RESEARCH PLAN PROPOSAL

on the title

"Physico - Chemical Characterization and Toxicological Evaluation of Liquid Effluents Generated by Health Care Establishments of Jaipur."

for registration to the degree of

Doctor of Philosophy

IN THE FACULTY OF SCIENCE

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Submitted by

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Introduction :-

In spite of the fact that the problem of waste management is a very urgent issue for every community around the world , health care establishments, research facilities and laboratories produce large quantities of waste that is duly monitored by few of them. There are some regulations and standards for preventing the pollution of rivers and underground resources (USEPA 1992; WHO 2004; AWWA 1990; APHA 2005 and Corbitt 2004).

Waste water composition refers to the actual quantity of physical, chemical and biological constituents present in the waste water. Chemical residues discharged into the sewerage system may have adverse effects on the operation of biological sewage treatment plants or toxic effects on the natural ecosystem of receiving waters. For instance, uncontrolled discharge of sewage from field hospitals treating cholera patients have been strongly implicated in cholera epidemics in some Latin America (Pruess *et al* ., 1999). Water genotoxicity studies are of interest because epidemiological investigators have shown a link between genotoxicity of drinking waters intake and risk of cancers (Koivusalo *et al* ., 1997).

Health care establishments, particularly hospitals consume an important volume of water per day. Indeed the consumption of domestic water, is an average 100 liters / person /day (Gadle, 1995) while the value generally admitted for hospitals varies from 400 to 1200 liters /day / bed (Delforre –Bonnamour ,1995; CCLIN ,1999).

Hospitals thus represent an incontestable release source of many chemicals compounds in their wastewaters, and which may have an impact on the environment and human health. Indeed, some of the substances found in wastewaters are genotoxic and are suspected to be a possible cause of the cancers observed in the last decades (Jolibois and Guerbet, 2005).

Hospitals effluents are serious problems in developing countries like India and when not treated adequately , can cause mutagenic effects on living organisms . In past decades , the scientific community has shown interest in amplifying their knowledge and control on hospital effluents. The impacts caused by toxic agents on the environments and humans , often are not capable of observed and measure directly (Bagatani *et al* .,2009). Genotoxic chemicals emissions from anthropogenic activities into environmental compartments require genotoxic assays for the assessment of the potential impact of these sources on the ecosystem. For example, some drugs like cytostatic agents are genotoxic .

Result of recent studies indicate the presence of low concentrations of antibiotics in municipal waste waters effluents and surface waters (Hartmann *et al*., 1998; Hartig *et al* ., 1999 ; Hirch *et al* ., 1999 ; Alder *et al* ., 2000 ; Meyer *et al* ., 2000 ; Nipales *et al* .,2000 ; Frick *et al*., 2001). With the passage of time , the problem of antibiotics resistance is increasingly alarming.

The application of Ames and Hanster cell test on hospital waste waters indicate that these effluents are potential mutagenic (Gartiser *et al* .,1996).The origin of this mutagenic potential remains to be investigated . The value of the total hospital waste

water show high toxicity as determined using the *Daphnia* and lumincisent bacteria tests (Leprat ,1998; Emmanuel *et al* ., 2001 and Jehanin , 1990).

In view of above mentioned content, this study focuses mainly on hospital waste waters of capital city of Rajasthan state - Pink city Jaipur , along with the waste waters from the diagnostic laboratories .

Short-term genetic bioassay have proved to be an important tool in these studies because of their simplicity , sensitivity to genetic damage , speed , low cost of experimentation and small amount of sample required.The present study is thus aimed at studying the mutagenic potential of final discharge from hospitals.

We also aimed to monitor the genotoxicity and mutagenicity of hospital waste water at different stages of treatment, to evaluate the efficiency of plants situated in hospitals along with this, we also evaluate the genotoxicity of Diagnostic Research laboratories situated in the middle of Jaipur .

Review of literature:-

In the last few decades , increasing attention has been paid to the presence of emerging pollutants in waste waters, surface waters and ground waters. (Daughton and Ternes ,1999 ; Heberer, 2002 ; Barcelo , 2003; Daughton, 2004).Emerging contaminants correspond in most cases to regulated pollutants ,which may be candidate for future regulation depending on research on their potential data regarding their occurrence .

Water is also convenient and versatile solvent that is often used to transport waste products away from the site of production and discharge. Unfortunately, the transport waste products are often toxic and their presence can seriously degrade the habitat of the river, lakes harbor or stream that receives them (White and Rasmussen , 1998). Consequently water comes that are located near environmentally impacted regions may have toxic characteristics that make them unfit for the irrigation of vegetables and fruit trees and may even be prohibitive for aquaculture and consumption by the animals that live there (Margarte *et al* ., 2011)

Hospital wastes could be dangerous to the ecological balance and public health. Pathological, radioactive, chemical, infectious, and pharmaceutical wastes, if left untreated, could lead to outbreaks of communicable diseases, diarrhea epidemics, water contamination, and radioactive pollution (Ajay Kumar *et al.,* 2006). However, the knowledge about the hospital waste water may have an impact on the environment and human health . Different review of literature have reported the fate of some pharmaceuticals compounds as well as their occurrence and effects in the aquatic environments (Richardson and Brown , 1985 ; Halling – Soreson *et al* ., 1998). Many of these chemical compounds resist normal wastewater treatment. They end up in surface waters where they can influence the aquatic ecosystem and interfere with the food chain. Humans are particularly exposed by the drinking water, produced from surface water (Pauwels and Verstraete, 2006).

Waste effluent from hospitals contains high numbers of resistant bacteria and antibiotic residues at concentrations able to inhibit the growth of susceptible bacteria (Grabow & Prozesky ,1973; Linton,1974) .Accordingly ,hospitals waste effluents could increase the number of resistance bacteria in the recipient sewers by bath mechanisms of introduction and selection of resistant bacteria (Al-Ahmad *et al* ., 1999). Due to heavy antibiotics use , hospitals waste water contains larger number of resistant organisms than domestic waste water.

The hospital liquid waste discharges have been analysed physico-chemically in many studies (Emmanuel *et al*., 2002; Mahvi *et al*., 2009). These studies reveal that organic matter can reach high concentrations in these effluents. However, very few literature is available regarding the evaluation of genotoxicity of hospital waste water. Even if no standard followed protocols for sample collection, sample processing, or selection of tests exist, all the studies done so far show that the hospital wastewater could have a genotoxic potential (Giuliani *et al*., 1996; Steger-Hartman *et al*., 1997; Hartman *et al*., 1999; Jolibois *et al*., 2005a; Emmanuel *et al*., 2002; Paz *et al*., 2006).

It is extremely difficult to quantify the risk associated with these chemical pollutants because they usually occur in the concentrations too low to allow analytical determination and putative mutagens, with few exceptions have never even been identified. Moreover, the composite effects of mixtures cannot be readily assessed via analytical methods. Thus, only physico-chemical analysis is not sufficient to estimate the potential harmful effects of hospital effluents.(Jolobis and Gilbiert , 2005). Thus the toxicity is often evaluate by means of biological tests ,e.g., bacterial genotoxicity test which do not require a *priori* knowledge of toxicant identify and physical – chemical properties (Jolobis and Gilbiert , 2004).

Hospital waste water effluents have been evaluated using a number of bioassays employing crustaceans like *Daphnia magna* (Emmanuel *et al*., 2002) and plants like *Allium cepa* (Paz *et al*., 2006; Bagatini *et al*., 2009). So far, study utilizing whole organism to study the genotoxic and cytogenetic potential of hospital effluents has never been reported. However, there exist two studies which had make use of animal cell lines such as V79 cell lines (Hartmann *et al.*, 1999) and primary rat hepatocytes (Ferk *et al*., 2009).However, the main disadvantages associated with animal and plant bioassays are: problems with standardization of the organisms, requirements for special equipment and skilled operators, long duration of the assay and lack of reproducibility.

Several *in vivo* and *in vitro* studies have been shown that chromium compounds damage DNA in a variety of ways , including DNA single and double stranded breaks generating chromosomal exchanges ; formation of DNA adducts and alterations in DNA replication and transcriptions (Zhitkloich *et al*.,1996 ; O'Brein *et al* ., 2001; Mustsumato *et al* .,2003 ;Mustsumato and Marian Morales ., 2004; Mutsumato *et al.,*2006). Therefore, evaluation of biological effects using a rapid, simple, sensitive and cost effective method could indicate specific information on toxicity and ecotoxicity and allow incorporation of toxicity parameters in the regulatory framework (Parvez *et al.*, 2006). In India, not much work has been done regarding the genotoxic potential of waste water generated from health care establishments.Till now only one report is available ,concerning genotoxicity evaluation of waste waters fielded by few prominent

Delhi hospitals .It is also first to look at the efficacy of onsite treatment in reducing mutagenic activity (Gupta *et al.,* 2009).

Genotoxicity and mutagenicity testing of waste water samples is important for human and environmental hazard evaluation .The proposed study , to estimate genotoxicity and mutagenicity of hospital effluents will provide an exact idea of the unfavorable effects generated due to waste waters from clinical laboratories and multi - speciality hospitals.

HYPOTHESIS:-

Short term microbial bioassays are successful in evaluating the genotoxicity of hospital waste waters.

OBJECTIVES:-

The proposed study is designed to:

1. Evaluate the genotoxic potential of waste effluents that are discharged from prominent hospitals of Jaipur.
2. Analysis of untreated and treated liquid waste generated by prominent hospitals and diagnostic laboratories of Jaipur for :
    - Physico-Chemical characteristics
    - Genotoxicity (Mutagenicity) and
    - Chromosomal Aberrations Assay
3. Compare the efficiency of short term microbial bioassays.
4. Development of a test battery of short term bioassays which could be significant in evaluating the genotoxicity in the treated and untreated liquid waste of hospitals and diagnostic laboratories.
5. To check the effectiveness of the liquid waste treatment plants functional in the Health care Establishments.

Methodology :-

I. Sampling of waste water :-.

Sites: Two main hospitals have been identified for the study.

(a) ***Santokba Durlabhji Memorial Hospital*** : Santokba Durlabhji Memorial Hospital (SDMH) is the largest private sector multi specialty hospital in Jaipur with the bed strength of 400. Operation theatres, ICUs laboratories, utility services and many more specialties are there.**The hospital has the treatment plant for the hospital liquid waste .**

(b) ***Fortis Hospital*** :- Fortis is a Multi Super-Specialty Hospital with major focus on super specialties of Cardiac Sciences, Neurosciences, Renal Sciences and GI Diseases backed up by a wide range of specialties. Its having 7 operation theatre and 210 operational in -patients beds with total bed strength of 350 . **The hospital has the treatment plant for the hospital liquid waste** .

Following **Two Diagnostic Laboratories** have been selected for the study .

(c) ***Dr. B. Lal's Clinical Laboratory :*** It has attained an exemplary reputation for its services in the field of clinical investigations. The centre has expanded the facilities for rare investigations in the field of infectious diseases, endocrinology, oncology, gastroentrology etc**.The laboratory do not have any treatment plant for liquid waste.**

(d) ***Getwell Daignostic Center*** : Getwell Poly Clinic & Hospital of Jaipur was originally associated with 20 doctors that specialize in various area of Diagnostic services for their patients. Over 200 doctors now utilize Medical Diagnostic Center's services. It is a fully accredited facility that offers state of the art multi modality diagnostic imaging (medical) equipment such as MRI 1.5 Tesla, Sonography, Mammography, Digital X-ray, General X-ray and CT Scan. **The laboratory do not having any treatment plant for liquid waste** .

II . ***Sampling :*** Samples of liquid waste from the above sites will be collected twice in a year between 8am to 6pm to cover the maximum hospital activities .

- Untreated sample – from the main sewer of the hospital where the entire water from the premises is collected .
- Samples after filteration and aeration : waste water.
- Finally the treated sample after chlorination

III. ***Characterization of liquid waste :***- The samples will be analyzed for various physico- chemical and biological parameters like pH, total dissolved solids(TDS) , Dissolved oxygen (DO), Biological oxygen demand (BOD),Chemical oxygen demand (COD) and bacterial count as colony forming units (CFUs) as per standards methods . (Grab Methodology,APHA,1995).

IV. ***Microbial Mutagenicity Assays*** :- Following Bioassay will be performed for all the collected samples .

(a) ***Salmonella Mutagenicity Test (Ames test)*** - This assay is used for detection of mutagenic material in waste and potable waters. The test uses several strains of the bacterium *Salmonella typhimurium* that carry mutations in genes involved in histidine synthesis i.e. it is an auxotrophic mutant, so that they required histidine for growth. The variable being tested is the mutagen's ability to cause a reversion in growth on a histidine-free medium. The tester strains are specially constructed to have both frameshift and pointmutations in genes required to synthesize histidine. *Salmonella* reversion assay will be conducyed using the plate incorporation procedures (Ames *et al* .,1975 and revised by Maron and Ames 1983) .The samples will be analysed with and without the hepatic S9 fraction , which incorporates an important aspect of mammalian metabolism into the *in vitro* test . The protocol used will be based on method described by Maron & Ames (1983).

(b) ***SOS Chromotest -*** The SOS Chromotest (EMBI ,Ontorio ,Canada) has been designed to test both for the presence of genotoxins in solution and genotoxins that may be transported into cells through direct contact. Potential applications of the SOS-chromotest that take advantage of the genetically engineered strain of *E. coli* include testing of effluents for possible mutagenic or carcinogenic genotoxic compounds. The test provides a colorimetric endpoint through which the presence of genotoxic materials can be determined and when done in solution and absorbance measured, allows the calculation of an SOS induction (factor or) potency (SOSIP) or slope factor by which the relative strength of genotoxic compounds or mixtures can be determined. The test employs a mutant PQ37 strain of *E. coli* in which the SOS gene complex repair promoter region of the genome that is responsible for activating the SOS genes, has been linked to the β gal (lacZ) gene responsible for the production of the β-galactosidase enzyme. The degree to which the cell is trying to repair DNA damage using the SOS gene repair complex is now directly linked to the production of β-galactosidase which is measured by the enzyme's reaction with a blue chromogen (Jolibois B, Guerbet M. 2005b).

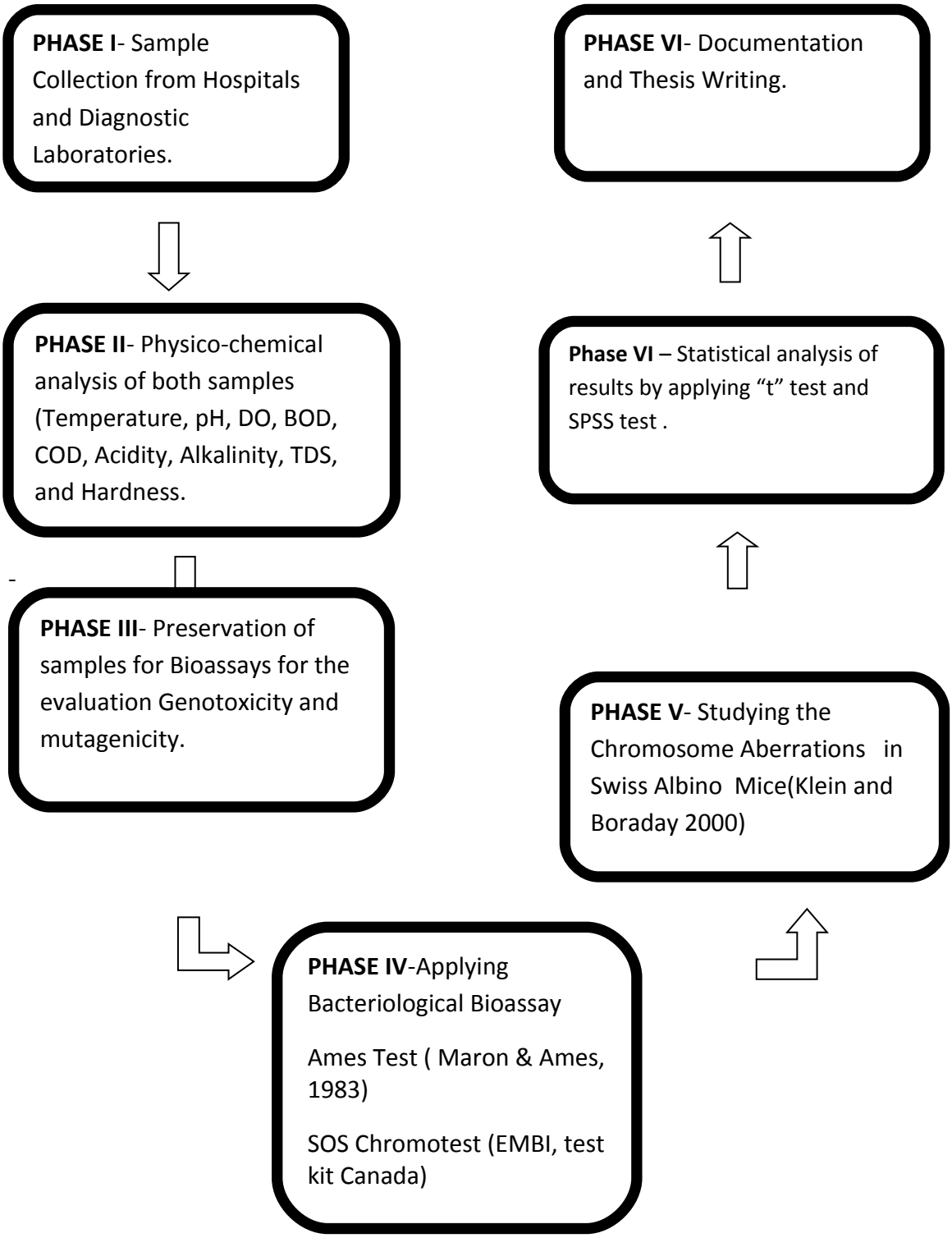
V. ***Chromosome Aberrations Assay***:- **Geimsa or Giemsa /trypsin** staining method with sub protocol of **preparation of metaphase chromosome spreads** will be performed for the study of chromosomal aberrations employing the protocol by Klein ,Borday and Costa, 2001. 10% (w/v) Geimsa solution (for Geimsa staining) or 5% (w/v) Geimsa solution (for trypsin/ Giemsa stain , dilute geimsa stain,1:9 (for 10%) or 1:19(for 5%) in Sorenson's phosphate buffer, pH 6.8 ,Prepare dilute stain fresh will be used to study .At different concentrations will be tested high dose to low dose with durations *viz* 24hrs, 48 hrs and 72 hrs as per the period protocol to get an insight into the fact that how drug will react to genetic material when exposed for longer duration .Two replicates will

be used for each concentration**.** Cyclophosphamid (CP) will be used as positive control and distilled water will be used as negative control. Colchicine will be added to the culture prior to harvesting at 68hrs of plantation. After colchicine treatment, cultures will be harvested and slides will be prepared by air- drying method. Giemsa (5%) in Sorenson's buffer (pH 6.8) will be used for staining. Screening of slides will be done at 1000X for visualization of structural anomalies. Chromosomal aberrations will be counted per 100 metaphase cells per concentration. Nomenclature for chromosome aberration will be followed according to the criteria given by the report of the standing committee on human cytogenetic nomenclature (ISCN, 1978).Statistical analysis will be performed employing student's t-test, using standard statistical table of Fisher and Yates (1963) for testing the level of significance.

VI. ***Statistical Analysis*** : To Test the subjective effects i.e samples sites ,doses and time , the general uni variate linear model (Draper and smith 1966; Moore and Felton ,1983) , inverse linear model (Draper and smith 1966); and Quadratic model(Draper and smith 1966); will be tested to the observed data. To compare all the possible groups (factors) ,which differs significantly , multiple post hoc comparison test (Woolson , 1987, Dawson – Saunders *et al.,* 1990) will be applied .

For mutagenicity testing and Chromosome aberrations, various concentrations of liquid waste , starting from the raw treated and untreated waste will be assessed.

Plan of work


PHASE I- Sample Collection from Hospitals and Diagnostic Laboratories.
PHASE II- Physico-chemical analysis of both samples (Temperature, pH, DO, BOD, COD, Acidity, Alkalinity, TDS, and Hardness.
PHASE III- Preservation of samples for Bioassays for the evaluation Genotoxicity and mutagenicity.
PHASE IV-Applying Bacteriological Bioassay
Ames Test (Maron & Ames, 1983)
SOS Chromotest (EMBI, test kit Canada)
PHASE V- Studying the Chromosome Aberrations in Swiss Albino Mice(Klein and Boraday 2000)
Phase VI – Statistical analysis of results by applying "t" test and SPSS test .
PHASE VI- Documentation and Thesis Writing.

SIGNIFICANCE OF THE WORK

Genotoxicity and mutagenicity testing of waste water samples is important for human and environmental hazard evaluation. This study to estimate the genotoxicity of hospital effluents will provide an exact idea of the unfavorable effects generated due to wastewaters from multi-specialty hospitals, since it is possible to carry out the ecotoxicological risk assessment of the hospital effluents by the use of standardized bioassays and physicochemical parameters.

The proposed scenario will allow a quantitative risk characterization of hospital wastewaters. Hence, this study will force hospital discharge sectors to apply genotoxicity testing of liquid effluent routinely before disposing it untreated (or after treatment) so that the necessity of setting up of Effluent Treatment Plant (ETP) in hospitals could be ascertained. A battery of bacterial and eukaryotic bioassays should be considered for any such study to be relevant.

Moreover, being simple, quick and relatively easy to perform, short term bioassays can assess harmfulness of effluents conveniently. The proposed study would also help in standardization of more short term bioassays as monitoring tools to screen waste waters for the presence of chemicals with mutagenic potential.

In case of positive genotoxic results in hospital waste water samples, from a scientific point of view, extensive monitoring programs should be performed in order to identify possible sources of genotoxic and mutagenic substances. This work would also be helpful in predicting the competence of hospital effluents to cause pollution of surface and underground water bodies and to lead to outbreaks and epidemics.

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