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Gene Therapy

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Abstract: This article aimed to educate the population from the reimbursement of gene therapy. Gene therapy is an experimental technique which used for medicinal purposes and eliminates the undesirable gene from the cell, tissue and organ. Early stages of the beginning of gene therapy functional DNA is used to replace mutated gene, then bacteria and recently viruses are used.

Key-words: Gene therapy, DNA, Bacteria, Viruses.

Introduction

With the passage of time the human is trying to get rid of disease. Up to some extent they success in their mission but in case of some diseases it is still a challenging target. The genetic disorder is a common problem found in every society of the world. The treatment of a disease on a molecular base is a very difficult task. Due to the emerging of gene therapy the genetic problems was solved up to some extent but still these challenges are life treating one. The preface of genes interested in human cells to renovate standard cellular activity is called gene therapy. During last two decades irregular impression of passion and stipulation transversely an extensive variety of disease. Two strategies that are ex vivo and in vivo are used for gene delivery and extensive gears which are used to support with gene transfers (Macpherson and Rasko, 2014). It is a variety of molecular medication support scheduled to the incorporation of an efficient gene interested in cells to accurate a cellular dysfunction or to present a new cellular activity (Soofiyani et al., 2013). Gene therapy is an experimental technique which used for
undesirables genes to eliminate them from the body or from a single cell. This technique also used to take care of or avoid disease, inserting a desirable gene into the patient (Genetics Home Reference, 2014). In gene therapy functional DNA is used to replace mutated gene (Sheridan, 2011). Neurologic diseases are prospective in the direction of extensive expression beneficial for diseases that are characteristically persistent in nature (Gelfand and Kaplitt, 2013; Simonato et al., 2013). All macro and microorganism were treated with the help of gene therapy as well as for their chromosomes modification which are ready to lend a hand for medicinal uses (Pezzoli et al., 2012). The insertion of a gene to a specific cell we need to concentrate the following factors that are target cell, transgene biology and pathophysiology for targeted genes (Hung et al., 2007). The identification and accessible ideologies still needed innovative approaches (Cannon et al., 2011). Genetic variation, frequently referred to as gene therapy, is a process whereby the genetic content (DNA sequence) of a cell, several cells or a complete organism is customized. The majority, nonfunctional or functioning lees genes are replace, manipulate or supplement with vigorous genes. There are two categories of genetic modification in human, somatic and germ line. Somatic gene therapy consists to replace or modified a gene segment into the entire cell, tissue and organs while germ line gene therapy, introduce new genes into germ line resolve consequence genetic changes to distress future generation (Isasi et al., 2006), but from 2009, germ line therapy is speculative (Peacock, 2010). In pasts microbiologist were transfer the gene to bacteria and then human cells, now new genetic technology introduced genes into viruses and they transmit proficiently these to human cell (Macpherson and Rasko, 2014).

Joshua Lederberg is the first one who introduced gene therapy in 1963. Then clinical gene therapy was done by Anderson in 1990, where they treated a young girl suffering from the deficiency of adenosine deaminase (ADA), he transfers the ADA gene into her white blood cells and she feel better development in her immune system (Soofiyani et al., 2013). Early accomplishment of gene therapy is the discovery of restriction enzyme, which are basically endonuclease having specific base sequences in the DNA and restriction enzymes in arrangement of restriction fragment length polymorphism (RFLP) method make the separation of genes and
other DNA molecules achievable (Parissis and Nikolaou, 2003). In the early stages human understood that some characters are transmitted from parent to their offspring, like the eye color, hear color and face structure are resemblance to their insisters (Misra, 2013).

Conflict of interest
None

References


Isolation and identification of Dengue virus in haematophagous and non-haematophagous mosquitoes using patient serum sample

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Abstract
Dengue virus is one of the medically important, positive-sense RNA arboviruses transmitted to humans by Aedes aegypti and sometimes by Aedes albopictus mosquitoes. Dengue virus causes classical dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Dengue has four serotypes [DEN-1, DEN-2, DEN-3 and DEN-4]. Since, there is still trial and error for the vaccine production, prevention and control of the Aedes mosquito is very important. Dengue virus in patient serum sample can be detected using various techniques like ELISA, PCR, IFA, cell lines etc. Using Toxo-IFA technique and conventional RT-PCR method for comparing haematophagous and non-haematophagous mosquitoes, it has been identified that non-haematophagous mosquitoes support for the growth of dengue virus when inoculated artificially. And also while comparing larvae and adult of non-haematophagous mosquitoes, larvae support the growth of virus than adult especially larvae inoculated at their heads. The larvae live upto 3-4 days while adults die within 24hrs when inoculated. Since this Toxorhynchites are non-haematophagous they are believed not to have any cross reactions during tests. The test is proved to be sensitive when serum sample is inoculated at the larval head and incubated till 48hrs. This technique is easily done using very minimum amount of sample, and also economical and pave the way for the development of cell lines using Toxorhynchites splendens larvae.
Materials And Methodology

Larval Collection

Toxorhynchites larva were collected from Madurai CRME (Centre For Research In Medical Entomology, Chinna Chokkikulam, Madurai.) and reared in our entomology lab at KIPMR (King Institute Of Preventive Medicine And Research,Guindy,Chennai.). Larva were grown individually in separate containers for each larvae. Culex larvae were collected and used as feed for Toxorhynchites larva. Water in a pot was kept as natural breeding source for the adult mosquitoes. After the larvae turns to pupa they were kept inside the dark cages and reared at 24˚C. Cotton dipped with honey were kept as feed for adults.

Larval Inoculation

Toxorhynchites larvae were inoculated with positive dengue serum sample at the intra-cerebral region of the larvae. Inoculated larvae were incubated at different time intervals such as 12hr, 24hr, 48hr. After incubation period larvae were crushed for IFA staining.

Adult Inoculation

Adult mosquitoes were collected using aspirators and given anesthesia using choloroform. Positive patient serum sample were inoculated at the head of the adult and incubated in a separate cage. After 24hrs the adult mosquito head was cut and crushed and used for IFA.

Preparation of Laraval Crush

Based on the different time duration of incubation of larvae inoculated with DenV, larval heads were pooled in eppendorf. 5 larvae were pooled in each eppendorf. 100µL BAPS solution was added and larva were crushed using small glass pestel. Then the crushed samples were sonicated and centrifuged at 4000rpm for 2 mins. After centrifugation supernatant were collected and processed for viral RNA extraction.

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Immunofluoresence Assay

Preparation of Test Slide

Toxorhynchites larvae were inoculated with positive dengue serum sample at the intra-cerebral region. Inoculated larvae were incubated at different time intervals such as 12hr, 24hr, 48hr. After incubation period larva were put in eppendorf and crushed with 1ml BAPS solution using pestel. Then the eppendorfs were sonicated for the destruction of unwanted cells. 20µL of the crushed solution were smeared onto the glass slide and air dried. The glass slides were fixed with chilled acetone for 10mins and air dried. The slides can be stored for several months at -20°C.

IFA staining procedure

20µL of dengue monoclonal antibody was added and incubated at 37°C for 30mins in humid chamber. After incubation slides are washed using PBST wash buffer and air dried. 20 µL of secondary antibody with FITC conjugate was added and incubated for 30mins at 37°C in humid chamber. After incubation period slides are washed using PBST wash buffer and air dried. 20 µL of 0.01% Evan’s blue was added and incubated for 10mins. Excess stain was removed using wash buffer. Glycerol was placed and covered using coverslip. Slides were viewed under fluorescent microscope.

Viral RNA Extraction

560 µl of prepared viral lysis buffer containing carrier RNA was pipetted into a 1.5 ml micro centrifuge tube. 140 μl larval crush supernatant was added to the viral lysis buffer–carrier RNA in the micro centrifuge tube and mixed by pulse-vortexing for 15 sec and incubated at room temperature (25°C) for 10 min. The tubes were briefly centrifuged to remove drops from the inside of the lid. 560 µl of ethanol (100%) was added to the sample, and mixed by pulse-vortexing for 15 sec. After mixing, the tubes were briefly centrifuged to remove drops from inside the lid. 630 µl of the solution from the above step was carefully added to the QIAamp Mini column (in a 2 ml collection tube) without wetting the rim. The cap closed, and centrifuged.
at 6000 x g (8000 rpm) for 1 min. The Qiagen Mini column was placed into a clean 2 ml collection tube, and the tube containing the filtrate discarded. Step 6 was repeated. To the Qiagen Mini column 500μl of Wash Buffer 1 was added and centrifuged at 6000 x g (8000 rpm) for 1 min. The Qiagen Mini column was placed in a clean 2 ml collection tube and the tube containing the filtrate discarded. 500 μl of Wash Buffer 2 was added to the Qiagen Mini column and centrifuged at full speed (20,000 x g; 14,000 rpm) for 3 min. The Qiagen Mini column was kept in a new 2 ml collection tube and the old collection tube with the filtrate discarded centrifuged at full speed for 1 min. The Qiagen Mini column was placed in a clean 1.5 ml microcentrifuge tube, the old collection tube containing the filtrate discarded.

To the Qiagen Mini column 60 μl of Elution Buffer was added and equilibrated to room temperature and incubated at room temperature for 1 min. centrifuged at 6000 x g (8000 rpm) for 1 min. Viral RNA is stable for up to one year when stored at –20°C until used for cDNA synthesis.

**Conventional RT-PCR**

4 samples are subjected to conventional reverse transcriptase PCR using sequence specific primers for gene separately. 25 μl reaction volume containing 12.5 μl of 2 X RT PCR master mixes, 0.5 μl of RT mix 1.0 μl of forward and reverse primers 5μl of Nuclease free water and 5 μl of RNA was taken into the reaction mix.

**Conventional RT-PCR Amplification Conditions For Gene**

**Table.1:** Cyclic conditions for amplification of gene using conventional RT-PCR

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<tbody>
<tr>
<td><strong>Reverse transcription</strong></td>
<td>50°C for 30 minutes</td>
</tr>
<tr>
<td><strong>Reverse transcriptase inactivation</strong></td>
<td>95°C for 5 minutes</td>
</tr>
<tr>
<td><strong>Denaturation</strong></td>
<td>95°C for 30 seconds(35 cycles)</td>
</tr>
</tbody>
</table>

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Annealing 55°C for 30 seconds (35 cycles)
Template extension 72°C for 30 seconds (35 cycles)
Final extension 72°C for 10 minutes
Cooling 4°C

Primer Sequencing For Conventional PCR

<table>
<thead>
<tr>
<th>ID</th>
<th>Primer</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MD1</td>
<td>134-TCAATATGCTGAAACGCGAGAGAAACCG</td>
<td></td>
</tr>
<tr>
<td>D2</td>
<td>TTGCAACCAACAGTCAATGTCTTCAGGTTC-616</td>
<td>511 (MD1-D2)</td>
</tr>
</tbody>
</table>

Product Analysis By Agarose Gel Electrophoresis

The PCR amplified products were analyzed on agarose gel electrophoresis. 1.5% agarose gel was prepared by mixing 100ml 0.5 X TBE buffer and 1.5gms of agarose. After heating in microwave oven the buffer was mixed with 4µl intercalating ethidium bromide dye. Then the gel is poured onto the casting tray and coombs were placed. Then gel is allowed to cool and solidify. Coombs were removed and wells were created. 5 µl of the amplified product was mixed with 1 µl of 10X loading dye and loaded into the wells along with 1 µl of molecular weight marker (100-1000 bp ladder). The electrophoresis was run at 100 volts in 0.5X TBE buffer. The gel was visualized under UV transilluminator and the bands were observed. The product was compared with molecular weight of the marker.

Result

Haematophagous mosquito larvae (Culex, Armigeres and Anopheles) and non-haematophagous mosquito larvae (Toxorhynchites), were inoculated with ELISA positive dengue serum sample. 30 larvae (Culex-5, Armigeres-5 and Anopheles-5, Toxorhynchites-15) and 5 adult Toxorhynchites were inoculated at the cephalothorax region of the head and 15 larvae were
inoculated at the thorax region. These larvae were inoculated at different time intervals such as 12hrs, 24hrs, 48hrs as explained in the table. After incubation larval samples were crushed and 20µL were taken for IFA. Remaining samples were sonicated centrifuged and supernatant were collected and used for conventional RT-PCR. Agarose gel electrophoresis was run and bands were observed and compared with markers.

**Immunofluorescence Assay**

The larval samples incubated at 24hrs and 48hrs showed positive results while the larval samples incubated at 12hrs showed negative results. The larval samples inoculated at the cephalothorax region showed positive result while the larval samples inoculated at thorax region showed negative results. While comparing haematophagous mosquitoes (*Culex, Aedes* and *Anopheles*) and non-haematophagous mosquitoes (*Toxorhynchites*), non-haematophagous (*Toxorhynchites*) mosquitoes showed positive results in IFA. *Toxorhynchites* larvae and *Toxorhynchites* adult mosquitoes were inoculated in the head and processed for IFA. In that larvae showed positive results when adult showed negative results.

Hence, this proves that *Toxorhynchites* larval cells support for the growth of dengue virus. This also proves that *Toxorhynchites* cell line supports for the cultivation of dengue virus. Toxo-IFA method is faster and easier method for the identification of dengue virus in patient serum sample using *Toxorhynchites*. This method is economical for isolation and identification of dengue. Since it requires only 20µL of patient serum sample this technique is very essential.

**Conventional RT-PCR**

Larval samples showed positive in IFA were pooled based on the incubation period, region of inoculation and genus used for the inoculation in eppendorfs. They were crushed using BAPS solution and pestel. After crushing they were sonicated and centrifuged. The supernatant were collected which contains viruses. The supernatant were processed for viral RNA extraction and
Table 2: Processing and results of samples in TOXO-IFA and Conventional RT-PCR

proceeded with conventional RT-PCR. After PCR the sample was taken and mixed with bromophenol blue. Agarose gel was prepared, ethidium bromide was added and wells were made using coombs. Sample mixed with dye was added into the wells and gel was made. After an hour bands were observed under UV transilluminator as shown in figure 7.
Lane 1- marker for dengue.
Lane 2- sample 1- Tx larval sample inoculated at the head and incubated for 12 hrs
Lane 3- sample 2- Tx larval sample inoculated at the head and incubated for 24 hrs
Lane 4- sample 3- Tx larval sample inoculated at the head and incubated for 48hrs
Lane 5- sample 4- Positive control
Lane 6- sample 5- Negative control

When the gel electrophoresis was run bands were observed in the 4th and 5th lanes. Hence this proves the positive in the larval sample inoculated at the head and incubated for 48hrs and positive control.

Fig. 1. Toxorhynchites larval cultivation

Fig. 2. Toxorhynchites larvae head inoculation

Fig. 3. Toxorhynchites adult head inoculation

A. Toxorhynchites, B. Armigeres, C. Culex
Fig. 5. Toxo-IFA-24hrs incubation

Fig. 6. Toxo-IFA-48hrs incubation

Fig. 7. Agarose gel electrophoresis

511 kbp marker
Conclusion

Hence, this shows that the growth of dengue virus is specific at the cephalothorax region and also brain cells of the larvae support the growth of the dengue virus. The cell line developed from the non-haematophagous mosquitoes will support the isolation and identification of dengue virus. Toxo-IFA is the simple, economical and easy way to identify positivity of dengue in patient using their least amount of serum sample.

References


Endocrine Disruptors: An emerging pollutant in pulp and paper mill wastewaters and their removal

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Introduction

Chemicals which can affect hormonal control and development of aquatic organisms, wildlife and even human beings are called as endocrine-disrupting compounds (EDCs). They include different groups of compounds, such as polychlorinated bisphenyls, dioxins, polyaromatic hydrocarbons (PAHs), phthalates, bisphenol A (BPA), nonylphenol (NP), pesticides, alkylphenols, as well as arsenic and heavy metals such as cadmium, lead or mercury (Balabanič et al. 2011). EDCs have been suspected to be associated with altered reproductive function in males and females; increased incidence of breast cancer, abnormal growth patterns and neuro developmental delays in children, as well as changes in immune function. Human exposure to EDCs occurs via ingestion of food, dust and water, via inhalation of gases and particles in the air, and through the skin. This write up briefly describes environmental contamination of EDCs and their health effect, and removal approaches.

EDCs in paper mill effluents

Pulp and paper mill effluents are major source of EDCs in the environment. Alkylphenols are degradation byproducts of alkylphenols polyethoxylates (APEs), which used as defoamers,
cleaners and emulsifiers in the pulp and paper industry. Alkylphenol ethoxylates (APEs) and related compounds recently have been reported to be estrogenic because it has been demonstrated in laboratory studies that they mimic the effects of estradiol both in vitro and in vivo.

β-sitosterol, a plant sterol widely distributed throughout the plants, which are used in pulping industry. Some raw materials used for paper production contain phthalates, which function as softeners in additives, glues and printing inks. Existing conventional wastewater treatment plants are incapable to remove EDCs and these micro-pollutants are continuously released into the environment via effluent discharge, so the latter are consequently consumed by aquatic organisms, and thereby represent a hazard to the whole food chain.

Table 1: Commonly found EDCs in pulp and paper mill effluent

<table>
<thead>
<tr>
<th>EDC Group</th>
<th>Compounds</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkylphenols</td>
<td>4-tert-octylphenol</td>
<td>Felty et al. (2007)</td>
</tr>
<tr>
<td></td>
<td>4-nonyphenol</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4-octylphenol</td>
<td></td>
</tr>
<tr>
<td>Phthalates</td>
<td>Dibutyl phthalate</td>
<td>Raj et al. (2007)</td>
</tr>
<tr>
<td></td>
<td>Dimethyl phthalate</td>
<td>Balabanic et al.(2012)</td>
</tr>
<tr>
<td></td>
<td>Diethyl phthalate</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Benzyl butyl phthalate</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bis(2-ethylhexyl) phthalate</td>
<td></td>
</tr>
<tr>
<td>Phytosterols</td>
<td>β-sitosterol</td>
<td>Xavier et al.(2009)</td>
</tr>
<tr>
<td></td>
<td>Stigmasterol</td>
<td></td>
</tr>
</tbody>
</table>
Campesterol
β-sitostanol
Stigmasterol
Campestanol

The occurrence of EDCs in paper mill effluent is evidenced by recent study of Felty et al. (2007). They have detected 4-tert-octylphenol, 4-nonylphenol, and β-sitosterol at concentration of 3.45, 6.62 and 19.92 μg/L, respectively. In another survey of 22 U.S. pulp and paper mills, the total sterol concentrations ranged from 71 to 535 mg/L, with β-sitosterol being the major plant sterol at all the sampling sites (Cook et al. 1996). Esterogenic activity of bleached kraft pulp mill effluents is well reported (Chamorro et al. 2010).

**Impact of EDCs on human health**

In recent years, there has been a growing concern about the increasing number of environmental pollutants that may disrupt normal endocrine function in exposed human health and wildlife populations. Endocrine disrupting chemicals (EDCs) comprise a large group of synthetic chemicals that mimic the action of natural hormones, act as antagonist or block their synthesis, release or metabolism. Toxic effects have been related to reproductive abnormalities, infertility and reduction in sperm count (Diamante et al. 2009). EDCs tend to be relatively bioaccumulative, persistent, and produce adverse developmental, reproductive, neurological, and immune effects in both humans and wildlife.
Fig. 1- Shows impact of endocrine disruptors on human health

**Removal of EDCs from wastewaters**

The fact that EDCs are capable of eliciting physiological responses at extremely low concentrations presents a unique challenge for the removal of such chemicals to safety levels in water treatment (Jobling et al. 1998; Huang. 2005). Oxidation by ozone and chlorine was shown to be effective in removing steroidal hormones from water (Hu et al. 2003; Bila et al. 2007). However, the efficiency of these processes under field conditions is limited by the low concentrations of the contaminants. Because chemical oxidation is nonspecific, the majority of oxidants and energy are in fact consumed by the nontargeted impurities. In addition, hazardous byproduct can be generated during the oxidation processes (Nakamura et al. 2006; Huber et al. 2004).

An environmentally friendly alternative for the elimination of EDCs may be the use of microorganisms. Among the different microorganisms, white rot fungi have been studied for their ability to not only to eliminate EDCs but also to reduce their estrogenic activity. EDCs reducing ability of fungi is usually related to the production and secretion of lignin modifying enzymes. However, use of fungi in practical treatment is not feasible due to their reduced enzymatic activity in real effluents. The bacterial community is able to dissipate EDCs, and
Biodegradation plays a major role in the elimination of BPA pollution in the environment, thus reducing the possible risk of EDCs (Zhang et al. 2013). Previous research works based on traditional culture-dependent approaches have provided a substantial amount of knowledge about the diversity of EDC degraders. However, culture approaches can typically select for microorganisms that are able to compete well under laboratory conditions (Zhang et al. 2012). Yang et al. (2014) reported the aerobic biodegradation of bisphenol A in river sediment and the associated bacterial community change. Recently, several estrogen-degrading bacteria were isolated from activated sludge, including *Novosphingobium tardaugens* (ARI-1) (Fujii et al. 2002), *Rhodococcus zopfii* and *Rhodococcus equi* (Yoshimoto et al. 2004) and *Achromobacter xylosoxidans* and *Ralstonia sp.* (Weber et al. 2005) were reported to degrade estrogen. However, there is a gap in knowledge regarding enzymes used by these systems for EDCs degradation. One promising approach to face this challenge consists in the use of enzymatic systems able to react with these molecules. Among the possible enzymes, oxidative enzymes are attracting increasing attention because of their versatility, the possibility to produce them on large scale, and to modify their properties (Macellaro et al. 2014). Horseradish peroxidase (HRP) and laccase are the enzymes that were most frequently studied for this purpose (Klibanov et al. 1983; Karam et al. 1997). Recent study demonstrated that lignin peroxidase (LiP) can also mediate effective reactions leading to the removal of estrogens (Mao et al. 2009). Earlier studies revealed that lignin peroxidase (LiP) mediates effective reactions of three steroid estrogens, estrone (E1), 17β-estradiol (E2), and estriol (E3) and one synthetic steroid 17R-ethinylestradiol (EE2), to form oligomeric products via radical coupling (Mao et al. 2010).

**Conclusion**

EDCs are emerging pollutants which contaminate environment through various sources including P&P mills effluents. Exposure to EDCs, significantly affect hormonal control, abnormal growth patterns and neuro developmental delays in children, as well as changes in

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immune function of exposed organisms. However, in India, the occurrence of EDCs in environment is not well documented. Hence, identification of EDCs and their removal is necessary for the safety of environment and public health.

References


Adjuvants in Veterinary Vaccines

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Introduction
Adjuvants are any chemicals, microbial components, or mammalian proteins that elicit the immune response of antigens. The word adjuvant comes from the Latin word *adjuvare*, which means to help or to enhance. Adjuvants enhance the efficacy of either killed or live attenuated vaccines. Along with killed antigens, adjuvants provide artificial signals that stimulate the immune system and can boost the efficacy of vaccines. In animal health, use of adjuvants have become a common way of reducing dependence on modified live antigens, which have the potential to prevent disease in immunocompromised animals.

Majority of vaccines developed at present are comprised of highly purified recombinant proteins, or peptides, which represent the subunits of pathogens. These vaccines lack the features of an original pathogen and are often poorly immunogenic. In such a situation the preferred strategy for the development of new-generation vaccines is to add highly purified synthetic adjuvants. Vaccines that comprise attenuated live organisms or whole inactivated organisms generally do not require adjuvants. Adjuvants alone are not licensed; a specific adjuvant/antigen formulation is licensed. This implies that the development of an adjuvant is strictly related to the antigen present in the specific formulation. As a general rule, two or more adjuvants with different mechanisms of action are combined to enhance the potency and type of the immune response to the vaccine antigen. For example, alum salts can be formulated in combination with other adjuvants such as Lipid A to increase immunogenicity. Similarly algammmulin which is the combination of γ-inulin and alum has increased absorptive capacity and increased ability to stimulate Th2 responses.

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History of Adjuvants

The concept of adjuvants arose in the 1920s from observations such as those of Ramon et al. who noted that horses that developed an abscess at the inoculation site of diphtheria toxoid generated higher specific antibody titres. They subsequently found that an abscess generated by the injection of unrelated substances along with the diphtheria toxoid increased the immune response against the toxoid. The adjuvant activity of aluminium compounds was demonstrated by Glenny et al. in 1926 with diphtheria toxoid absorbed to alum. To this day, aluminium-based compounds (principally aluminium phosphate or hydroxide) remain the predominant. In 1936, Freund developed an emulsion of water and mineral oil containing killed mycobacteria, thereby creating one of the most potent known adjuvants, Freund's complete adjuvant (FCA). Despite being the gold standard adjuvant, FCA causes severe local reactions and is considered too toxic for use. The oil in water emulsion without added mycobacteria is known as Freund's incomplete adjuvant (FIA) and, being less toxic, has been used in human vaccine formulations. In the 1950s, Johnson et al. found that lipopolysaccharides (LPS) from Gram-negative bacteria exhibited adjuvant activity and detoxified LPS or related compounds such as lipid A have since been used as adjuvants in human studies. In 1974, Lederer et al. identified muramyl-dipeptide (MDP) as a mycobacterial component with adjuvant activity contained in FCA. Bacterial components are often potent immune activators although commonly associated with toxicity, for example, bacterial DNA with immunostimulatory CpG motifs is one of the most potent cellular adjuvants. Immunostimulatory CpG are unmethylated cytosine-guanine dinucleotides found in bacterial DNA but absent in mammalian DNA.

Uses of adjuvants in veterinary vaccines

Currently licensed adjuvants such as aluminum compounds (e.g. Alum), squalene-in-water emulsions (MF59 and AS03), monophosphoryl lipid A (MPL), Ribi adjuvants, combined with alum (AS04); adjuvants in pre-clinical development (e.g. Montanides, polymeric microparticles, saponins (e.g. Quil A QS-21, ISCOMS, ISCOMATRIX), immunostimulatory nucleic acids (e.g. 

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CpG oligodeoxynucleotides, poly IC:LC); other toll-like receptor-agonists (e.g. flagellin, imidazoquinolines, small molecules), cationic liposome formulations (CAF) combined with immunestimulators such as trehalose dibehenate (TDB) virus-like particles, nanoparticles,19-21 and other procedures or emulsions such as subcutaneously-implanted chambers, TiterMax, EMULSIGENS, Syntex Adjuvant Formulation (SAF), and Specol. Currently, none of the US-licensed vaccine contains the adjuvants, MF59 or AS03. It could be authorized for emergency use only. The multinational use of adjuvant vaccines requires manufacturers to submit to each national regulatory agency for approval and product release. All adjuvants used in animal research must be approved by the Institute/Center (IC) Animal Care and Use Committee (ACUC), and use of adjuvants that could induce a severe reaction must be scientifically justified.

**Mechanism of action of different adjuvants in veterinary use**

**Depot adjuvants** delay the elimination of antigens thereby permits an immune response to last longer. The rate of antigen elimination can be slowed by mixing it with an insoluble, slowly degraded adjuvant. Ex: aluminum salts. When antigen is mixed with this salts and injected into an animal, a macrophage-rich granuloma forms in the tissues. The antigen within this granuloma slowly leaks into the body and provides a prolonged antigenic stimulus. These depot adjuvants influence only the primary immune response and have little effect on secondary immune responses.

**Particulate adjuvants** can trap and process particles such as bacteria or other microorganisms much more efficiently than soluble antigens. These adjuvants include emulsions, microparticles, immunestimulating complexes (ISCOMs), and liposomes, and all are designed to deliver antigen efficiently to antigen-presenting cells. The particles are usually of similar size to bacteria and are readily endocytosed. Liposomes are lipid-based synthetic microparticles containing encapsulated antigens that are effectively trapped and processed yet are also protected from rapid degradation. They are not yet widely employed in veterinary vaccines. The potency of liposomes depends on the number of lipid layers, electric charge, composition and method of preparation. They
enhance both humoral and cellular immunity to protein and polysaccharide antigens. Liposomes have been used widely in experimental vaccines.

**Tensioactive adjuvant** such as saponins induces a strong adjuvant effect to T-dependent as well as T-independent antigens. Saponins also induce strong cytotoxic CD8+ lymphocyte responses and potentiate the response to mucosal antigens. Quil A has been used successfully for veterinary applications.

**Adjuvant emulsions** include oil in water or water in oil emulsions such as FIA, Montanide, Adjuvant 65, and Lipovant. Formation of depots at the injection site, enable slow release of antigen and the stimulation of antibody producing plasma cells. Montanide is a family of oil-based adjuvants that have been used in experimental vaccines in mice, rats, cats and dogs, using natural, recombinant and synthetic antigens. Montanide Gel was used in cattle, pigs and dogs. Nevertheless, the uses of such adjuvant need validation in avian and fish vaccines.

**Complete Freund's adjuvant (CFA)**, is effective in potentiating cellular and humoral antibody responses to injected immunogens. Sustained release of antigens from the oily deposit and stimulation of a local innate immune response, results in enhanced adaptive immunity.

**Immunostimulatory adjuvants** exert their effects by promoting cytokine production. Many of them are complex microbial products that often present PAMPS, and they are designed to target specific PRRs. As a result, they activate dendritic cells and macrophages through TLR and other PRRs and stimulate the production of key cytokines such as IL-1 and IL-12. These cytokines in turn promote helper T cell responses and drive and focus the adaptive immune responses. Depending on the specific microbial product, they may enhance either Th1 or Th2 responses. TLR ligands alone are not usually effective adjuvants since they induce excessive inflammation. Double-stranded RNA (dsRNA) is the ligand for TLR3, and synthetic dsRNA (e.g., polyIC) is an effective adjuvant. TLR4 ligands such as bacterial lipopolysaccharides (or their derivatives) have long been recognized as having adjuvant activity. Their toxicity, however, has limited their use. Lipopolysaccharides enhance antibody formation if given at about the same time as the antigen. They have no effect on cell mediated responses, but they can break T cell tolerance, and they
have a general immunostimulatory activity. Killed anaerobic corynebacteria, especially *Propionibacterium acnes*, have a similar effect. When used as adjuvants, these bacteria enhance antibacterial and antitumor activity. The TLR5 ligand bacterial flagellin is an adjuvant that promotes mixed Th1 and Th2.

**Commonly Used Adjuvants in Veterinary Vaccines**

<table>
<thead>
<tr>
<th>Sl No</th>
<th>Vaccine</th>
<th>Strain</th>
<th>Adjuvant</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Raksha Ovac</td>
<td>FMD(O,A, C and Asia 1)</td>
<td>Mineral oil</td>
</tr>
<tr>
<td>2</td>
<td>Raksha Biovac</td>
<td>FMD(O, A and Asia 1) + HS</td>
<td>Double emulsion oil adjuvant(Mineral oil)</td>
</tr>
<tr>
<td>3</td>
<td>Raksha Monovalent</td>
<td>FMD O strain</td>
<td>Aluminium hydroxide and saponin</td>
</tr>
<tr>
<td>4</td>
<td>Raksha bivalent</td>
<td>FMD(O and A)</td>
<td>Aluminium hydroxide and saponin</td>
</tr>
<tr>
<td>5</td>
<td>HS vaccine</td>
<td>Inactivated <em>Pasteurella multocida</em> organism</td>
<td>Aluminium hydroxide</td>
</tr>
<tr>
<td>6</td>
<td>Raksha HS+ BQ vaccine</td>
<td>Inactivated culture of <em>Pasteurella multocida</em> and <em>Clostridium chauvoei</em></td>
<td>Aluminium hydroxide</td>
</tr>
<tr>
<td>7</td>
<td>ET vaccine</td>
<td><em>Clostridium perfringens</em> type D and Epsilon toxoid</td>
<td>Aluminium hydroxide</td>
</tr>
<tr>
<td>8</td>
<td>Botulinum vaccine</td>
<td>Toxoid of <em>Clostridium</em> type C and D</td>
<td>Aluminium hydroxide</td>
</tr>
<tr>
<td>9</td>
<td>Raksharab</td>
<td>inactivated rabies virus</td>
<td>Aluminium hydroxide</td>
</tr>
<tr>
<td>10</td>
<td>Botuthrax</td>
<td>inactivated alum precipitated toxoids of <em>Clostridium botulinum</em> types C and D</td>
<td>Aluminium hydroxide</td>
</tr>
</tbody>
</table>
Conclusion

Selecting the adjuvant is one of the key for the success of the vaccine in the veterinary field. A flexible adjuvant will fit with several vaccines dedicated to one or more animal species is a source of economical efficiency. Few adjuvants like Montanid Gel have been tested with more than one animal species and found fitting. Next generation veterinary vaccines like subunit or inactivated bacteria/viral vaccines would require optimal adjuvants and delivery system to accord long-term protection from infectious diseases in animals.

References


Viral Pathogenesis

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Pathogenesis is Greek word pathos ("disease") and genesis ("creation") means the mechanism by which viruses produce disease through a complex interaction between the virus and the infected organism. Pathogenesis is the process by which an infection leads to disease. Viral pathogenesis is all about understanding the tropism of virus, type of organ involved and type of lesions produced, how viruses spread from the initial site of infection to become systemic and cause symptoms in a host is an important aspect of viral pathogenesis. By studying the pathogenesis one can understand the process of development of disease and accordingly plan its management.

Steps in viral pathogenesis are as follows:

1. Entry of virus into host
2. Primary replication of virus at the site of entry
3. Spread of virus to lymph nodes
4. Entry of virus into blood circulation
5. Primary viremia
6. Secondary replication in spleen, liver, bone marrow and endothelium
7. Secondary viremia
8. Cell/tissue tropism
9. Mechanisms of virus-induced cell injury
10. Lesions in cells/organs or organ invasion
11. Response of immune system
Entry of Virus into Host

Viruses are obligate intracellular parasites that can infect hosts by many routes, common routes are as follows:

A) **Respiratory tract**: Respiratory tract is the most common route of entry. There are some barriers which minimize the risk of disease like mucociliary blanket, alveolar macrophage, mucus produced by goblet cells, specialized lymphoid aggregates like NALT (Nasal associated lymphoid tissue) and BALT (Bronchus associated lymphoid tissue). After inhalation most of the virions are trapped in mucus and then by ciliary action carried from airways to pharynx, and finally either swallowed or coughed out from the host. For disease development viruses must overcome these defense mechanisms.

B) **Gastrointestinal tract**: Ingestion of virus contaminated food and water is an important route. The Gastrointestinal tract is protected from viruses by many factors like acidic pH of stomach, mucus found in intestine and stomach, enzymes found in digestive juices, bile, immunoglobulins and specialized lymphoid aggregates like GALT (Gut associated lymphoid tissue). Virus spread by this route must be adapted to the hostile environment of gastrointestinal tract. Lymphoid follicles are scattered throughout the intestinal mucosa. Intestinal mucosa is covered with a specialized follicle-associated epithelium on the luminal side consisting mainly of columnar absorptive cells and M cells (membranous epithelial cells). M-cell transcytosis is the mechanism by which viruses gain entry to deeper cells of the host from the intestinal tract. Viruses cause enteric infections are Rotavirus, Parvovirus, Coronavirus, Astrovirus, Torovirus.

C) **Skin**: Skin is the most effective barrier against viral infections, as the keratin layer of epidermis cannot support viral growth. Entry via skin occurs primarily when the integrity of the skin is breached by breaks, cuts, abrasions and punctures. In this case viral replication is usually restricted to the site of entry because the epidermis is devoid of blood or lymphatic supply for further spread. Viruses infect the epidermis produce local lesions (e.g.,
papillomaviruses) and viruses infect the dermis and deeper tissue produce generalized infection.

D) **Bite of Arthropod vectors**: Some viruses gain entry to the dermis or even direct into blood circulation through the bites of arthropod vectors such as mosquitoes, sand flies, mites, ticks and many other dipteran flies.

E) **Urogenital tract**: Some viruses gain entry through the urogenital tract. The urogenital tract is protected by various physical barriers, including mucus and low pH of the vagina. Some viruses gain entry as a result of sexual activities. Even normal sexual activity can also result in minute tears or abrasions in the vaginal epithelium or urethra which allows viruses to enter and produce diseases.

F) **Eyes**: There is usually less opportunity for viruses to infect through this route. Infection of eye occur only when it is injured or by abrasion. The route of entry for viruses is the epithelium covering the exposed part of the sclera and the conjunctivae. Direct inoculation into the eye may occur during ophthalmologic procedures or from environmental contamination. In most cases, replication of virus is localized and results conjunctivitis.

**Primary Replication of Virus**

The site of primary replication is the place where virus replicates after gaining entry to the host. It determines whether the infection will be localized or spread to become a systemic infection. Primary replication of viruses depends on virus ability to overcome host immune defense mechanism otherwise viruses are thrown out to the host without any replication in association with mucus or trapped in macrophages.

**Spread of Virus to Lymph Nodes**

Following entry and Primary replication, local spread of virus from cell to cell occurs frequently. Dissemination of virus is influenced by the manner of budding and site of budding of viruses from the cells. When viral progeny release from the apical surface of mucosal cells it favours the...
localized infections of tubular structures (air passages and the intestine). Whereas, release of virus from the basal surface into the subepithelial tissues favours the systemic infection.

Absence of suitable cell receptors and lack of permissive cells limit the ability of viruses to spread systemically. In subepithelial tissues, the progeny viruses enter the lymphatic system and transported to the regional lymph nodes by lymphatic vessels either as free virions or trapped in infected macrophages.

**Entry of Virus into Blood Circulation**

Viruses that escape from local defense mechanism produce infection by entering the bloodstream, this is known as hematogenous spread. Hematogenous spread starts when newly replicated viral particles are released into the extracellular fluids, which are taken up by the local lymphatic capillaries. These capillaries are more permeable than circulatory system capillaries which facilitates entry of viruses. In the lymphatic vascular system, virions pass to the lymph nodes. From the lymph nodes, viruses transported to the efferent lymphatics and then from thoracic duct and finally opens into the left subclavian vein. By this way virus gain entry in the blood stream, which is the most important route of dissemination.

**Primary Viremia**

Term viremia means the presence of infectious virus particles in the blood. These virus particles may be found free in the blood or found within the cells like lymphocytes. Enteroviruses, Parvoviruses, Flaviviruses and Togaviruses are circulate free in the plasma.

**Viraemia is of two types:**

**Active viremia-** when viremia is produced by virus replication in blood.

**Passive viremia-** when virus particles are introduced into the blood without any viral replication.

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The concentration of virions during primary viremia is low. Viruses carried in white blood cells like lymphocytes or monocytes, are not cleared from the host as viruses that circulate in the plasma. Cell-associated viruses are protected from immunoglobulins, complements and other plasma components, and they can be carried as “passengers” by these cells. Viruses emigrate along with white blood cells when these cells emigrate into tissues. Individual viruses exhibit tropism to special leukocyte populations.

Example - Monocyte-associated viremia → Canine distemper
Lymphocyte-associated viremia → Marek’s disease and bovine leukosis.
Erythrocyte-associated viremia → African swine fever and bluetongue virus.
Platelets- associated viremia → Equine infectious anemia virus, BVDvirus

Secondary Replication
Primary viraemia leads to spread of viruses in visceral organs like liver, spleen etc. where secondary replication takes place. These tissues are characterized by presence of sinusoids which are lined by macrophages. These organs are the primary constituents of reticulo-endothelial system. Function of reticulo-endothelial system is to filter the blood and remove foreign particles. Along with circulating leukocytes which provide a site for virus replication, viremia is usually maintained by infection of the parenchymal cells of target organs like lymph nodes, liver, spleen, endothelium, and bone marrow.

Secondary Viremia
After secondary replication in spleen, liver, bone marrow and endothelium there is massive secondary viremia, which may lead to generalized systemic spread. The concentration of virions during secondary viremia is very high. Virions invade the target organs and shedding of virus is seen in this stage. Shedding of infectious virions is important for maintenance of infection in
populations. Viruses that replicate only at epithelial surfaces there shedding usually occur from the same organ system involved in virus entry. In generalized viral infections, exit of infectious virions can occur from several sites. The amount of virus shed in secretion and excretion is important in relation to transmission. Body fluids like urine, saliva, semen and milk may contain viruses and help in propagation of disease.

Cell/Tissue Tropism
Viruses do not infect all the cells of a host but are infect to specific cells of certain organs. Viral affinity for specific body tissues is termed as tropism. For example, an enterotropic virus replicates in the gastrointestinal tract, whereas neurotropic viruses are replicates in cells of the nervous system.

Tropism is determined by
1. Receptors of Cells for virus.
2. Physical barriers.
3. Ability of the cell to support virus replication inside the cell.
5. Temperature, pH, and oxygen tension.
7. Digestive enzymes and bile in the gut that may inactivate some viruses.

Mechanisms of Virus-Induced Cell Injury
A general mechanism by which virus induces cell injury and death after cell/tissue tropism includes:
- Inhibition of Nucleic Acid Synthesis of Host Cell
- Inhibition of RNA Transcription of Host Cell
- Inhibition of Processing of Messenger RNAs of Host Cell
Inhibition of Protein Synthesis of Host Cell
- Interference with Cellular Membrane Function of Host Cell
- Cytopathic Effects of “Toxic” Viral Proteins on Host Cell
- Lysis of Host Cell

Lesions in Cells/Organs or Organ Invasion

Respiratory Tract
Viruses exhibit different levels of tropism for the respiratory tract, from the nasal passages to the terminal airways and alveoli. Lesions vary from mild inflammation to severe damage. For example, bovine rhinoviruses cause mild rhinitis, whereas respiratory syncytial virus causes bronchiolitis and interstitial bronchopneumonia. Some viruses cause injury to the type I pneumocytes or type II pneumocytes which lines the alveoli. Extensive injury to type I pneumocytes may lead to acute respiratory distress syndrome, whereas extensive injury to type II pneumocytes delays and impairs repair and healing in the affected lung. Initial injury is followed by progressive infection of epithelial cells of airways within the mucosal layer, and the severity of inflammation is increasing with exudation of inflammatory fluid and infiltration of inflammatory cells. Necrotic cellular debris and fibrin-rich inflammatory exudate accumulates in the lumen of the affected airways leading to subsequent obstruction and, in severe cases, resulting in hypoxia and various levels of respiratory distress. Regardless of the level of involvement of respiratory system viral infection leads to focal loss of integrity of the mucous layer lining, local cessation of ciliary activity and multifocal destruction of epithelial cells.

Gastrointestinal Tract
Infections with Enteric viruses usually result in rapid onset of gastrointestinal system diseases followed by a short incubation period, whereas systemic infections have a longer incubation period and are accompanied by clinical signs that are not confined to dysfunction of...
the gastrointestinal tract only. Diarrhea induced by virus results from the infection of enterocytes.

Astroviruses, coronaviruses, Rotaviruses and toroviruses specially infect the mature enterocytes lines the intestinal villi, whereas parvoviruses and pestiviruses specially infect and destroy the immature and dividing enterocytes present in the intestinal crypts.

Enteric virus infections begins from the stomach or proximal part of small intestine (Duodenum), and then spread towards the distal parts in form of a wave sequentially affecting the jejunum, ileum, and then large bowel (caecum, colon and rectum). As the infection progresses through the intestine the absorptive cells (enterocytes) destroyed by the virus are quickly replaced by immature enterocytes from the intestinal crypts. These immature enterocytes contributes to malabsorption and intestinal hypersecretion leading to diarrhea which results in fluid and electrolyte loss.

Severe diarrhea may lead to development of pronounced dehydration, hemoconcentration and acidosis. Diarrhea can be fatal in very young animals because of systemic electrolyte disturbances like decreased sodium and increased potassium ion concentration.

**Central Nervous System**

Central nervous system is susceptible to serious, often fatal injury by certain viral infections. Viruses travel from distal sites of entry to the brain via nerves or blood. To spread from the haematogenous route viruses must overcome the blood–brain barrier.

After entry within the central nervous system, viruses quickly spread to cause infection of neurons and glial cells like microglia, astrocytes, and oligodendrocytes. Infections of neurons caused by herpesviruses, togaviruses, flaviviruses, or other neurotropic viruses, leads to encephalitis or encephalomyelitis characterized by necrosis of neurons, neuronophagia, and perivascular cuffing. Rabies virus infection of neurons is non-cytocidal and produces very little inflammatory reaction. In canine distemper infection of glial cells in dogs leads to progressive demyelination of neurons. In prion diseases like bovine spongiform encephalopathy (BSE) in

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cattle and scrapie in sheep there is slowly progressive degeneration of neurons along with vacuolization.

**Skin**

The skin may be either invaded primarily as being a site of initial infection or secondarily via the haematogenous route. Skin lesions may be either localized like papillomas, or generalized. Commonly described virus-induced lesions that affect the skin are papillomas (warts), macules, papules, vesicles, pustules and scabs. Some times cutaneous lesions are found frequently in association with similar lesions in the oral and nasal mucosa, teats and at the coronary band (Junction of the hooves and skin) of ungulates which are suggestive of specific diseases. Vesicles are characteristic of foot- and- mouth diseases which are discrete blisters that result from accumulation of edema fluid within the epidermis, or separation of epidermis from the underlying dermis, or separation of mucosal epithelium from the submucosa. Rupture of vesicles leave focal ulcers which heal simultaneously. Papules may be either localized like in contagious ecthyma (orf) or disseminated as in lumpy skin disease. In poxvirus infections there are characteristic epithelial proliferations leads to raised lesions which become extensively encrusted with inflammatory exudate.

Virus infections result from widespread endothelial injury in blood vessels throughout the body resulting in subcutaneous edema, erythema and hemorrhages in the skin. Erythema of the skin caused by systemic viral infections is most commonly seen on hairless, exposed and non-pigmented areas such as the ears, paws, snout, scrotum, and muzzle and teat.

**Hemopoietic System and Immune response**

Viral infections of hemopoietic system have profound effects on immunity. Viruses like arenaviruses, hantaviruses, orbiviruses, filoviruses and bunyaviruses exhibit tropism for hemopoietic system. After initial invasion, viruses are phagocytized by dendritic cells and macrophages in thymus, bone marrow, lymph nodes, Peyer’s patches, and spleen.
Then infection spread in these tissues, leading to destruction of adjacent lymphocytes resulting in immune dysfunction.

Viral infections of hematopoietic system result in two forms:

- Immunodeficiency
- Generalized immunosuppression.

Systemic infections, in which mononuclear phagocytes or lymphocytes are affected, there is suppression of adaptive immune responses either humoral or cell mediated or both. Affected immunosuppressed animals are predisposed to various diseases caused by other infectious agents.

Immunosuppression induced by viruses lead to enhanced virus replication, like the reactivation of latent viral infections like herpes virus, adenovirus, or polyoma virus infections.

**Viral Infection of the Fetus**

Most viral infections of the dam have no effect on fetus, but in severe infections of the dam may lead to harmful effect on fetus like death and abortion even in the absence of fetal infection with virus. Some viruses like herpesviridae (varicellovirus), retroviridae (deltaretrovirus), reoviridae (orbivirus), bunyaviridae (bunyavirus) and flaviviridae (Pestivirus) can cross the placenta to infect the fetus.

In severe cytolytic infections of fetus, especially in early gestation period there is fetal death and resorption of fetus.

Teratogenic viruses produce developmental defects *in-utero* after infection. These viral infections are occurring during critical stages of organogenesis in the developing fetus and resulting in destruction of progenitor cells before they differentiate in organs like brain. For example: Cache Valley virus, bovine viral diarrhea virus, Akabane virus, and bluetongue virus cause congenitally teratogenic brain defects in ruminants.
Evasion of immune system

Viruses evade immune response by following methods:

a. By producing Latent Infection
b. By rapid Mutation
c. Inhibition of recognition by immune system
d. Targeting Host Cytokines
e. Lysis of cells of Immune system
f. Inhibition of Complement system

Inflammatory responses accompanying the most viral infections also contribute to viral disease pathogenesis. Immune-mediated tissue injury caused by viruses involves the four types of hypersensitivity reactions which are called as immunopathologic reactions.

In general, cell-mediated immunity plays the major role in clearing of virus infection from the host. Whereas humoral immunity protects the host against reinfection. Majority of viral infections are cleared from the host but certain viruses may cause persistent infections. There are two types of persistent infections of chronic nature.

**True Latency**—In this the virus remains completely latent e.g. Herpes virus

**Persistence** - In this the virus replicates continuously in the body at a very low level e.g. HIV.

**Conclusion**

Viral pathogenesis helps us to understand the disease progression and accordingly their diagnosis and treatment can be done. Thus by understanding the pathogenesis of viral infection one can manipulate their path ways of infection for controlling the disease. The treatment strategies may vary virus to virus dependent on the pathogenesis of disease. Interest in viral pathogenesis stems from the desire to treat or eliminate viral diseases that affect humans. This goal can be achieved in part by identifying the viral and host genes that influence the production of disease.
A fair knowledge about viral pathogenesis may help us in nearly accurate diagnosis and prompt treatment in order to control the deadly viral diseases. This can further help us to develop new vaccines and treatment methods for cure.

References

Outer Membrane Vesicles in Gram Negative Bacteria: Biogenesis, Functions and Isolation

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In prokaryotes vesicle formation was reported several decades ago; first in Gram-negative and recently in Gram-positive bacteria and also in Archaea and this vesicles are known by different names, like, membrane vesicles, extracellular vesicles, outer membrane fragments or blebs. The bacteria were shown to release outer membrane and periplasm through the production of outer membrane vesicles (OMVs) ranging in size from 20-250nm. The vesicles secreted by Gram negative bacteria were studied extensively owing to its multiple biological roles.

Components of OMVs

OMVs, the spherical bilayered structures are produced when a small portion of the outer membrane (OM) bulges away from the cell entrapping periplasmic content. Therefore, OMVs are mainly composed of LPS, periplasmic and outer membrane proteins and phospholipids. However, biochemical and proteomic studies have repeatedly shown the presence of components from the plasma membrane and cytoplasm. The presence of such components has been reported in \textit{Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Helicobacter pylori, Haemophilus influenzae, Francisella novicida and Neisseria meningitidis}. It is also proposed that the selection of cytoplasmic proteins in the MVs involves some specific sorting mechanism.
Biogenesis of OMVs

Since many years, the formation of OMVs was thought to be a physical process associated with the routine wear and tear of the outer membrane of the cell. However, recent discoveries showed the energy expenditure required for the sorting of biomolecules to OMVs, which contradicts the concept of OMVs as the products of mere material shearing of the membrane. The exact pathway of OMV biogenesis is not known till date. There are several mechanisms proposed for the biogenesis of OMVs. Some scientists consider OMV formation as a result of cell wall turn over or due to a stress response. A bilayer couple model for biogenesis of OMVs has been proposed in Pseudomonas aeruginosa, which emphasizes on the need of Pseudomonas Quinolone Signals (PQS) for the production of OMVs. The proteins PagC and OmpX in Salmonella enterica is also known to accelerate OMV formation. The formation of OMV can also result from the interplay between peptidoglycan, LPS and the outer membrane. The concept of ‘nanopods’ for OMV production is also gaining wide acceptance in case of organisms living in water scarce environment. However, none of the mechanism has proven conclusively for the biogenesis of OMVs, but it may be possible that all of them work together for the formation of OMVs.

Functions of OMVs

Many different functions have been demonstrated for OMVs, making them indispensable for the survival of these bacteria. The different functions furnished by OMVs include the secretion and delivery of proteins, membrane lipids and toxins at high concentrations and in close proximity to the targets. OMVs aid in bacterial survival via nutrient acquisition, biofilm formation and also they act as decoys for the phage and antimicrobials. OMVs are important in adhesive and invasive mechanisms of pathogens and in carrying antimicrobials targeting other species as well as toxins and virulence factors affecting host cells. All of this entitles OMVs as ‘bacterial bullets or virulence bombs’. OMVs are considered as an effective communication tool which enables intermicrobial communication, cross kingdom dialogs and ecological determinants as well.
Isolation and Purification of OMVs

The foremost step for OMV preparation includes cultivation and removal of intact bacteria, which can be furnished by differential centrifugation. A combination of sterile filtration and ultracentrifugation ensures better elimination of any contaminants. Purification of isolated OMVs can be done by density gradient centrifugation and gel filtration chromatography. Protein identification is done by usual procedures like SDS-PAGE and mass spectrometry.

Vesicle biogenesis is engineerable. Genome engineering enables sophisticated optimization of vesicle production and it is possible to tailor OMVs for specific applications such as OMV based vaccines and adjuvants.

References


Bacterial Profile: *Azotobacter*

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**Introduction**

*Azotobacter* is a genus of Gram-negative rod-shaped bacterium, belonging to the family Azotobacteraceae, according to the Bergey’s Manual of Bacterial Classification. The bacteria belonging to this family are characterized by their ability to convert atmospheric nitrogen (N₂) into biologically available form in their free-living habitat. *Azotobacter* is thus, a non-symbiotic diazotroph. The genus consists of seven species viz. *A. chroococcum*, *A. beijerinckii*, *A. vinelandii*, *A. armeniacus*, *A. paspali*, *A. nigricans* and *A. salinestri*. However, new species in this genus are being discovered.

**Discovery**

*Azotobacter* was first isolated by the Dutch Microbiologist and Botanist- Martinus Beijerinck, in 1901. The first species so studied was named *Azotobacter chroococcum*. The name is derived from “azoto”, which was the name ascribed to nitrogen gas by Lavoisier, for its inability to sustain life (in stark contrast to oxygen) and “bacterium”. The name, thus, relates to the ability of this organism to fix atmospheric nitrogen.

**Distribution**

*Azotobacter* is widely distributed in soil. The cells are most commonly isolated from neutral to alkaline soils. Some species are present to a greater extent in the rhizosphere than in root-less soil. The association between *A. paspali* and the grass *Paspalum notatum* is found to be specific. They are also found in the phylloplane, as commensal epiphytes on leaf surface. It has been
theorized that the carbohydrate-rich but nitrogen-poor exudates from the leaves act as enrichment for *Azotobacter*. Besides, these organisms are found in aquatic habitats as well. *A. chroococcum* and *A. vinelandii* also exist in marine environment.

**Cell Structure**

The cells of *Azotobacter* spp are rod shaped but have been described as oval to blunt as well; the cell size and shape differ as per species, strain and age of culture and growth conditions. A good example is the tapered ends found in *A. chroococcum*. The cell size varies from 2.0-7.0μ x 1.0-2.5μ. The G-C content of the genome is 63-67.5%. The cells produce extracellular slime, which is found to be correlated to the nutritional state of the organism, such as the type of carbon and nitrogen source.

*Azotobacter* spp are free-living, aerobic and motile organisms. The motility is conferred to the cell by peritrichous flagella. *A. chroococcum* is found to have the highest motility among the species so described. The cells occur in pairs but can occur singly or even in chains. The cells contain poly-β-hydroxybutyrate particles. The aged cells are produce melanin, a dark brown to black pigment. The mechanism involves the oxidation by tyrosinase.

The organism is found in two distinct states:

- **Vegetative state:**

  The cells are rod-shaped and are under the normal stage of metabolism, without environmental stress factors. This is more prominent in younger cells.

- **Microcyst:**

  Microcysts are spherical resting structures, which develop from the vegetative cells upon the advent of environmental stress such as dessication, radiation and sonication. The microcyst arises from a single vegetative cell and has a contracted cytoplasm. It is covered by a cyst wall consisting of an exocystorium and an exine and is able to withstand harsh conditions. Unlike a spore, the cellular content in the contracted central body undergoes no cytological changes.
before germination and is very similar to the vegetative cell. Upon germination under favorable conditions, a single cell exits the cyst, leaving behind a hollow covering.

**Isolation**

*Azotobacter* spp are isolated and identified using morphological and physiological characters in combination. *Azotobacter* spp are found in soil and are isolated by inoculating the soil sample in a nitrogen-free medium. The lack of nitrogen is selective for diazotrophs, which can fix the dinitrogen in the ambient air. The media generally used are *Azotobacter* media, Ashby’s mannitol agar, Burk’s medium and so forth. The colonies appear soft, white and mucoid. The organism is capable of utilizing a wide variety of carbon sources such as alcohols, sugars, organic acids and their salts. The organism is mesophilic, with an optima being in the 25-30°C range. The cells require high humidity, similar to the requirements of higher plants, to grow. The organism is cultured aerobically and is catalase positive. The optimum pH range is 7.2-7.6.

In culture, the efficiency of N₂ fixation can be improved by reducing the oxygen tension or the concentration of carbohydrates to mimic the conditions pertaining naturally in soil. *Azotobacter* is differentiated from Rhizobia based on the fact that unlike the latter, *Azotobacter* spp are not symbiotic and can fix N₂ under higher oxygen tension. Also the Rhizobia exist as pleomorphic bacteroides in the root nodules from where they are isolated, most prominently observed under the microscope as cells with variable shape, unlike *Azotobacter*. Furthermore, Rhizobia require a greater number of organic growth factors. Similarly, the *Azotobacter* spp are differentiated from the *Azomonas* spp (also members of the same family) simply due to presence of microcysts.

**Benefits for Plants**

- **Nitrogen Fixation:**

The most significant contribution of *Azotobacter* is the conversion of biologically unavailable dinitrogen from the atmosphere to biologically available forms such as ammonia and amino
acids. The most important enzyme involved in this process is nitrogenase. Mo-Fe associated nitrogenase is the basic version of nitrogenase used for nitrogen fixation. The process is highly energy-demanding and utilizes many moles of Adenosine Triphosphate (ATP) to fix a single mole of dinitrogen. The process is impeded by high oxygen concentration; thus, the cell employs nitrogenase-protective proteins along with a very efficient metabolic machinery to reduce intracellular oxygen level. *Azotobacter* has several types of nitrogenases. An alternative form of this enzyme used by the organism is a Vanadium-dependent nitrogenase, more active at lower temperatures.

- **Growth Promoters:**
  *Azotobacter* exudes B vitamins into the soil, including riboflavin, thiamine, nicotinic acid and pantothenic acid and biotin in different plant rhizospheres. It also produces plant growth hormones such as auxins, including indole acetic acid (IAA), gibberlins, cytokinins, which promote germination of the seed along with shoot and root growth and leaf expansion. Thus, *Azotobacter* is taken as a plant growth promoting rhizobacterium (PGPR).

- **Antifungals:**
  *Azotobacter* has been known to produce fungistatic and fungicidal compounds against plant pathogens such as *Fusarium* spp, *Alternaria* spp, *Trichoderma* spp, *Colletotrichum* spp, *Rhizoctonia* spp, *Cephalosporium* spp and so forth. The organism is known to produce siderophore, which binds Fe$^{3+}$ ions in the rhizosphere, making it unavailable to the pathogens. Its antibiotic property is also attributed to the production of hydrogen cyanide (HCN), and to competition for substrate and space.

**Use in Agriculture**

A carrier-based *Azotobacter* inoculant has been applied to soils. The carriers so used include lignite, a mixture of charcoal and soil and also farmyard manure. Similarly, liquid cultures have been used for foliar application, especially in combination with other known nitrogen-fixers such as *Beijerinckia*. Seedling-inoculation (onto the roots) using a carrier-slurry has also been
suggested. Seed-inoculation with carrier-based slurry is a popular method, which attaches the diazotroph directly onto the surface of seeds. Such application of the diazotroph has augmented the rates of germination of seeds and the development of leaves, roots, flowers and fruits. This has been ascribed to both nitrogen-fixation as well as the release of growth-promoting factors by the organism. The increase in leaf surface area increases the rate of photosynthesis. The favorable effects of the organism have also been ascribed to increased uptake of nitrogen, phosphorus, along with micronutrients such as iron and zinc by the plant. The supply of nitrogen is especially well-pronounced in nitrogen-deficient soils. *Azotobacter* inoculants have also been shown to raise the tolerance of the plant towards reduced water availability.

According to Shende and Apte (1982), plant growth responses to *Azotobacter* inoculation ranged from 6.7-71.7% rise in crop yield, in different cereal and cash crops such as cotton, maize and sorghum. Paul and Verma (2005) have noted marked rise in crop yield in paddy, wheat, fingermillet, sorghum, onion, potato, cotton and mustard. Baral and Adhikari (2013) have noted a 15-35% increase in the yield of maize using *Azotobacter* inoculants over non-inoculated sample. They also discovered that the effect of the organism was more pronounced when the field was not treated with chemical fertilizers.

The rate of N₂ fixation in a given patch of soil has been found to increase in mixed cultures of *Azotobacter* with other microbes compared to the diazotroph alone. Verma and Neelakantan in 1967 found such an increase in mixed cultures with *Rhodopseudomonas capsulatus* and *Cylindropermum muscicola*. Here, it has been hypothesized that a micro-ecosystem of blue-green algae, *Azotobacter* and the other heterotrophs and autotrophs is established. Similarly, the combined effect of arbuscular mycorrhizal fungi and *Azotobacter* is more prominent in soils of low fertility compared to a single organism. A similar effect is found in case of *Azotobacter* and a phosphate solubalizer.

The long-term deteriorative effects brought about by continued use of chemical fertilizers call for the better use of biofertilizers instead. *Azotobacter*, the non-symbiotic diazotroph, therefore,
holds a lot of promise in this regard. Judicious use of the organism can not only help increase crop yields but also improve soil health as a whole.

References

Mycorrhiza as Biocontrol Agent

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It is studied that microorganisms in soil and in other substrate growing around the plants roots and hence create rhizosphere. Rhizosphere develops at the interface of root and soil so microorganism interacts with both plants as well as with soil. The interaction between roots, soil and microorganism are by chemical, biological and sometime physical. So, the interaction between these organisms can be categorized into three major categories Interaction of a plant with other plant due to interaction of rhizospheres. Due to this interaction competition for nutrients increases among plants. Interaction of plant roots with microorganisms. It is due to activities of plants that enhance the activity of microorganism to grow in rhizosphere and to infect plants for beneficial or to cause disease. Interaction of microbes with one another.

Biological control is defined as to protect plants against pathogen by controlling factors influencing growth of plants. Commonly plants have an association with their roots known as mycorrhizae. Mycorrhiza is defined as a mutualistic association of plants with soil borne fungi. Arbuscularmycorrhiza is considered to be more common type of such association. This association saves plants from different pathogens by acting as biocontrol agents.
Mycorrhiza as biocontrol Agent

Protection against pathogens

Many agricultural practices are used to control soil borne pathogens. These techniques include resistant cultivars, seed certification, crop rotation, fumigation of soil, and many chemicals like fungicides. Due to less resistance in plants against pathogen and less chances of control of pathogen inoculum it’s difficult to control plant pathogens. Due to these reasons, researchers trying to use other alternatives against these pathogens like microorganism added to the soil and used fungus inoculum these organisms increase resistance in plants against pathogen by competing with soil borne pathogens for nutrients and space by secreting adding antibiotics or act like parasites for those pathogens or induced resistance in plants.

It is studied that using chemicals against soil borne pathogens also damages plants growth as well so creating an alarming situation that’s why scientists suggest and uses alternative agents for controlling pathogens like fungi and other microbes like bacteria. Many researchers studied that arbuscular mycorrhizal association is very beneficial against soil borne pathogens. There is a complex interaction between soil pathogen, plant and fungal partner which reduced the disease effect cause by pathogens in host plant which is colonized with arbuscular mycorrhizal fungi.

It is reported that when tomato roots were associated and colonized by *Glomus mosseae* it reduced the disease effect of pathogen *Phytophthora parasitica* as compared to non mycorrhizal associated tomato roots. It is also reported that when oil palm plant were colonized by arbuscular mycorrhizal fungi it reduced the disease effect of *Ganoderma boninense* and seedlings of oil palm plant are more resistant to *Ganoderma*. 
Arbuscular mycorrhizal fungi interact with soil borne pathogen and reduced their effect on plant by antagonism, mycoparasitism and antibiosis. During this association AMF causes certain changes like biochemical changes in host plant, microbial changes in rhizosphere, status of nutrients in soil, change the anatomy of plant cells, changes morphology of roots and reduced stress.

**Damage Compensation**

Arbuscular mycorrhizal fungi also increase resistance in plants against pathogen by compensating the damage caused by pathogen by increasing root growth and development. The root biomass loss and damages in roots by fungi and nematodes reduces by this way. Arbuscular mycorrhizal fungi when colonized the plants they reduce these loses by increasing root surface area for better uptake of mineral nutrition for better growth and development of plants by fungal hyphae which growing outside in the soil as well as inside the root cell by formation of arbuscules.
Competition with Host Photosynthates

It is revealed by many studies that the plant photosynthates are the major reason for the growth of soil borne pathogens and arbuscular mycorrhizal fungi that’s why both these pathogen and fungi compete for these photosynthates like carbon compounds getting from roots. In this competition arbuscular mycorrhizal fungus is better than pathogens for taking carbon compounds from plant that’s why inhibit the growth of pathogens. It is studied that 4-20% of carbon compounds are transferred to AMF rather than pathogens while there is not much data in support of this concept.

Interactions of Soil Microbial Population

The interaction of arbuscular mycorrhizal fungi with pathogens and their ability to increase plant nutrition is studied with reference to the growth of host plant. There is little information that how these interaction effects plants. It is studied that plants with mycorrhizal association differ from the plant having no such association in the rhizosphere, due to these interactions quality of respiration of root and the quantity of the chemicals secreted from roots altered. These interactions between pathogens and fungi also effect the growth of fungi. In the presence of bacteria in soil, the hyphae emerging from the germination of fungal spore developed smoothly
and form small vesicles and more branched and longer than the hyphae grow in the absence of such bacteria.

AMF changes the mycorrhizosphere by changing the number of such pathogens as well as their activity as a pathogen. Plants with arbuscularmycorrhizal association with their roots show different in their effect on bacterial community composition within rhizosphere. It is studied that many biotic and abiotic factors affect the efficiency of arbuscularmycorrhizal fungi as a biocontrol agent. These factors are Soil moisture, Soil content, Genotype of Host, Inoculum of Mycorrhiza, Inoculation time of mycorrhiza, Virulence of Mycrohhizal species, Inoculation potential of pathogen, Microflora of soil.

It is studied that the protection of plants against all such type of pathogens is totally depend upon the rate of AMF colonization of plant roots. There are two zones of interaction between pathogens and AMF and these two are mycosphere and rhizosphere. There are some bacteria present in the rhizosphere known as Mycorrhization helper bacteria (MHB) which increase the ability of mycorrhizal fungi to colonize the plant roots.
Competition for Colonization and Infection site

There is a competition between pathogens and arbuscular mycorrhizal fungi in rhizosphere for occupying more space and it is important for explaining the interaction between pathogens and AMF. One phenomenon as already mentioned is that mycorrhizal fungi depend upon the host photosynthates like carbon compounds which are the major cause of suppression of pathogens growth in rhizosphere. It is also noticed through several studies that presence of arbuscules in root cells hinderance the penetration of pathogens in the cells. So there is a competition for space between pathogens and mycorrhizal fungi to penetrate inside the cells of cortex and epidermis.

Morphological and Anatomical changes

The roots of plants which are in mycorrhizal association with AMF have some morphological and anatomical modification in their root systems. Colonized roots are highly branched as compared to non-colonized roots and adventitious roots also have large diameter in mycorrhizal associated plants. Due to such modification in root system morphology certain pathogens attacks are reduced these changes also include highly lignified epidermal cells of roots. High rate of lignification in cells prevent the penetration of other pathogens and it also increases the phenolic metabolites inside the host plant. Other changes include leads to a bigger size of roots, dense branching, and increase number of root tips, length, surface area and root volume.
Changes in Root Exudates

It is studied that some toxic chemicals like phytoalexins released and produced by arbuscular mycorrhizal fungi but it also noticed that such chemicals are not observed during early stages of formation of arbuscular mycorrhizal fungi but observed in later stages of this association. But activity of peroxidases observed in early stages. For example development of *Phytophthoraparasitica* decreases in tomato roots when these roots are colonized by *Glomus mosseae* and in non-colonized parts show defense responses and accumulate phenolic compounds. Cortical cells of the same plant also show localized immune response so they are immune to pathogens.

During this study it is noticed that some corresponding proteins are also involved in the plant defense mechanism in plants associated with AMF. These include glycoprotein rich with hydroxyproline, chitinases, phenolics peroxidases, PR-pathogenesis proteins and B-1-3 glucanases-callose proteins. In establishment of arbuscular mycorrhizal association root exudates play an important role. Growth and germination of many pathogens inhibited in the presence of such exudates. For example the sporulation of *Phytophthora fragariae* is suppressed by root exudates released from strawberry plant which is
associated with AMF. It is suggested that phenolic compounds bound with the cell wall is the indirectly related to the resistance of plant roots which are colonized with AMF to pathogenic fungi so the resistance of cell wall against the action of digestive enzymes increased.

**Nutrients uptake:**

The actual role of mycorrhizal association is the increase the yield of production of plants. AMF is important in increasing the rate of uptake of mineral nutrients for the proper growth and development of plants. Due to increased rate of uptake of mineral nutrients plants have ability to stand against the pathogen and reduce uptake of toxic compounds to plant growth. AMF association provide greater root surface area which cover greater volume of soil and these roots penetrate in the depletion zone for better uptake of nutrients and water. In mycorrhizal roots, their weight, length, number and layer diameters increased than non mycorrhizal. The average diameter of fungal hyphae is less than the root hairs that’s why these hyphae penetrate soil pores and contact with soil where root hairs unable to reach. Am roots increased the nutrients uptake from the soil and it is studied that the phosphorous uptake is more enhanced by AMF than other nutrients. Absorption of phosphorous from soil is complete and faster in mycorrhizal associated plants than in non mycorrhizal because the distance of diffusion of $\text{HPO}_4^{2-}$ and $\text{H}_2\text{PO}_4$ ions in the soil is shorter for fungal hyphae than the roots of plant. Due to increase rate of mineral nutrition uptake there is many physiological changes in root.
It is consider that increase in phosphorous uptake is one of the most important benefits of mycorrhiza. Phosphate present in soil is converted into polyphosphate by polyphosphate kinase enzyme in vacuole and then transported from fungal hyphae tip to the sink of symbiotic interface. The rate of translocation is affected by the net efflux of phosphorous at hyphal tips and net uptake. AMF is also increasing efficiency of nitrogen uptake and growth in legumes. Due to high uptake of phosphorous, nitrogen uptake of nitrogen also increases to accomplish high demand of nitrogen. Hyphae of AMF have ability to uptake nitrogen from the soil and transported to the plants. The regulation of nitrogen uptake by fungal hyphae is regulated by the demand of plants for nitrogen. When nitrogen presents in the soil in the form of NH$_4^+$ and NO$_3^-$ then AMF uptake these minerals and transported them to the plants. When nitrogen is applied to the soil in the form of (NH$_4$)$_2$SO$_4$ then fungal hyphae uptake about 40% of this nitrogen and some nitrogen transported over a distance of 5cm. other than phosphorous and nitrogen AMF also increases the uptake of many other minerals like Zn, S, Fe and Ca. when plants associated with AM grown in low pH then the Fe uptake increases than when grown in high pH. But it was noticed that Manganese uptake is relatively lower in AM as compared to non AMF.

**Bioremediation**

AMF plants also show effect on uptake of trace elements. When AMF associated with plants grown in a soil having low concentration of Cu and Zn their uptake is increases by AMF. The accumulation of Cu and Zn is increases in shoot and leaves in mycorrhizal plants as compared to non AMF.
non mycorrhizal. Uptake of Sulphar also increases in plants associated with AMF. In acidic and alkaline soil uptake of Boron also increases on the other hand uptake of potassium, calcium and magnesium also enhanced by AMF as compared to non mycorrhizal. In acidic soil uptake of toxic Aluminium is lower in AM than in non AM. AMF also increases the acquisition of trace elements from the soil like Zn, Mg, Mn, Cd, Cu, Co, Cs, Ni, and B etc.

**Mycorrhizosphere**

Mycorrhizosphere is defined as the zone of soil associated or in interaction with mycorrhizal fungi. It is observed that the rhizosphere soil extract of mycorrhizal plants reduced the formation of sporangia of pathogenic fungus than the rhizosphere soil extract of non mycorrhizal. The reason behind this is may be the absence of sporulation inducing microorganisms or the number of sporulation microrganisms increased. Microbial communities around the roots are affected by changes in exudates of roots, leading to the formation of mycorrhizosphere. In mycorrhizosphere the mycorrhiza influence the microbes of rhizosphere. The microflora in mycorrhizosphere differ quantitatively as well as qualitatively as compared with non mycorrhizal. Mycorrhizosphere has two components a layer of soil surrounded the mycorrhizal roots and a layer of soil surrounded the arbuscular mycorrhizal hyphae in the soil known as hyphosphere. There are two possible interactions between soil borne pathogens and AMF, these interactions may be positive (synergistic) or negative (antagonism). In positive interaction, AMF interacts with Plant Growth Promoting Bacteria, nitrogen fixing bacteria, phosphorous solubilizing bacteria which enhance the germination of AMF spores. In negative interaction is the ability of AMF to reduce the presence of soil borne pathogens.

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Thanks,

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