HBA1 and HBA2 was found at chromosomal position 16p13.3. Whereas, HBB was located at 11p15.4. All of the genes consist of at least three exons. Each exon is further separated by two introns. All introns differ in size and found closely to each other. Mostly, the genes with conserved introns can easily shuffle the exons during evolution of various proteins (Doolittle, 1972). Moreover, the introns differ in globin genes in some vertebrates which has no effect or change on vertebrate evolution (Hardison, 1998). The human β-globin gene cluster consists of five genes arranged in chromosome 11 (Lawn et al., 1978). The gene expression can be regulated particularly in a way, that alpha and beta chains can be regulated equally. Many modifications like transcriptional, posttranscriptional, and posttranslational are controlled by single β-globin gene (Smithies et al., 1978).

All genes which are involved in regulation are not active or functional every time. For activation, the transcriptional machinery will arrange on its promoter. This happens even if the gene is defective and unable to produce any protein. β-globin gene synthesis is decreased if there is any defect in β-globin gene. Studies shown expression of α and β globin genes is regulated differently in cells. A balanced production of α-globin and β-globin in erythroid cells is required for the efficient formation of hemoglobin (Weatherall and Clegg, 2008). The type of expression and time of occurrence is different in all genes encoding alpha and beta chains of globin genes. The evolutio nary pathways of globin genes have undergone multiple conversion events during mamma lian evolutionary process (Song et al., 2011; Song et al., 2012). Also, the expression patterns of all globin are different for specific lineages (Hoffmann et al., 2008).

In addition, the hemoglobins produced at various stages have different affinities for oxygen and are subject to complex regulation by certain cofactors. The importance of hemoglobin in oxygen transport through blood vessels is perhaps a real support for oxidative metabolism and regulatory processes. However, the globin polypeptides can bind heme leading to successful binding of hemoglobin to oxygen by transporting it from lungs to respiring tissues

The study involves analysis of HBB gene, its transcripts, protein domains and genetic variants found in different species. This was done by using various bioinformatics tools and software's. The predicted domains in protein encoded by HBB gene can be further studied to analyze the 3D structure and function of protein.

MATERIALS AND METHODS

Several computational tools was used for identification and analysis of human HBB gene.

Sequence Retrieval

NCBI's is a useful data repository with tremendous sets of various genes with homologs, orthologues, protein domains and gene expression data. The nucleotide sequence of HBB gene was retrieved from NCBI-Genbank data repository and translated to get a protein sequence.

Analysis of Chromosome Map

Chromosome map was obtained for HBB gene from ensemble software. The map shows the exact chromosomal location of HBB at chromosome 11.

Transcript Identification

The transcript map of HBB gene was analyzed by using ensemble software. Transcript mapping can help to conquer genes with identified locus. The transcripts on total genomic regions are analyzed with annotated gene regions and splice variants.

Analysis of Splice Variants for Identification of Protein Domains

The transcript map for HBB gene was analyzed for identification of putative domains and protein signatures within the protein sequence. The Conserved Domain Database (CDD_NCBI) is a resource for the annotation of functional units in proteins. Study of these domain models will help to predict 3D structure and function of protein. Identification of protein domains is an important step for better understanding of protein function. The HBB proteins were analyzed by using InterPro protein domain analysis software.

RESULTS

The coding region of human HBB gene was obtained from NCBI. The complete sequence was 73308 bp in size. The coding region of gene was 1430bp in size. The highlighted area shows the coding region of HBB gene (Fig. 1).

```
ATGGTGCATTTTACTGCTGAGGAGAAGGCTGCCGTCACTAGCCTGTGGAGCAAGATGAATGTGGAAGAGGC
TGGAGGTGAAGCCTTGGGCAGGTAAGCATTGGTTCTCAATGCATGGGAATGAAGGGTGAATATTACCCTAG
CAAGTTGTTGGGAAAGTCCTCAAGATTTTTTGCATCTCTAATTTTGTATCTGATATGGTGTCATTTCATAGAC
TCCTCGTTGTTTACTGGACCCAGAGATTTTTTGACAGCTTTGGAAACCTGTCGTCTCCCTCTGCCATCCTGG
GCAACCCCAAGGTCAAGGGCAAGAAGGTGCTGACTTCCTTTGGAGATGCTATTAAAAACATGGACAACCTC
AAGCCCGCCTTTGCTAAGCTGAGTGAGCTGCACTGTGACAAGCTGCATGTGGATCCTGAGAACTTCAAGGT
GAGTTCAGGTGCTGGTGATGTGATTTTTTGGCTTTATATTTTGACATTAATTGAAGCTCATAATCTTATTGGA
AAGACCAACAAAGATCTCAGAAATCATGGGTCGAGCTTGATGTTAGAACAGCAGACTTCTAGTGAGCATA
ACCAAAACTTACATGATTCAGAACTAGTGACAGTAAAGGACTACTAACAGCCTGAATTGGCTTAACTTTTC
AGGAAATCTTGCCAGAACTTGATGTGTTTATCCCAGAGAATTGTATTATAGAATTGTAGACTTGTGAAAGA
AGAATGAAATTTGGCTTTTGGTAGATGAAAGTCCATTTCAAGGAAATAGAAATGCCTTATTTTATGTGGGTC
ATGATAATTGAGGTTTAGAAGAGATTTTTGCAAAAAAAAATAAAAGATTTGCTCAAAGAAAAATAAGACAC
ATTTTCTAAAATATGTTAAATTTCCCATCAGTATTGTGACCAAGTGAAGGCTTGTTTCCGAATTTGTTGGGG
ATTTTAAACTCCCGCTGAGAACTCTTGCAGCACTCACATTCTACATTTACAAAAATTAGACAATTGCTTAAA
GAAAAACAGGGAGAGAGGGAACCCAATAATACTGGTAAAATGGGGAAGGGGGTGAGGGTGTAGGTAGGT
AGAATGTTGAATGTAGGGCTCATAGAATAAAATTGAACCTAAGCTCATCTGAATTTTTTGGGTGGGCACAA
ACCTTGGAACAGTTTGAGGTCAGGGTTGTCTAGGAATGTAGGTATAAAGCCGTTTTTGTTTGTTTGTTTGTTT
TTTCATCAAGTTGTTTTCGGAAACTTCTACTCAACATGCCTGTGTGTTATTTTGTCTTTTGCCTAACAGCTCC
TGGGTAACGTGATGGTGATTATTCTGGCTACTCACTTTGGCAAGGAGTTCACCCCTGAAGTGCAGGCTGCCT
GGCAGAAGCTGGTGTCTGCTGTCGCCATTGCCCTGGCCCATAAGTACCACTGAGTTCTCTTC
```

Fig. 1: Shows the nucleotide sequence of HBB gene obtained from NCBI. The coding regions are highlighted above.

Translated Sequence of HBB gene

The HBB gene coding region was translated to obtain a protein sequence. The translated sequence was analyzed to check the protein encoded by particular gene. The proteins expressed by HBB gene was importantly involved in various functions with specific features and also to control different expression levels. The translated protein sequence is shown in Fig. 2.

"MVHFTAEEKAAVTSLWSKMNVEEAGGEALGRLLVVYPWTQRFFDSFGNLSSPSAILGN
PKVKAHGKKVLTSFGDAIKNMDNLKPAFAKLSELHCDKLHVDPENFKLLGNVMVIILAT
HFGKEFTPEVQAAWQKLVSAVAIALAHKYH"

Fig. 2. Shows the translated protein sequence of HBB gene starting with a start codon.

Chromosome Map

The chromosomal map was obtained from ensemble software. The HBB gene was located on chromosome number 11. The map is given below in Fig. 3.

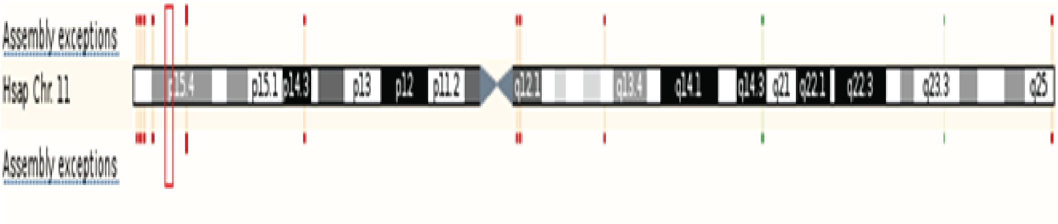


Fig. 3. Shows the chromosomal map of HBB gene located on chromosome 11.

Transcript Analysis

The transcripts covered by HBB gene were analyzed by using transcript analysis software. Five different types of software's were found. Each with different size. Two of them were protein coding only. These are given in the Table 1 below.

Table 1: HBB Transcripts obtained from ensemble.

Sr. No.	Name	Transcript	Protein	Size	Type
1.	HBB-001	ENST00000335295	147aa	628	Protein Coding
2.	HBB-004	ENST00000380315	90aa	502	Protein Coding
3.	HBB-005	ENST00000633227	55aa	609	Nonsense mediated decay
4.	HBB-002	ENST00000485743	No Protein	680	Retained intron
5.	HBB-003	ENST00000475226	No Protein	319	Retained intron

Analysis of Splice Variants

Splice variants lying within HBB gene was analyzed. All of them was associated with different protein profiles and domains. The conserved regions was compared from different protein databases. The protein signatures and profiles were also compared which shows evolutionary relatedness. The protein profiles from three different databases namely, Pfam, PROSITE and Prints were showed maximum matches with little variation. The results are shown in Fig. 4.

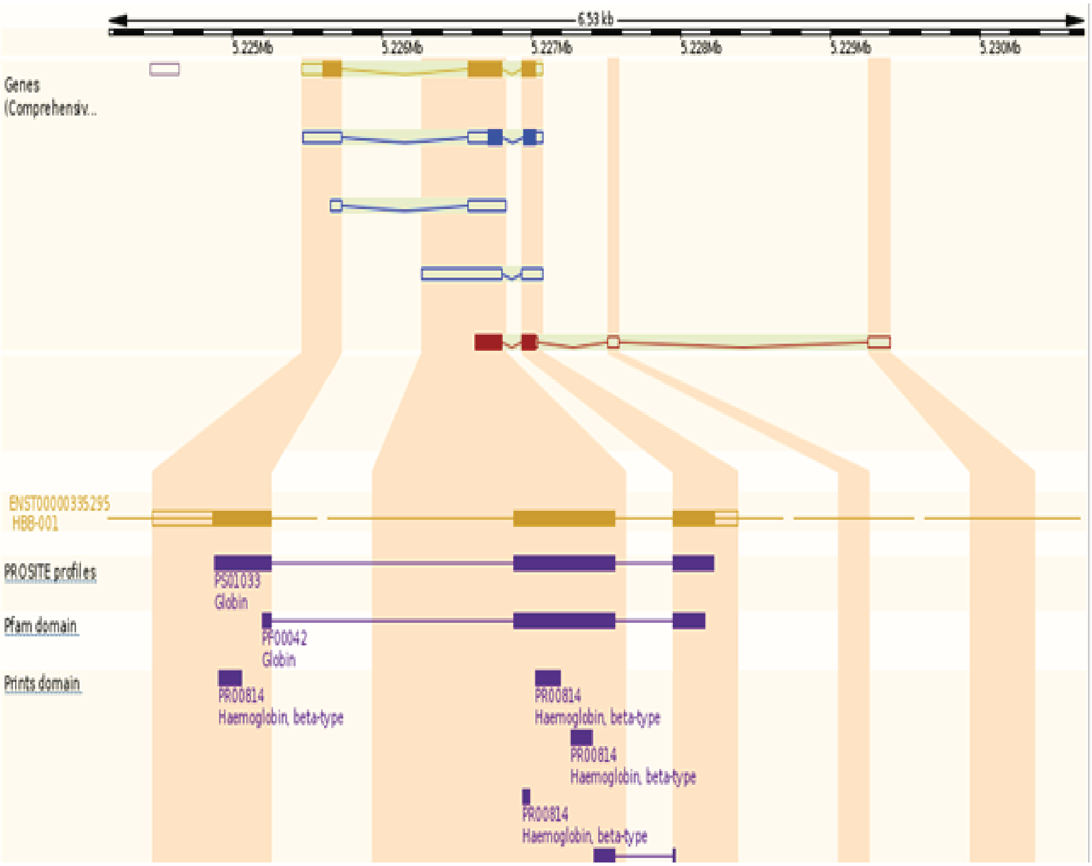


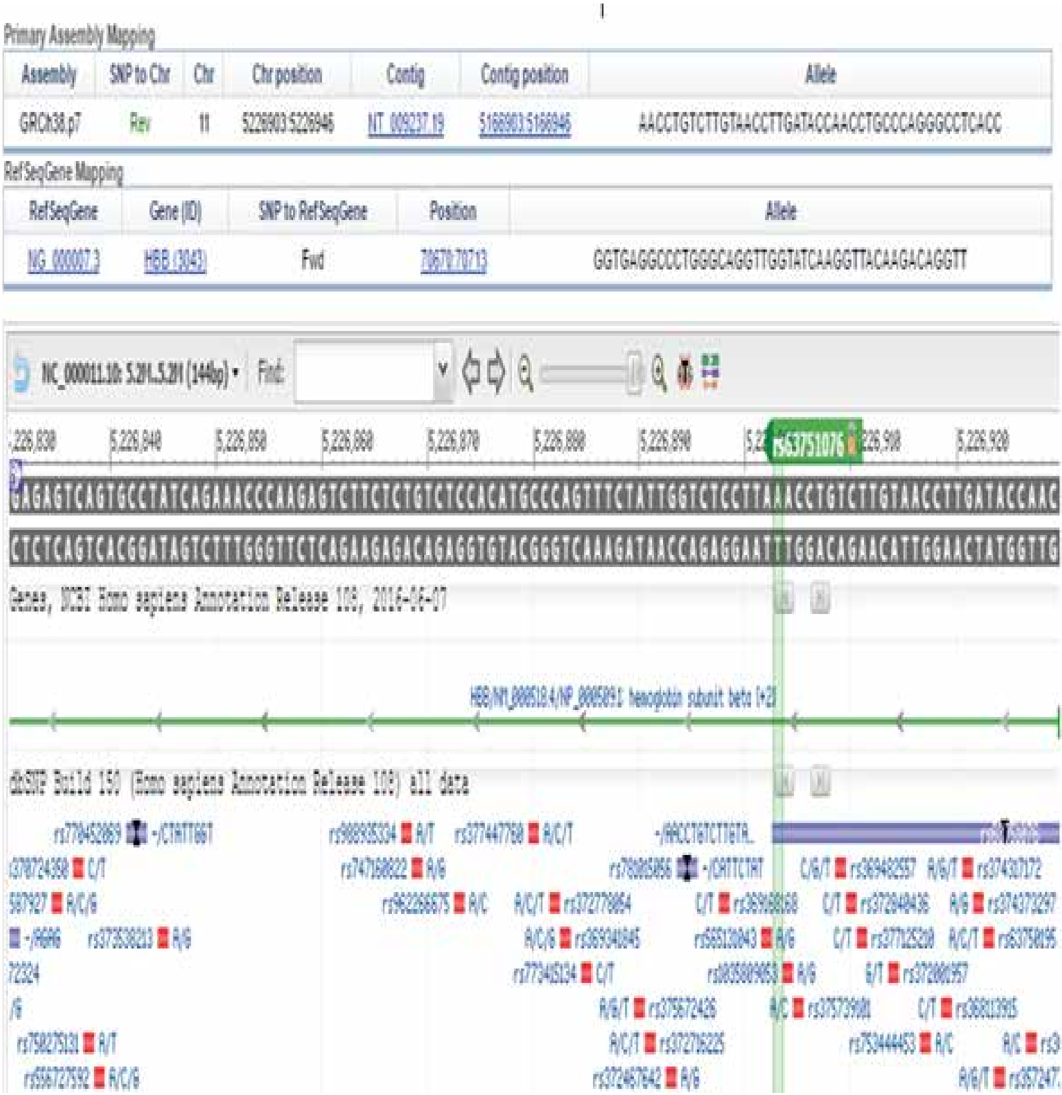
Fig. 4. Shows HBB Protein profiles and signature with unique identity.

Reported SNP within 100 bases of HBB gene

The genetic variations of HBB gene showed various SNP's within the coding region of gene. All reorted SNP were having insertions/deletions. The position of contig specific to its allele was verified by contig map by using dbSNP variation viewer. The results are shown in Table 2 and Fig 5.

Table 2: SNP for rs63751076 (within 100 bases)

Distance (Bases)	rs	Map Weight	Contig Accession	Contig Position
-100	rs750005117	1	NT_009237.19	5166803
-100	rs755025590	1	NT_009237.19	5166803
-97	rs779006439	1	NT_009237.19	5166806
-96	rs752631303	1	NT_009237.19	5166807
-95	rs758422235	1	NT_009237.19	5166808
-95	rs768952155	1	NT_009237.19	5166808
-90	rs777373323	1	NT_009237.19	5166813
-89	rs35456885	1	NT_009237.19	5166814



Primary Assembly Mapping

Assembly	SNP to Chr	Chr	Chr position	Contig	Contig position	Allele
GRCh38.p7	Rev	11	5226903:5226946	NT_009237.19	5166903:5166946	AACCTGTCTTGTAACCTTGATACCAACCTGCCCAGGGCCTCACC

RefSeqGene Mapping

RefSeqGene	Gene (ID)	SNP to RefSeqGene	Position	Allele
NG_000007.3	HBB (3043)	Fwd	70670:70713	GGTGAGGCCCTGGGCAGGTTGGTATCAAGGTTACAAGACAGGTT

Fig. 5. Shows position of rs63751076 located in intron region of HBB gene by Gene view contig annotation system.

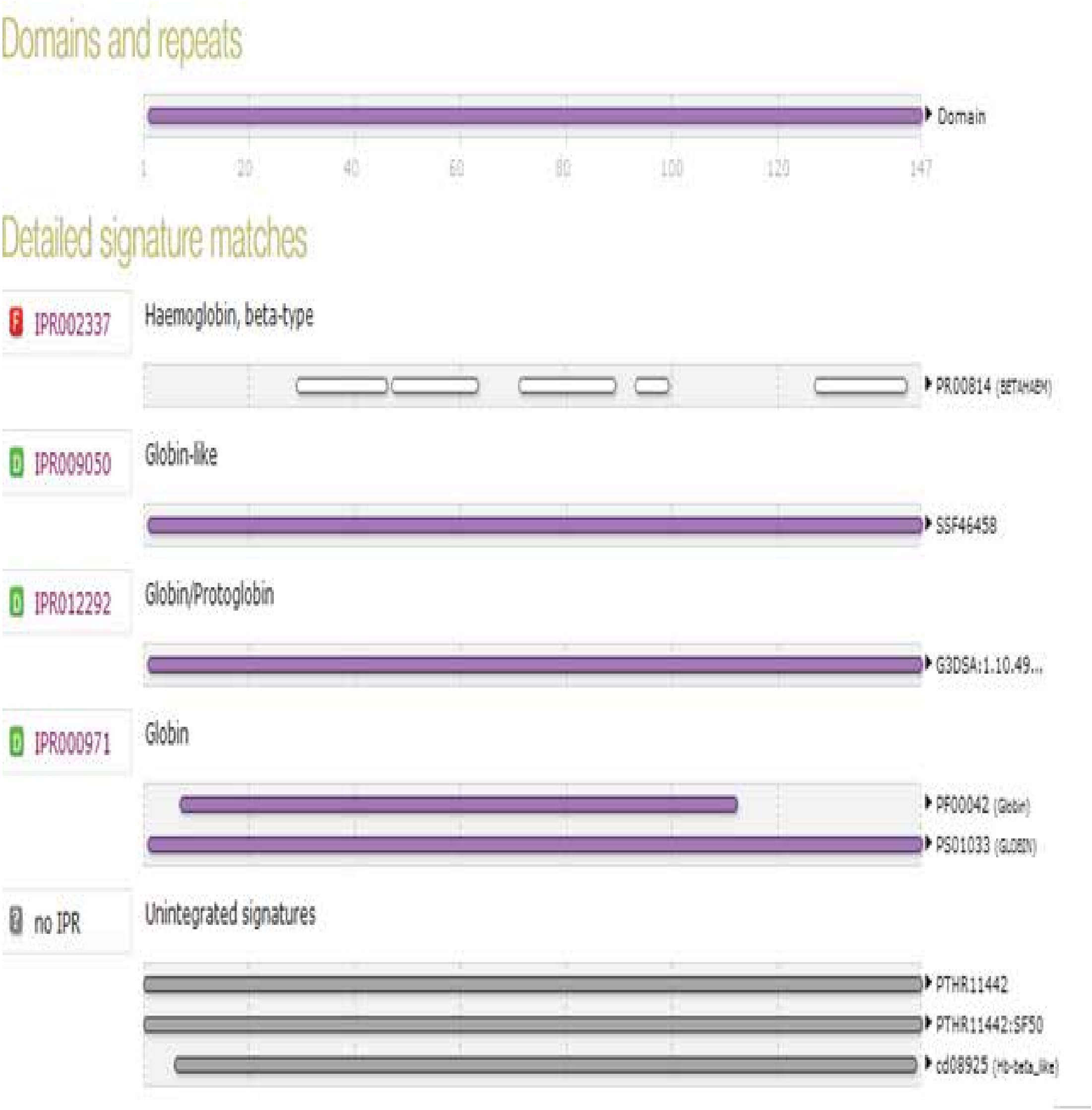


Fig.6. Shows the protein signatures obtained through InterPro database

Analysis of Protein Domains and Signatures

Domains are highly conserved regions of proteins. They can be used to study protein functions. Also, structure prediction can be performed by analyzing various domains. The HBB protein was analyzed for identification of conserved regions by using InterPro database. The conserved regions for globin chain of HBB protein were found with little variation at site PF00042-PSO01033. The results are shown in figure 6.

DISCUSSION

Hemoglobins are commonly found in many organisms like protozoa, the cyanobacteria and the green alga (Gardner et al., 1998; Iwaasa et al., 1989; Takagi et al., 1993; Thorsteinsson et al., 1996; Kaneko et al., 1996). Similarly, family of bacterial and yeast hemoglobin shows various unique characteristics (Couture et al., 1994; Dewilde et al., 1996). The hemoglobin is a hetero tetramer made up of two α-globin and two β-globin polypeptides, with an associated heme group. Both of these are encoded by the duplicated HBA1 and HBA2 genes and by the specific HBB gene. However, hemoglobins are produced only in erythroid cells, where they act as a major and essential protein. A number of different variants of hemoglobin were discovered resulting in multiple transcripts (Burmester et al., 2002; Trent and Hargrove, 2002). All behave and expression in a different fashion. Some exhibit only tissue specific expression pattern. However, the most distantly related globin found in the human genome is named as neuroglobin, encoded by NGB (Burmester et al., 2001). It was abundant in many tissues but mainly expressed in brain tissues.

Previous studies showed, the existence of five types of globin genes located on five different chromosomes: HBA1 and HBA2 at chromosomal position 16p13.3, HBB at 11p15.4, MB at 22q12.3, CYGB at 17q25.1, and NGB at 14q24.3. MB, CYGB, and NGB. These genes exists as single copy genes a compare with HBB gene which occurs in clusters with multiple genes. All of the genes consist of at least three exons separated by two introns. Presence of genes of any specific genome within its genomic region can be useful to find some flanking gene clusters for HBB gene (Flint et al., 2001; Gillemans et al., 2003). In addition, several beta globin genes were found to be linked with alpha globin genes at defined locus (Fuchs et al., 2006; Hellsten et al., 2010).

Up till now, more than 730 variants of the β-globin chain have been characterized (Weatherall and Clegg, 2001). These variants exhibits range of different types of mutations not only limited to point mutations but also includes large deletions. Such mutations can affects synthesis of HBB gene, its transcription, translation and protein structure (Patrinos et al., 2004). The levels of expression of globin gene in different reactions controlled by promoters is found very low. The promoters regulates the activity of HBB gene in humans and other organisms. The activity depends upon level of regulation and modifications. A number of different mutations were detected in HBB gene. These mutations are categorized as insertions, deletions and frameshifts. A bioinformatics approach for sequence analysis can be used for identification of genetic variations with mutations.

CONCLUSION

The HBB gene analysis showed various functions occurring at different times during regulation and process of development. The gene transcripts showed different patterns, signatures and protein profiles. All profiles covers the maximum area of HBB gene with number of introns and splice variants. The analysis predicted several conserved regions with integrated signatures of globin chain. These regions are covered and enriched with various beta globin chains. The transcripts of HBB gene found in different organism were not all protein coding. In spite of the fact, only few of them were protein coding. Hence, analysis of HBB gene performed by these computational tools was found useful to study the gene expression and regulation process. Also, to study the globin chain at macro level for 3D structure prediction of hemoglobin beta type genes.

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A Chemical Approach Against *Aspergillus paraciticus* and Aflatoxins Production for Feed Safety During Storage

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ABSTRACT: *The anti-aflatoxigenic activity of citric acid, acetic acid, ascorbic acid and sodium bicarbonate were investigated in Aspergillus parasiticus inoculated broiler feed. The commercial broiler feed was treated with these synthetic additives (1%, 3% and 5% w/w) and stored for the period of six months at ambient conditions of 16% moisture and 28^{o}C. Aflatoxins (B1, B2, G1 and G2) levels were analyzed at the end of each month by HPLC technique and compared with control. Sodium bicarbonate (1%) inhibited aflatoxins production up to 100% during six months of storage, whereas ascorbic acid (5%) inhibited aflatoxins production up to 4 months. Results revealed that Sodium bicarbonate is a potential source of anti-aflatoxigenic activity, which could be employed to inhibit the aflatoxins production of feed and other commodities during storage.*

Keywords: Aflatoxins, Aspergillus parasiticus, broiler feed, inhibition, sodium bicarbonate, ascorbic acid, citric acid.

INTRODUCTION

Aflatoxins are toxic metabolites which are especially produced by certain fungal strains like *Aspergillus flavus* and *Aspergillus parasiticus*. As fungal strains are distributed worldwide and can produce toxins under favorable environmental conditions (Hassan et al., 2017). Various types of aflatoxins are well known, but AFB1, AFB2, AFG1, AFG2, AFM1 and AFM2 are the most prominent among them (Krishnamurthy and Shashikala, 2006). AFB1 is the most toxic type followed by AFG1 > AFB2 > AFG2 (Aliabadi et al., 2013). As Aflatoxins (B1, B2, G1 and G2) are sorted out as Group I human carcinogens whereas M1 is categorized as Group 2B human carcinogen (Krishnamurthy and Shashikala, 2006).

Aflatoxins have been detected in various agricultural commodities like oil seeds, cheese, cereal grains, fruit juice, milk, meat, nut products (Shirurkar et al., 2012). Aflatoxins destroy the nutritive value of food and feed during storage. So, it is most important to protect food and feed products from aflatoxins production (Hassan et al.

2017). To overcome this scenario, various strat egies such as physical, chemical and biological have been established to convert the toxic materials into less toxic products with less mutagenic effects (Safara et al., 2010). The use of chemicals is a well-known strategy to prevent fungal growth in poultry feed during storage. Different organic acids and salts of organic acids are considered as more promising synthetic additives to prevent mould growth in feed (Akande et al., 2006; Salgado et al., 2011).

As aflatoxins are found to be unavoidable contaminants of poultry feed products. Keeping this in view, the present research work was designed to elucidate the potential of nontoxic inorganic salts and organic acids to prevent the growth of *Aspergillus paraciticus* as well as effect of these chemicals to control aflatoxins build up in during storage.

MATERIALS AND METHODS

Collection of synthetic additives

All chemicals and reagents used in research work were of analytical grade and were procured from Merck (Darmstadt, Germany). Synthetic additives (chemicals) like citric acid, acetic acid, ascorbic acid and sodium bicarbonate were also of analytical grade. Standards of aflatoxins were of Supelco (Bellefonte, PA, USA).

Inoculum preparation

The aflatoxins producing fungal strain of *Aspergillus paraciticus* was isolated from grains of wheat and corn. Then the *A. paraciticus* was again cultured on potato dextrose agar slant (PDA) (Oxoid, UK) and incubated at 28^{o}C for 7 days. The spores were harvested in sterilized distilled water with 0.1% Tween 80. The suspension was further diluted to adjust spore density at 10^{12} spores/mL. Spore suspension was preserved at 4^{o}C.

Effect of chemical additives on aflatoxins production in broiler feed during storage

Collection of broiler feed samples/ pretreatment

Freshly prepared antitoxin free broiler feed samples (3 Kg) were taken from Punjab Feed Corporation, Lahore road, Sheikhupura. The broiler feed was dried at 40-70^{o}C in an oven. The broiler feed was divided into 13 lots separately, each lot having 200g. Each feed sample was autoclaved to kill microorganism and was packed under laminar air flow in an air tight plastic bag aseptically. The broiler feed samples were moistened (16%) with sterilized distilled water and mixed thoroughly by shaking. The inoculation of broiler feed samples was done by adding 4 mL of spore suspension (10^{12} spores/mL) of *A. paraciticus* to each bag separately under laminar air flow chamber.

Stabilization of broiler feed with chemical additives

Each chemical additive (1%, 3% and 5%) was added to inoculated broiler feed samples and were mixed properly by shaking thoroughly. Untreated broiler feed sample (without chemical additive) was taken as control. The controlled and treated feed samples at 16% moisture were stored at 28^{o}C for a period of 6 months. The analysis of aflatoxins was carried out by HPLC at regular interval of time of one month.

Analysis of aflatoxins

Extraction of Aflatoxins

Aflatoxins from broiler feed sample were extracted by following the procedure of Beltran et al. (2011) with slight modifications. 5 g broiler feed sample was taken in conical flask and was mixed with 20 mL of acetonitrile:water (84:16). Then the mixture was shaked in an orbital shaker for 90 min at ambient conditions (average temperature 30 °C). Whatman filter paper No. 4 was used to filter the extract and filtrate obtained was concentrated by evaporation at 50°C to final volume of 2-5 mL under reduced pressure.

Quantitative analysis by High Performance Liquid Chromatography

Clean-Up

2-5 mL of concentrated sample was diluted with 20 mL of deionized water and was passed through Vicam (waters) Aflatest WB immunoaffinity column at a flow rate of 2 mL min−1 with the help of suction pump. Then the immunoaffinity column was further washed with deionized water (20 mL) and was dried by air streaming. The retained aflatoxins in column were eluted by passing 2 mL of methanol. The samples were dried under N2 blanketing.

Derivatization

To perform the pre-column derivatization of aflatoxins, n-hexane (200 µL) was added to the tested samples and then 50µL Trifluoro acetic acid was also added and mixed by vortex for 30s and 1.95 µL of water:acetonitrile (9:1) was added. Finally, the sample mixture was vortexed for 20s and finally analysed by high performance liquid chromatography (HPLC). First of all, the instrument was validated to see its accuracy, precision and limit of detection. Calibration curve was drawn using a series of calibration solutions of aflatoxins (B1, B2, G1 and G2) in acetonitrile with concentration of 0.05, 0.1, 0.5, 1.0, 5.0, 10 µg L^{-1}.The resolution pattern of aflatoxins was assayed by reverse phase HPLC column. Aflatoxins content was expressed in terms of ppb and aflatoxin inhibition was calculated by using the following formula; Percentage of inhibition= [Y-X/Y] × 100

Where "X" is the concentration of aflatoxins in treated samples and "Y" is the concentration of aflatoxins in control.

Conditions for HPLC Analysis

Quantitative estimation of aflatoxins was performed on an HPLC apparatus (ProminanceTM, Shimadzu®, Kyoto, Japan) having Shimadzu LC software package, Mediterranae Sea 18® column (5 mm; 25 × 0.46 cm) Serial No. N45074, LC-20AT® pump and CTO-20A® column oven. An isocratic system with methanol, acetonitrile and water (22.5:22.5:55) was used. The separation was performed at a flow rate of 1 mL min−1. Injection volume was 20 µL. The spectrofluorometer detector was operated at excitation wavelength and emission wavelength of 360 nm and 440 nm respectively.

Statistical analysis

All data from three independent replicate trials was subjected to statistical analysis using Minitab 2000 Version 13.2 statistical software (Minitab Inc. Pennsylvania, U.S.A) at 5% significant level and reported as means ± standard deviations. Analysis of variance was performed using ANOVA procedures.

RESULTS AND DISCUSSION

Inhibitory effect of chemical additives against aflatoxin synthesis:

Potential of various chemical additives to prevent aflatoxins (B1, B2, G1 and G2) synthesis in broiler feed produced by *A. paraciticus* over a storage period of six months is given in Tables 1-6. Results demonstrated that about all the additives possessed antiaflatoxigenic activity against *Aspergillus paraciticus* but with varying potential. All the treatments revealed variable inhibitory potential on aflatoxin biosynthesis over control in concentration dependent manner. By comparing the antiaflatoxigenic potential of all additives applied; sodium bicarbonate showed more promising results (even at concentration of 1%) in controlling fungal growth and significantly ($p< 0.05$) prevented the aflatoxins (B1, B2, G1 and G2) biosynthesis upto 100% during complete storage period of six months.

Among remaining additives, ascorbic acid (5%) was also found to be comparatively better inhibitor (100%) of aflatoxins (B1, B2, G1 and G2) upto 4th month of storage. On the other hand, acetic acid at 1% of concentration inhibited aflatoxin production (100%) upto 3 months. However, citric acid (5%) can inhibit the aflatoxins production for only two months of storage.

Overall results showed that all the organic acids and inorganic salts were found effective in inhibiting the aflatoxins (B1, B2, G1 and G2) production as compared to control; however, the antiaflatoxigenic activity of these chemical additives was different at different concentration.

It is reported that the intake of contaminated feed is very harmful for animals as mycotoxins are present in feed. The contamination of animal feed, food products and agricultural commodities with aflatoxins is a serious worldwide issue. Animal feed samples (243) from manufacturers companies revealed that 81% samples were contaminated with aflatoxins and 58% samples showed the aflatoxins levels beyond the permissible limits. Furthermore, 85% samples exhibited positive results from urban dairy farms and among which 70.50% samples showed aflatoxins levels beyond the permisile level. Likewise, feed samples from agrochemical shops also showed positive results and aflatoxins were present in 87% samples and in 60% samples aflatoxins level was found to be above the acceptable limit set by FAO/WHO (FAO/WHO, 1992; Kangethe and Langa, 2009). A report from EU countries showed that feed raw materials and feed products from Poland, Portugal, Eastern Europe, Slovakia, Germany, Southern Europe, Italy, Greece, Spain, Croatia, , Bulgaria, Czech Republic, Switzerland and Romania (Streit et al., 2012). Results of present study demonstrated that synthetic additives under investigation are able to inhibit the aflatoxins contamination and can be used to control contamination of feed during storage. On the other hand, the chemical-control is an economic technique and these findings support the results of present investigation that synthetic additives could possibly be used as antifungal agents (Palou et al. 2001; Alvindia et al. 2004).

CONCLUSION

This study accomplished the objectives outlined in the introduction that organic acids and inorganic salts are effective against *A. paraciticus*. Moreover, they are safe for consumption and these non-toxic chemicals are also effective during storage of broiler feed up to six months. In summary, sodium bicarbonate can be used in minimum quantity to protect stored broiler feed from fungal growth. These non-toxic and edible additives can be used as easily applicable and cost effective strategy to store the animal feed and other commodities in developing countries.

Table 1: Aflatoxin content (ppb) in broiler feed treated with chemicals at incubation period of 1st month.

Treatment	Conc. (g/100gm)	*A. paraciticus* ^{Y}			
		B1 ^{a}	B2 ^{c}	G1 ^{b}	G2 ^{d}
Control	00	4.21±0.06	1.23±0.04	2.14±0.01	0.29±0.02
Citric acid^{B}	01	1.64±0.06 (61.1)	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)
	03	1.33±0.03 (68.4)	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)
	05	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)
Ascorbic acid^{A}	01	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)
	03	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)
	05	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)
Acetic acid^{A}	01	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)
	03	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)
	05	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)
Sodium bicarbonate^{A}	01	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)
	03	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)
	05	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)

Values are mean ± SD of three samples analysed individually in triplicate at $p<0.05$. Superscript small alphabets within the row indicated significant difference ($p<0.05$) among different aflatoxins while superscript capital alphabets within the column depicted significant difference ($p<0.05$) among different chemicals.

Table 2: Aflatoxin content (ppb) in broiler feed treated with chemicals at incubation period of 2nd month.

Treatment	Conc. (g/100gm)	*A. paraciticus* ^{Y}			
		B1 ^{a}	B2 ^{c}	G1 ^{b}	G2 ^{d}
Control	00	7.78±0.03	2.47±0.05	3.17±0.08	1.25±0.03
Citric acid^{B}	01	3.35±0.06 (56.9)	0.00±0 (100)	0.04±0.01 (98.7)	0.00±0 (100)
	03	2.41±0.07 (69.1)	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)
	05	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)
Ascorbic acid^{A}	01	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)
	03	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)
	05	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)
Acetic acid^{A}	01	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)
	03	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)
	05	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)
Sodium bicarbonate^{A}	01	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)
	03	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)
	05	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)

Values are mean ± SD of three samples analysed individually in triplicate at $p<0.05$. Superscript small alphabets within the row indicated significant difference ($p<0.05$) among different aflatoxins while superscript capital alphabets within the column depicted significant difference ($p<0.05$) among different chemicals.

Table 3: Aflatoxin content (ppb) in broiler feed treated with chemicals at incubation period of 3rd month.

Treatment	Conc. (g/100gm)	*A. paraciticus* ^{Y}			
		B1 ^{a}	B2 ^{c}	G1 ^{b}	G2 ^{d}
Control	**00**	**10.47±0.01**	**4.51±0.06**	**6.86±0.09**	**2.39±0.09**
Citric acid ^{B}	**01**	**4.16±0.07 (60.2)**	**0.92±0.04 (79.6)**	**2.61±0.08 (61.9)**	**0.19±0.03 (92.1)**
	03	**3.72±0.02 (64.4)**	**0.74±0.02 (83.5)**	**1.85±0.04 (73.1)**	**0.15±0.04 (93.7)**
	05	**1.03±0.04 (90.1)**	**0.17±0.02 (96.2)**	**0.00±0 (100)**	**0.00±0 (100)**
Ascorbic acid ^{A}	**01**	**0.00±0 (100)**	**0.00±0 (100)**	**0.00±0 (100)**	**0.00±0 (100)**
	03	**0.00±0 (100)**	**0.00±0 (100)**	**0.00±0 (100)**	**0.00±0 (100)**
	05	**0.00±0 (100)**	**0.00±0 (100)**	**0.00±0 (100)**	**0.00±0 (100)**
Acetic acid ^{A}	**01**	**0.00±0 (100)**	**0.00±0 (100)**	**0.00±0 (100)**	**0.00±0 (100)**
	03	**0.00±0 (100)**	**0.00±0 (100)**	**0.00±0 (100)**	**0.00±0 (100)**
	05	**0.00±0 (100)**	**0.00±0 (100)**	**0.00±0 (100)**	**0.00±0 (100)**
Sodium bicarbonate ^{A}	**01**	**0.00±0 (100)**	**0.00±0 (100)**	**0.00±0 (100)**	**0.00±0 (100)**
	03	**0.00±0 (100)**	**0.00±0 (100)**	**0.00±0 (100)**	**0.00±0 (100)**
	05	**0.00±0 (100)**	**0.00±0 (100)**	**0.00±0 (100)**	**0.00±0 (100)**

Values are mean ± SD of three samples analysed individually in triplicate at *p<0.05*. Superscript small alphabets within the row indicated significant difference (*p<0.05*) among different aflatoxins while superscript capital alphabets within the column depicted significant difference (*p<0.05*) among different chemicals.

Table 4: Aflatoxin content (ppb) in broiler feed treated with chemicals at incubation period of 4th month.

Treatment	Conc. (g/100gm)	*A. paraciticus* ^{Y}			
		B1 ^{a}	B2 ^{c}	G1 ^{b}	G2 ^{d}
Control	**00**	**18.58±0.03**	**8.37±0.04**	**12.41±0.03**	**3.24±0.03**
Citric acid ^{C}	**01**	**7.56±0.03 (59.3)**	**1.42±0.07 (83.1)**	**3.84±0.07 (69.1)**	**0.36±0.01 (88.8)**
	03	**5.42±0.09 (70.8)**	**1.31±0.05 (84.3)**	**2.32±0.08 (81.3)**	**0.28±0.05 (91.3)**
	05	**1.78±0.05 (90.4)**	**0.23±0.02 (97.2)**	**0.54±0.02 (95.6)**	**0.08±0.03 (97.5)**
Ascorbic acid ^{AB}	**01**	**1.24±0.04 (93.3)**	**0.00±0 (100)**	**0.00±0 (100)**	**0.00±0 (100)**
	03	**1.03±0.03 (94.4)**	**0.00±0 (100)**	**0.00±0 (100)**	**0.00±0 (100)**
	05	**0.00±0 (100)**	**0.00±0 (100)**	**0.00±0 (100)**	**0.00±0 (100)**
Acetic acid ^{B}	**01**	**1.17±0.02 (93.7)**	**0.24±0.04 (97.1)**	**1.05±0.06 (91.5)**	**0.07±0.01 (97.8)**
	03	**1.14±0.03 (93.8)**	**0.12±0.02 (98.5)**	**0.81±0.02 (93.4)**	**0.00±0 (100)**
	05	**0.86±0.02 (95.3)**	**0.00±0 (100)**	**0.00±0 (100)**	**0.00±0 (100)**
Sodium bicarbonate ^{A}	**01**	**0.00±0 (100)**	**0.00±0 (100)**	**0.00±0 (100)**	**0.00±0 (100)**
	03	**0.00±0 (100)**	**0.00±0 (100)**	**0.00±0 (100)**	**0.00±0 (100)**
	05	**0.00±0 (100)**	**0.00±0 (100)**	**0.00±0 (100)**	**0.00±0 (100)**

Values are mean ± SD of three samples analysed individually in triplicate at *p<0.05*. Superscript small alphabets within the row indicated significant difference (*p<0.05*) among different aflatoxins while superscript capital alphabets within the column depicted significant difference (*p<0.05*) among different chemicals.

Table 5: Aflatoxin content (ppb) in broiler feed treated with chemicals at incubation period of 5th month.

Treatment	Conc. (g/100gm)	*A. paraciticus* ^{Y}			
		B1 ^{a}	B2 ^{c}	G1 ^{b}	G2 ^{d}
Control	00	30.43±0.08	12.54±0.05	19.51±0.03	6.21±0.04
Citric acid^{C}	01	12.51±0.02 (58.8)	2.37±0.06 (81.5)	5.28±0.07 (72.9)	0.53±0.02 (91.4)
	03	8.16±0.08 (73.2)	1.90±0.06 (84.8)	3.15±0.08 (83.8)	0.52±0.04 (91.6)
	05	3.12±0.09 (89.7)	0.62±0.02 (95.1)	1.01±0.03 (94.8)	0.16±0.03 (97.4)
Ascorbic acid^{AB}	01	2.91±0.07 (90.4)	0.00±0 (100)	0.00±0 (100)	0.06±0.02 (99.0)
	03	1.95±0.04 (93.5)	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)
	05	0.78±0.02 (97.4)	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)
Acetic acid^{B}	01	2.33±0.07 (92.3)	0.52±0.01 (95.8)	2.18±0.07 (88.7)	0.18±0.04 (97.1)
	03	2.23±0.06 (92.6)	0.23±0.05 (98.1)	1.47±0.04 (92.4)	0.08±0.02 (98.7)
	05	1.53±0.04 (94.9)	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)
Sodium bicarbonate^{A}	01	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)
	03	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)
	05	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)

Values are mean ± SD of three samples analysed individually in triplicate at $p<0.05$. Superscript small alphabets within the row indicated significant difference ($p<0.05$) among different aflatoxins while superscript capital alphabets within the column depicted significant difference ($p<0.05$) among different chemicals.

Table 6: Aflatoxin content (ppb) in broiler feed treated with chemicals at incubation period of 6th month.

Treatment	Conc. (g/100gm)	*A. paraciticus* ^{Y}			
		B1 ^{a}	B2 ^{c}	G1 ^{b}	G2 ^{d}
Control	00	45.19±0.05	21.41±0.04	30.13±0.07	11.47±0.01
Citric acid^{D}	01	21.13±0.01 (53.2)	3.51±0.04 (83.6)	7.82±0.06 (74.1)	0.94±0.04 (91.8)
	03	15.21±0.09 (66.3)	2.52±0.07 (88.2)	5.10±0.03 (83.1)	0.91±0.03 (92.1)
	05	5.34±0.03 (88.1)	1.01±0.02 (95.2)	1.94±0.06 (93.5)	0.23±0.03 (97.9)
Ascorbic acid^{B}	01	3.24±0.07 (92.8)	0.48±0.01 (97.7)	1.93±0.04 (93.6)	0.12±0.03 (98.9)
	03	3.17±0.09 (92.9)	0.31±0.04 (98.5)	1.58±0.07 (94.7)	0.11±0.02 (99.1)
	05	1.35±0.06 (97.1)	0.14±0.02 (99.3)	0.64±0.02 (97.8)	0.00±0 (100)
Acetic acid^{C}	01	4.71±0.07 (89.5)	1.08±0.05 (94.9)	3.35±0.04 (88.8)	0.32±0.05 (97.2)
	03	4.06±0.01 (91.1)	0.49±0.02 (97.7)	2.74±0.06 (90.9)	0.17±0.03 (98.5)
	05	3.28±0.08 (92.7)	0.12±0.02 (99.4)	0.94±0.04 (96.8)	0.00±0 (100)
Sodium bicarbonate^{A}	01	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)
	03	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)
	05	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)	0.00±0 (100)

Values are mean ± SD of three samples analysed individually in triplicate at $p<0.05$. Superscript small alphabets within the row indicated significant difference ($p<0.05$) among different aflatoxins while superscript capital alphabets within the column depicted significant difference ($p<0.05$) among different chemicals.

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Mediterranean Diet Pyramid: a Pivot Role in Dilemma of Anti-Aging

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ABSTRACT: *Mediterranean diet reflects traditional dietary habits and foods of mediterrranean people, historically associated with good health.Mediterranean diet pyramid having the basis of dietary habits and food pattern more likely according to Crete, Greece and Italy, where life expectancy ratio among adults is greatest in the world. The diet is categorized by plentiful plant foods, fresh fruits as the usual daily dessert, olive oil as the major source of fat, dairy products particularly cheese and milk,fish and poultry in moderate to low amount, zero to four eggs consumed weekly with the consumption of red meats and wine in low amounts.All research studies included in this study emphasize on the fact that combinations of the foods in mediterrranean diet impart beneficial impact on the chronic disease prevention as well as their management primarily due to the nutraceutical nature of the ingredients rich in substances like omega 3 & 6, vitamins, minerals, polyphenols and functional fibers likely to be effective in cardiovascular disease risk factors and biomarkers of metabolic syndrome.*

Keywords: Mediterranean diet, anti-aging, chronic disease prevention,

Aboriginal foods vs processed foods and chronic diseases(CVD's)

"Nutrition, nourishment, or aliment, is the supply of materials - food - required by organisms and cells to stay alive."Food is the basic necessity of life, not only food but the quality of food affects the life. Healthy and nutritious food is needed by the body. Good nutrition is the basic requirement for everyone. And the deficiency and excess of any nutrient is bad for our health. The diet plays major role in the development and prevention of chronic diseases. Diet is key factor which we can change that will affect all other factors related to chronic diseases. Aboriginal foods are rich in nutrients and much healthy food a person then processed foods. Processed foods contain much quantity of sodium in form of sodium carbonate, sodium benzoate. Processed foods contain food preservatives that are much harmful for our health and are threatening to our life.

To check the effect of Mediterranean diet on plasma level of C protein a study was conducted having 1514 men and 1528 women in which it was observed that in both the genders there was a reduction in concentration of inflammation and coagulation marker reducing risk of cardio vascular disease, showing the beneficial effects of diet in cardio vascular system(Chrysohoou et al. 2004).

To check the association of fish consumption and cardio vascular events a study was conducted men and women which were reporting to eat fish >300 grams in a week and when test results were observed it was concluded that consumption of fish was independently associated with the low inflammatory markers reducing risk of hypertension and cardio vascular events(Zampelas et al. 2005).

A modified diet was given with extra virgin oil and nuts and it was reported that they helped in reducing the cardiovascular disease and proved beneficial for healthy hearts. It was observed that this diet was helping in maintaining blood volume in the body. Eating nuts were observed to reduce LDL level in the body and increasing HDL level in the body(Kopel et al. 2013).

Serum cholesterol

According to a study conducted on two group of people one inter vented group and one was control group. The inter vented group were given fruits, milk, almonds, meat organ soya bean olive oil it and control group was given nothing of it was observed that there was decrease in cardiac deaths as well as their blood pressure was normal as compared to control group. Serum cholesterol level were also being reduced in the inter vented group(Singh et al. 2002).

Cardiovascular Diseases

The one of the risk factor for cvd's is processed foods that leads towards hypertension high blood pressure, heart attack, heart strokes and ultimately death. Sp our much concern should be on the healthy diet that is not from processed foods. Aboriginal foods are healthy for us because they are rich of nutrients, contains raw milk, contains unrefined salts and unrefined sugars, lacto fermented vegetables and contains unrefined food items, whereas processed foods contains refined food items which are rich in salt and food preservatives and causing.

The attention to Mediterranean diet in CVD is increasing day by day. A study was conducted in men and women (9408) both were CVD patient and were given food according to the plan, data was collected through food frequency questionnaire and there was 4 year follow up. It was observed that both group showed good results in decrease n CVD risk as well as increases on HDL level in their body(Núñez-Córdoba et al. 2009).

A study was conducted on Mediterranean diet score was given to effect of diet on lowering hypertension. There was a positive predictive score on hypertension 45 % as well as hypercholesterolemia 46%, results showed that effect of Mediterranean diet on blood was good (Panagiotakos et al. 2007).

Hypertension

Hypertension leads to Cardiac problems mainly heart attack or any other diseases of hearts, food is now what is being consider the most variable part of this cause a study was conducted on different types of diet and it was concluded that diets low n saturated fats and sodium and rich in fruits and vegetables with adequate amount of potassium, calcium and magnesium are effective in treatment for hypertension(Kokkinos et al. 2005).

The Mediterranean diet was found to be have inverse relationship with both systolic and diastolic blood pressure. They found that intake of vegetables, olive oil and fruits were having inversely effect on both diastolic and systolic blood pressure as well as meat and meat product were also having positive effect on the blood volume showing beneficial effect on the arterial blood pressure(Psaltopoulou et al. 2004).

Raw milk and dairy products are used in diet of traditional people diet which will help in the prevention of many chronic diseases. There are following studies which shows that milk is very important for prevention of chronic diseases especially cardiovascular disease.

Metabolic Syndrome

Calcium is considered as one of the main nutrient have beneficial impact of milk and milk products on blood pressure control. Other minerals as magnesium and potassium. Also help to regulate BP. bioactive peptides containing components of milk can regulate the blood pressure. These all studies are also supporting the same effect of milk consumption on the heart diseases, stroke, ischemic heart disease, diabetes and metabolic syndrome.

According to a study the postmenopausal women who consumed more calcium both from dietary sources and supplemental calcium not particularly from dairy products and vitamin D sources are relatively at decreased risk of ischemic heart disease and mortality (Bostick et al. 1999).

The cow milk contains an enzyme named as IGF-1 which is responsible for the growth of the baby cow but if a human is consuming cow milk IGF-1 performs some inflammatory function which initiates the complication of acne. Milk and Dairy products cause an insulin production which leads to even more production of IGF-1 resulting in more acne. Utilization of milk and dairy products causes excessive secretion of sebum resulting in the blocking of pores as a result more acne occurs and a favorable condition is generated for the production of P.acnes bacteria as a result more severe acne takes place.

According to the study which concluded that the individuals' mostly adolescent girls and boys who consume more skimmed milk are at high risk of acne. This study suggest milk has several bioactive molecules and hormonal constitutes in it e.g. androgenic compounds in milk which have many physiological effects on individuals (Adebamowo et al. 2006).

Fast Foods

More consumption of fast food and soft drinks leads towards obesity and weight gain and both of these factors leads towards chronic diseases and especially cvd’s.

With increasing age and school grades

from elementary to junior high and middle school their consumption of breakfast, fruits, vegetables and milk decreased but consumption of fast foods and soft drinks increased. The results of this cohort study described that consumption of breakfast in third grade was 99% and in eighth grade this consumption reduced to 85%. The reduction in consumption of fruits and vegetables was 41% and 25% respectively from third grade to eighth grade. There was significant reduction in milk as compared to three times increased proportion of soft drinks. (Lytle et al. 2000).

According to a study the consumption of soft drinks in youth from ages of 6-17 years increased up to 48% while this % age was 37 in 1977 and 56 in 1988. The consumption of soft drinks is associated with energy intake which may result in excessive weight gain and obesity in children. Secondly the soft drinks consumption can affect the dietary quality by decreased consumption of milk and can result in deficiency of calcium.(French et al. 2003)

Milk contains pre-biotic and probiotics which are very helpful in treating diarrhea and can also rehabilitate the beneficial microorganisms present in the colon of the GIT. And can also act as a rehydrating drink in diarrhea. According to the study which suggested that those people who consumed raw milk are mostly victim of chronic diarrhea. So people with increased intake of raw milk are at high risk of chronic diarrhea with secretory mechanism (Belongia et al. 1993).

Soy milk is a type of milk which contains soy protein and it is very effective in different types of cancers for example prostate, colon or colorectal cancer. According to a study which concluded that men with increased intake of soy milk are relatively at less risk of prostate cancer. So this study suggests that increased intake of soy milk can prevent from prostate cancer in men (Jacobsen et al. 1998).

In a study either to check intake of DASH diet 412 random participants were selected and they were given traditional diets 30 days consecutively after one month their blood examination was taken and it was found that they were having low blood pressure low diastolic as well as systolic pressure due to less intake of trans fat as well as sodium (Sacks et al. 2001).

Cardiovascular disease is growing worldwide day by day as there are several studies claiming foods to be the cause of that. Hypertension, obesity, diabetes are leading causes for heart disease, it was observed that certain food elevate this risk and certain show protection against risk like food high in fat causes obesity a and food high in poly unsaturated fats reduces this risk, foods that are high in sodium causes hypertension and food high in potassium causes low risk for hypertension(Reddy and Katan 2004).

Healthy Diet

The healthy diet of children is of much importance because the future of the nation depends upon the children of that nation and the best growth and development of children is only possible when they are consuming healthy diet and bad habits and unhealthy dietary practices is mostly seen in school going children it will leads towards obesity weight gain and also malnutrition and it will further cause chronic diseases in children.

According to study Obese children and adolescents were consuming more amount of meat, grain products, foods away from home, sugar sweetened drinks and potato chips. Consumptiom of theses things will contribute to higher calorie intake and higher percent body fat. Boys were consuming more amount of sugar sweetened drinks than girls. So obese childen and adolescents should not consume foods away from home and sugar sweetened drinks because there is a relationship between these food items and percent body fat(Gillis and Bar-Or 2003).

This study was conducted on children of age group 4 to 9 years old in the united states. According to study 30 percent of children were consuming fast food on a typical day irrespective of gender. The consumption of fast foods in children have very bad effeects on their health and it also effects th dietary quality. Fast foods consumption in children also leads to chronic diseases and causing obesity in school going children (Bowman et al. 2004).

This study was conducted on breakfast habits, nutritional status, academic performance and body weight of children and adolescents. Breakfast skipping habit is mostly seen in United States and Europe. The children who consumed breakfast daily tend to have higher nutritional status and show good academic performance than those children who do not consume breakfast daily. The children who consume breakfast daily consume higher calories daily and are not overweight than breakfast skippers. There is a relationship between breakfast skipping and obesity and poor cognitive function. Breakfast consumption habit may enhance cognition related to memory and develop a healthy life style (Rampersaud et al. 2005).

This study was conducted on US school going children. According to this study there is relationship between food insecurity and children nutritonal and nonnutritional staus. Food insecuirty was measured by interveiweng the parents of children. Academicperfomance,weight and height of children were dierctly measured and social performance of chlidren was taken from teachers. So mostly food insecure children were poor in studies and were showing poor academic performance and they were also socialy not good. So this study concluded that food insecuirty has poor impact on nutrional and nonnutrioinal status of chlidren (Jyoti et al. 2005).

According to a study is was found that family who were indulging themselves in good meal practices in front of their children, they were more likely to have good eating habits in their adulthood, they were consuming more fruits and green leafy vegetables as well less intake of soft drinks (Larson et al. 2007).

According to a study 20 children of school were checked half of them were given glucose drinks half of them no breakfast and half were given cereals that were rich in carbohydrates. They found that those children who were consuming glucose drinks and no breakfast were more likely to have less attention and much memory problem as compared to the children who were consuming cereals they all were having great attention and good memory control. They concluded that complex carbohydrates rich diet in morning can help to increase children mental health (Wesnes et al. 2003).

According to a research from all the households about 7.5% were found food insufficient after considering many demographic factors differnce between food sufficient and food insufficient low income groups. Children of both the groups consumed almost similar amount of macronutrients and micronutrients irrespective of their family groups but there was some differnce in consumption of total cholesterol and higher cholesterol in food sufficient group.(Casey et al. 2001)

A study was conducted on Japanese people as they have highest life longevity in the world as it was due to nitrite that cause vascular integrity resulting in less cardiovascular diseases these nitrites are produced from nitrates that were found in vegetables that all the Japanese consume. Due to high intake of these vegetables they were less likely to be prone to cardiovascular events(Sobko et al. 2010).

American heart Association Company is critically working on people dietary behavior to reduce cardio vascular failure events among population. They suggested that people should consume whole grains, fish, meat organ and fruits and vegetables and should avoid refined foods food containing high amount of salt in order to have a healthy life and controlled hypertension(Lichtenstein et al. 2006).

A study was conducted on hypertensive patient 436 participants were taken as they were given DASH diet for 3 weeks including fruits, vegetables, low dietary fats, low saturated fats, foods containing less amount of sodium and high amount of potassium after testing it was observed that they were having LDL values in lesser amount and HDL values in higher amount(Obarzanek et al. 2001).

CONCLUSION

Mediterranean diet is the reflection of culture and traditional dietary habits and foods of Mediterranean people. Traditionally associated with good health. Mediterranean diet pyramid includes the basis of dietary habits and food pattern more likely according to Crete, Greece and Italy, where life expectancy ratio among adults is greatest in the world. The diet is recognized by plentiful plant foods, fresh fruits as the usual daily dessert, olive oil as the major source of fat, dairy products particularly cheese and milk, fish and poultry in moderate to low amount, zero to four eggs consumed weekly along with the consumption of red meats in low amounts. All research studies included in this study highlightened the fact that combinations of the foods in Mediterranean diet has beneficial impact on the chronic disease prevention as well as its management caused by the nutraceutical nature of the ingredients rich in substances like omega 3 & 6, vitamins, minerals, polyphenols and functional fibers likely to be effective in cardiovascular disease risk factors and biomarkers of metabolic syndrome.

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