PERPETUATION OF NEWCASTLE DISEASE VIRUS AT DIFFERENT TEMPERATURE IN NATURAL WATER BODIES

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Jotan Kar

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Department of Microbiology and Veterinary Public Health

Faculty of Veterinary Medicine

Chittagong Veterinary and Animal Sciences University

Chittagong- 4225, Bangladesh

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This is to certify that we have examined the above Master's thesis and have found that the thesis is complete and satisfactory in all respects.

Dr. Abdul Ahad

Professor

Department of Microbiology and Veterinary Public Health

Supervisor

Dr. Abdul Ahad

Professor and Head

Department of Microbiology and Veterinary Public Health

Chairman of the Examination Committee

Chittagong Veterinary and Animal Sciences University
Chittagong- 4225, Bangladesh

December, 2016

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The Author

December, 2016

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The Author

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List of abbreviation

APMV-1	Avian Paramyxovirus Serotype 1
BCRDV	Baby Chick Ranikhet Disease Vaccine
C	Celsius
CI	Confidence Interval
DSC	Differential scanning calorimetric
EBT	Erichrome Black-T
F	Fusion Protein
FAO	Food and Agriculture Organization
HA	Hemagglutination
HN	Haemagglutinin- Neuraminidase Protein
ICPI	Intra cerebral Pathogenicity Index
M	Matrix Protein
ND	Newcastle Disease
NDV	Newcastle Disease Virus
P	Phosphoprotein
PBS	Phosphate-Buffered Saline
NP	Nucleoprotein
NT	Non Treated
PPM	Parts per million
R	Relative Humidity
SPF	Specific Pathogen-Free
SD	Standard Deviation
UV	Ultra Violet
WHO	World Health Organization

Abstract

Survivability of Newcastle Disease virus (NDV) in environment depends on several physiochemical factors. The study was conducted aiming to investigate the effect of pH, bi-carbonate and hardness of water on NDV and the thermo-stability of the virus at different temperature (22°C, 30° and 37°C) in laboratory condition. Five water samples were collected from 5 different upazilla of Chittagong and Dhaka districts. Each sample was divided into two group; non-treated (NT) and treated (T). Water samples of treated group were boiled at 100°C for 15 minutes and then filtered using bacteriological filter. Later pH, bi-carbonate and hardness of each sample of both groups were analyzed. Between two groups of samples there was no significant difference in terms of pH and bi-carbonate except hardness of water. Viral inoculums were prepared from previously characterized velogenic NDV and mixed with water sample and treated at 22°C, 30° and 37°C. Every six hours interval 2 ml exposure virus samples were removed up to 96 hours. To know the survival period of virus in each sample (both in NT and T), 0.1ml virus suspension was inoculated to embryonated chicken egg for different temperature, time and categories and incubated at 37°C where allantoic fluid was harvested after 48 hours of inoculation. Slide hemagglutination test was performed to know the presence of virus in allantoic fluid. Later plate hemagglutination (HA) test was done to know the virus titre. In every sample, virus survived longer period in treated than the non-treated water samples. Highest viral survival period was seen 84 hours for T_4 sample and lowest was 66 hours for NT_1, NT_2, NT_5 (except NT_4) samples at 22°C and the HA titre was 2^2. At 30°C it was highest 78 hours for T_4 and lowest 48 hours for NT_1 with HA titre 2^2. Finally in case of 37°C virus survived lowest 24 hours for NT_3 and highest 48 hours for T_4 sample where HA titre was 2^2. The study revealed that higher the environmental temperature lower the viral survival period. Again hardness of water is an important factor that affects the survival of NDV in water. So, survivability of NDV in environment depends on geographical distribution.

Key words: Survivability, NDV, thermo-stability, pH, bi-carbonate and water hardness.

Chapter-1: Introduction

Newcastle Disease (ND) is reported as the most important viral disease of poultry in the world including developing countries (Adene, 1990 and Spradbrow, 1997). In Africa and Asia it is a major constraint against the development of both industrial and village poultry production (Alders *et al.*, 2001a). Newcastle Disease (ND) viral infections of poultry range from latent to rapidly fatal depending upon the pathotype of virus involved (Alexander, 2003). The disease causes high economic losses due to high mortality, morbidity, stress, decreased egg production and hatchability (Alexander, 2000). Before the advent of avian influenza, ND was considered as the cause of highest economic loss in village chickens in most developing countries including Bangladesh (Alexander, 2001).

Newcastle disease (ND) is caused by Newcastle disease virus (NDV), a member of the *Paramyxoviridae*, genus Avulavirus and serogroup Avian Paramyxovirus 1 (Lamb *et al.*, 2005). The virus is enveloped and has a negative- sense single-stranded genome of approximately 15 kb, in length which codes for six structural proteins including Nucleoprotein (NP), Phosphoprotein (P), Matrix Protein (M), Fusion Protein (F), Hemagglutinin-Neuraminidase (HN), Large RNA depended polymerase protein(L) **(Figure 1)** and two non-strucural protein that are additionally created within the p gene during transcription of mRNA at editing site by insertion of guanines (Alexander, 2003).

The virus affects 27 of the 50 orders of the birds (Madadger *et al.*, 2013). Depending upon the pathotype and susceptibility the mortality varies from zero to 100% (Nanthakumar *et al.*, 2010). There are nine serotypes of paramyxoviruses i.e.PMV1 to PMV9. Newcastle Disease Virus (NDV) was categorized into three main pathotypes depending on the severity of disease produced by the isolate in chickens. Lentogenic isolates do not usually cause disease in adult birds and are considered avirulent. Viruses of intermediate virulence that cause respiratory disease are termed as mesogenic, while virulent viruses causing high mortality are termed as velogenic. Neurotropic and viscerotropic forms of velogenic viruses have been reported worldwide. Velogenic viscerotropic NDV is endemic in Bangladesh and causes

mortality in both village chickens (Barman *et al.*, 2010) and commercial poultry farms (Islam *et al.*, 1998; Talha *et al.*, 2001).

The virulence of virus depends on different physical and chemical factors. The virus is inactivated by being heated at 56°C (132.8°F) for 3 hours, or 60°C (140°F) for 30 minutes by acidic pH < 2. The virus remained active at pH 4 and 9 up to 24 hours but lost its visibility at pH 1 and pH 13 within six hours. The virus remained active following exposure of ultra violates light for 45 minutes. Among the chemical factors, formalin at 0.4% concentration inactivated virus in 30 minutes while it's 0.24 and 0.12% concentration did not activate the virus in 45 minutes. Phenol crystal at 0.4% and aldekol at 0.5% concentration inactivate the virus within 15 and45 minutes, respectively. Bromosept at 1 and 0.5% concentration inactivate the virus within 15 and 30 minutes respectively. Iosan at both the concentration i.e.0.5 and 1% inactivate the virus within 15 minutes. (Rizwan *et al.*, 1999)

The disease is a highly contagious disease of poultry and causes a huge economic loss in different parts of the world. The virus can survive in poultry premises for 120 days (Jordan, 1990) and remains a source of infection for susceptible chicks in the vicinity. This disease is still one of the most important diseases in poultry production worldwide and remains a major constraint against the development of both industrial and village poultry production in Asia in spite of control measures including vaccination. The disease is spread worldwide affecting various species of poultry and other birds. However, chickens appear to be the most susceptible to the disease whereas aquatic birds, including geese and ducks are relatively resistant. The disease can vary from clinically in apparent to highly virulent forms, depending on the virus strain and the host species. The ND virus is shed during the incubation period of the disease, the clinical phase and for a period during recovery. The rate of spread of NDV is determined by the organs in which the virus multiples. Newcastle Disease Virus is present in air respired through the trachea, as well as the cloacal discharges in infected fowls. Live poultry, carcass and offal, poultry show and markets are important in the spread of the virus, but the current practice of moving exotic birds around the world has helped the international transport of the disease.

The virus can also spread though direct contact with infected birds, contaminated poultry products consumed by other birds, people with contaminated clothes or shoes, equipment or vaccines (Alexander, 1988). Infection from ingestion of contaminated feed (Alexander *et al.*, 1984), water (Saber *et al.*, 1978) and flies found in a poultry house may also occur.

Wild web-footed birds may act as a carrier of organism as it may cause inapperent infection. They shed the virus through fecal droppings in water where they are mixed up with native ducks and this duck acts as a source of NDV.

Objectives of the study

a. To identify the survival time of Newcastle disease virus at different temperature in laboratory condition using natural source of water.

b. To identify the influence of Natural water's parameters such as pH, bi-carbonates and water hardness for the survival of Newcastle disease virus.

c. To identify the influence of treated natural water on the survival of Newcastle disease virus.

Chapter-2: Review of Literature

2.1 Definition of Newcastle Disease

Although it is likely that the vast majority of birds are susceptible to infection with Newcastle Disease viruses of both high and low virulence for chickens, the disease seen with any given virus may vary enormously from species to species. Many other factors also affect the course of disease. Newcastle Disease viruses show a considerable range of virulence for susceptible hosts such as chickens. Generally, variation consists of clusters around the two extremes in tests used to assess virulence, but, for a variety of reasons, some viruses may show intermediate virulence (mesogenic). Equally, the very virulent viruses may infect and replicate in vaccinated birds without causing clinical disease (Parede & Young, 1990; Guittet *et al.*, 1993; Capua *et al.*, 1993). This enormous variation in virulence and clinical signs means that none can be regarded as pathognomonic and that it is necessary to define carefully what constitutes ND for the purposes of trade, control measures and policies.

The current OIE definition (OIE, 2000a) is Newcastle disease is defined as an infection of birds caused by a virus of avian paramyxovirus serotype 1 (APMV-1) that meets one of the following criteria for virulence:

a) The virus has an intra cerebral pathogenicity index (ICPI) in day-old chicks (*Gallus gallus*) of 0.7 or greater.

Or

b) Multiple basic amino acids have been demonstrated in the virus (either directly or by deduction) at the C-terminus of the F2 protein and phenylalanine at residue 117, which is the N-terminus of the F1 protein. The term 'multiple basic amino acids' refers to at least three arginine or lysine residues between residues 113 to 116. Failure to demonstrate the characteristic pattern of amino acid residues as described above would require characterization of the isolated virus by an ICPI test.

In this definition, amino acid residues are numbered from the N-terminus of the amino acid sequence deduced from the nucleotide sequence of the F0 gene, 113-116 corresponds to residues -4 to -1 from the cleavage site." NDV is a paramyxovirus that causes Newcastle disease in a wide variety of birds (most notably, in chickens)

(Csatary *et al.*, 1999; Nelson *et al.*, 1999 and seal *et al.*, 2000). This is often fatal disease is characterized by inflammation of respiratory tract and of either the brain or the gastrointestinal tract.

2.2 Current world situation

In many respects, it is extremely difficult to assess the prevalence of ND in the world at any given time. In some countries or areas disease is not reported at all or only if it occurs in commercial poultry, while its presence in village chickens or backyard flocks is ignored. Even in poultry reared commercially, estimations of the geographical distribution of NDV are confused by the use of live vaccines in all but a few countries throughout the world. In some countries the distribution is especially complicated by using, as live vaccines, viruses that are considered sufficiently virulent in other countries to warrant the current definition of ND.

When countries or areas are declared free of ND, further complications are caused by the definition of the type of ND virus described as harmless although this is being be addressed by the new definitions and codes to be adopted by the OIE. Even in countries that have long been recognized as free of ND, monitoring surveys often reveal symptomless infections with avirulent viruses which have presumably spread from waterfowl or other wild birds. However, there can be little doubt that the highly pathogenic form of ND is a serious problem, either as an enzootic disease or as a cause of regular, frequent epizootics throughout Africa, Asia, Central America and parts of South America (Copland, 1987; Spradbrow, 1988; Rweyemamu *et al.*, 1991 and Alders & Spradbrow, 2001a). In other areas such as Europe, the situation appears to be one of sporadic epizootics occurring despite vaccination programmes (Kaleta & Heffels-Redmann, 1992).

In Western Europe there was a marked increase in reported outbreaks during the early 1990s, peaking with 239 outbreaks in European Union countries in 1994. The distribution over time suggests a single epidemic from the early to mid 1990s, but, in fact, antigenic and phylogenetic evidence indicates that several strains of virus were responsible for these outbreaks. During 1991-1995 the majority of outbreaks in the EU occurred in the Benelux countries and Germany, predominantly in backyard poultry and most of the outbreaks since 1995 have been in these types of birds. One of

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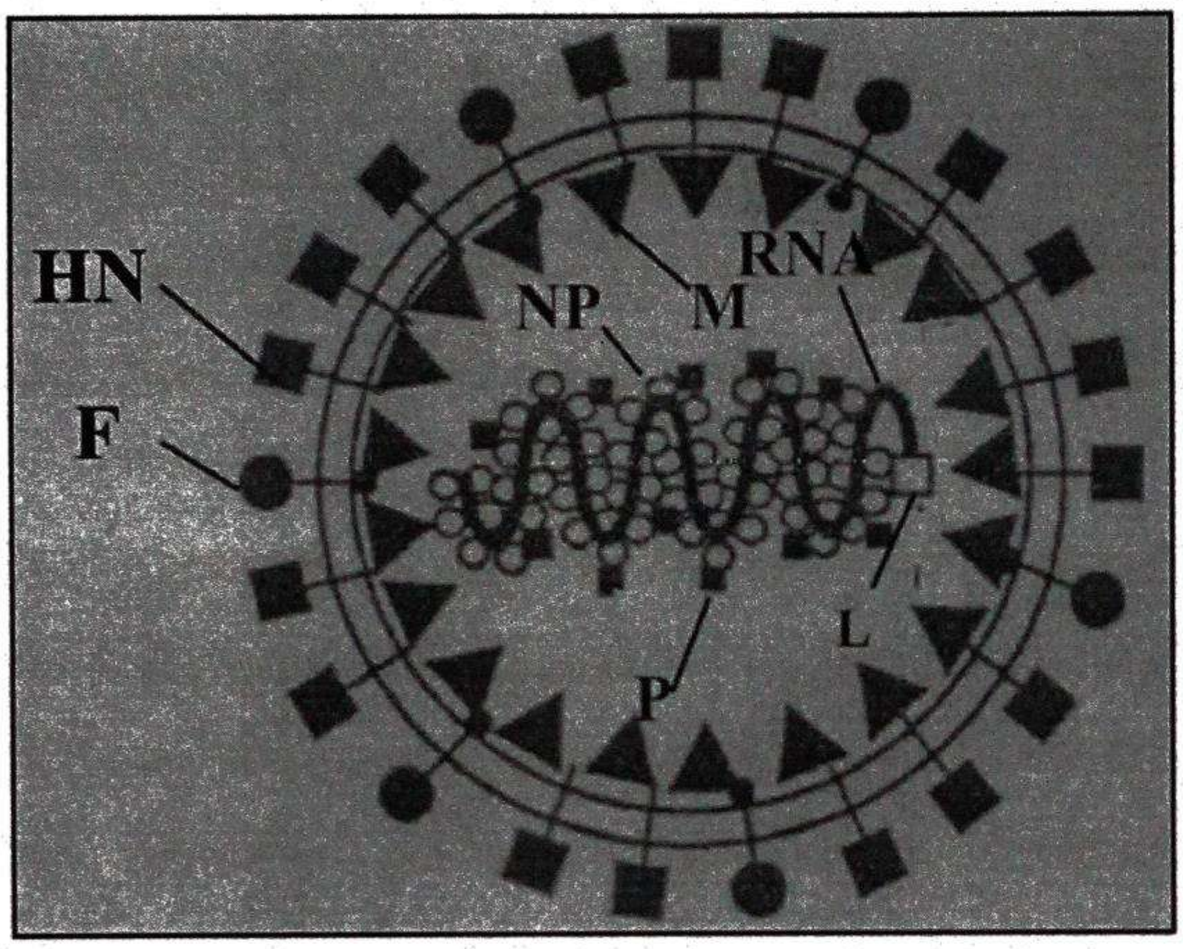
the most extensive epidemics in Western Europe occurred in Italy in 2000 when 254 outbreaks of ND were confirmed, again mainly in backyard poultry. One notable aspect of the outbreaks during the 1990s concerned those that occurred in countries that had been free of the disease for many years. Between 1995 and 1999, there were 18 outbreaks in Denmark, 2 in Finland and 27 in Northern Ireland. There was also 1 in Sweden, 1 in Norway and 1 in the Republic of Ireland.. These were all areas of Western Europe that had been declared free of ND and which were monitored regularly by serological testing and had no evidence of ND virus infections other than occasional incursions of avirulent viruses typical of spread from wild birds.

From the time of the 1932 outbreak (Albiston & Gorrie, 1942) to 1998, Australia had been free of virulent ND virus. Since 1966, however, it has been recognized that viruses similar to those placed in the "asymptomatic enteric" pathotype group (Westbury, 1981; Spradbrow, 1987) are present in wild birds in Australia and on occasions have spread to commercial poultry flocks. Two outbreaks of virulent ND occurred in Australia in 1998 and further outbreaks were reported in 1999 and 2000 (Kirkland, 2000; Westbury, 2001).

2.3 Aetiology

The three virus families *Rhabdoviridae*, *Filoviridae* and *Paramyxoviridae* form the order Mononegavirales; i.e. viruses with negative sense, single stranded and non-segmented RNA genomes. ND is caused by avian paramyxovirus serotype 1 (APMV-1) viruses, which, with viruses of the other eight APMV serotypes (APMV-2 to APMV-9), have been placed in the genus *Avulavirus*, sub-family *Paramyxovirinae*, family *Paramyxoviridae*, in the current taxonomy (Lamb *et al*., 2000; Mayo, 2002). Antigenic variation of ND viruses (APMV-1) detectable by conventional haemagglutination inhibition (HI) tests has been reported, although only rarely (Arias-Ibarrondo *et al*., 1978; Hannoun, 1977, Alexander *et al*., 1984). One of the most noted variations of this kind has been the virus responsible for the panzootic in racing pigeons. This ND virus, referred to as pigeon APMV-1 (PPMV-1), was demonstrably different from standard strains in haemagglutination inhibition tests, but not sufficiently different antigenically that conventional ND vaccines were not protective (Alexander and Parsons, 1986). In recent years antigenic variations detected by monoclonal antibodies and genetic variations detected by nucleotide sequencing of

the virus genome have proved invaluable in understanding the epidemiology of ND (Alexander *et al*., 1997; 1999; Herczeg *et al*., 1999; 2001).



F- fusion protein,
HN-haemagglutinin-neuraminidase protein,
M- matrix protein,
P –phosphoprotein,
NP-nucleoprotein,
L- RNA dependent RNA polymerase

Figure 1. A schematic representation of ND virion. (Gurib *et al.*2003)

2.4 Epidemiology

2.4.1 Host Range

ND viruses have been reported to infect animals other than birds, ranging from reptiles to man (Lancaster, 1966; Kaleta and Baldauf, 1988) concluded that NDV infections have been established in at least 241 species of birds representing 27 of the 50 Orders of the class. It seems probable that all birds are susceptible to infection but, as stressed by Kaleta and Baldauf, the disease seen with any given virus may vary enormously from one species to another.

2.4.1.1 Wild birds

NDV isolates have been obtained frequently from migratory feral waterfowl and other aquatic birds. Most of these isolates have been of low virulence for chickens and similar to viruses of the "asymptomatic enteric" pathotype. The most significant outbreaks of virulent NDV in feral birds have been those reported in double-crested cormorants (*Phalacrocorax auritus*) in North America during the 1990s. Earlier reports of ND in cormorants and related species had been in the late 1940s in Scotland (Blaxland, 1951) and in Quebec in 1975 (Cleary, 1977). Recent outbreaks in cormorants in North America were first seen in 1990 in Alberta, Saskatchewan and Manitoba in Canada (Wobeser *et al*., 1993). In 1992 the disease re-appeared in

cormorants in western Canada, around the Great Lakes and North mid-west USA, in the latter case spreading to domestic turkeys (Mixson & Pearson, 1992; Heckert, 1993). Antigenic and genetic analyses of the viruses suggested that all the 1990 and 1992 viruses were very closely related despite the geographical separation of the hosts. Disease in double-crested cormorants was observed again in Canada in 1995 and in California in 1997 and in both instances NDV was isolated from dead birds; as before, these viruses appear to be closely related (Kuiken, 1998).

Thirty-eight outbreaks of ND in commercial poultry were confirmed in 1997 in the United Kingdom (Alexander *et al.*, 1998). There were also outbreaks caused by genetically similar viruses in Scandinavian countries in 1996 (Alexander *et al.*, 1999). These, linked to the unusual patterns of movement of migratory birds at the end of 1996 and the beginning of 1997, suggest that migratory birds may have been responsible for the primary introduction of the causative virus into Great Britain (Alexander *et al.*, 1998).

2.4.1.2 Caged "pet birds"

Virulent NDV isolates have often been obtained from captive caged birds (Senne *et al.*, 1983). (Kaleta and Baldauf, 1988) thought it unlikely that infections of recently imported caged birds resulted from enzootic infections in feral birds in the countries of origin. They considered that the infections probably originated at holding stations before export, either as a result of enzootic NDV at those stations or of spread from nearby poultry such as backyard chicken flocks. (Panigrahy *et al.*, 1993) described outbreaks of severe ND in pet birds in six states in USA in 1991. Illegal importations were assumed to be responsible for the introductions of the virus.

2.4.1.3 Domestic poultry

Virulent NDV strains have been isolated from all types of commercially reared poultry, ranging from pigeons to ostriches.

2.4.1.4 Racing and show pigeons

In the late 1970s, an NDV strain, PPMV-1, showing some antigenic differences from classical strains, appeared in pigeons. It probably arose in the Middle East and

subsequently produced a true panzootic, spreading in racing and show pigeons to all parts of the world (Alexander, 1997).

2.4.2 Introduction and spread

2.4.2.1 Transmission between birds

Apart from predatory birds or the practice of feeding poultry with untreated swill containing poultry meat, spread from bird to bird appears to occur as the result of either inhalation of excreted droplet particles or the ingestion of infective material such as faeces. Although it is clear from the administration of live vaccines by aerosol that infection may be established via the respiratory route, there is remarkably little experimental evidence that infected birds pass on the virus to susceptible birds in this way, even over short distances. The success of this route of transmission depends on many environmental factors, such as temperature, humidity, and stocking density. In contrast, it is easily demonstrated that virus infection can be passed from one bird to another via contaminated faeces. It seems most likely that the pigeon variant virus, the "asymptomatic enteric" viruses, and other viruses which fail to induce significant respiratory signs in infected birds, are transmitted primarily in this way (Alexander *et al.*, 1984). In animal studies, NDV infection has been accomplished by intratumoral, intraperitoneal, intravenous, intramuscular, or subcutaneous injection. (Lorence et al., 1994; Lorence *et al.*, 2001; Reichard *et al.*, 1992); Schirrmacher *et al.*, 2001; Heicappell *et al.*, 1986; Schirrmacher *et al.*, 2000). NDV-infected, whole cell vaccines have been given to animals by intraperitoneal, (Plaksin *et al.*, 1994) intradermal, (Bier *et al.* 1989) or subcutaneous injection,or by a combination of subcutaneous and intramuscular injection (Heicappell *et al.*, 1986); (Schirrmacher *et al.*, 1987).

Several reviews have dealt with the way in which the ND virus may be introduced into a country or area and then subsequently spread from flock to flock (Lancaster, 1966; Lancaster and Alexander, 1975; Alexander 1988b, 1995). In summary, the main methods by which virus can be spread are equipment and people transmission, different poultry products transmission, transmission of different live birds, different food and water contamination.

2.4.2.2 Movement of live birds

Migratory feral birds may be responsible for the primary introduction of infection, but nearly all NDV isolates obtained from feral birds have been of low virulence. A more significant role of such birds may be the spread within an area once NDV infections have already occurred in poultry. Exceptions to the presence of the virus of low virulence in migratory birds have been discussed in the Host Range section above. World trade in captive caged birds is enormous and in many countries virulent NDV has been isolated frequently from such birds held in quarantine. For example, 147 virulent NDV isolations were made from 2 274 lots of captive birds held in quarantine in the USA during 1974-1981 (Senne *et al.*, 1983). Some infected psittacines have been shown to excrete virulent virus intermittently for extremely long periods, in some cases for more than one year (Erickson *et al.*, 1977). This further emphasises the potential role of these birds may have in the introduction of NDV to a country or area. There is also considerable international trade in game birds, which are often imported for immediate release. The potential for racing pigeons to carry and introduce ND into a country or area has been highlighted by the panzootic in such birds over the last ten years. Trade in backyard flocks and other birds kept for recreational purposes (hobby birds) have been implicated in the introduction and spread of ND in the outbreaks in European Union countries during 1991-1994.

Modern methods of slaughter of commercial poultry, marketing of poultry meat and veterinary inspection, have reduced the movement of live commercial poultry (with the exception of day-old chicks) in many European and other developed countries. However, in many countries, the normal method of trade is by live poultry markets. Such markets, where birds of many different species may be placed in close contact with each other, represent ideal opportunities for viruses to be disseminated. The movement of village chickens from one village to another, whether directly or through live bird markets, is the main method of spread of ND. (Alexander et al., 2004)

2.4.2.3 Movement of people and equipment

Secondary spread during most epizootics of ND in recent times has been the result of the movements of personnel or equipment. Human beings may be infected with NDVs, but their most likely role is the transfer of infective poultry faeces from one

site to another via clothing, footwear, crates, feed sacks, egg trays or vehicles. (Alexander et al., 2004)

2.4.2.4 Movement of poultry products

In the past, poultry meat has been seen as the main vehicle for the introduction and spread of NDV. Modern methods of poultry carcass preparation as well as legislation on the feeding of untreated swill to poultry have greatly diminished the risk from poultry products, but the possibility of spread in this way still remains. (Alexander et al., 2004)

2.4.2.5 Contaminated poultry food or water

In the British Isles, outbreaks of ND in commercial poultry have been associated with food contaminated with infective faeces from feral pigeons infected with the ND virus (Alexander *et al.*, 1985; O'Reilly *et al.*, 1994). Similarly, water contaminated with infective faeces may introduce NDV to a flock.

2.4.2.6 Airborne spread

In recent years, the significance of airborne transfer of viruses has been the subject of some debate. During the 1960s and 1970s, this was considered a major method of spread and (Smith, 1964) considered it the most logical explanation of spread in outbreaks occurring in 1960 and 1962 in Great Britain. In the same country, Dawson (1973) considered windborne spread to be of major significance during the 1970-1972 outbreaks that were noted for the severe respiratory signs and unusual patterns of spread. But in the 1971-1973 epizootic in California, with ostensibly the same virus, respiratory signs were not especially prominent and utter back and Schwartz (1973) considered airborne spread to be of little significance. There have been few attempts to assess the survival of airborne virus, but Hugh was able to detect virus 64 meters but not 165 meters downwind of infected premises. These authors stressed the importance of environmental conditions, particularly relative humidity, with regard to the likelihood of airborne spread.

It is possible that when climatic conditions have been right and poultry farms sufficiently concentrated, as in Northern Ireland in 1973 (McFerran, 1989), airborne spread may have played a significant role in epidemics of ND. But in recent years,

airborne spread has not been an issue in reported outbreaks and there has nearly always been an alternative and more likely cause, particularly the movement of poultry and humans.

2.4.3 Vaccines

Good manufacturing practices should ensure that vaccines are highly unlikely to be carriers of virulent ND virus. However, in the past, birds have become infected by vaccines for other diseases being contaminated with ND and also as a result of failure to properly inactivate vaccines prepared from virulent ND virus. In 1996-1997, a series of ND isolates of low virulence were obtained from poultry flocks in Denmark, a country which pursues a non-vaccinating policy for ND. It was demonstrated that these viruses were the result of contamination of avian virus vaccines with vaccinal ND viruses (Jorgensen *et al.*, 2000). This episode further emphasizes the potential of spread of ND in this way.

2.4.4 Non avian hosts

This is likely to be by mechanical transfer of infective faeces, for example, by insects, rodents or scavenging animals. In hot countries, reptiles may enter poultry houses and should not be ignored as potential spreaders of NDV, as their susceptibility to infection has been reported.

2.5 Disease and pathogenicity

2.5.1 Virulent factor

The clinical signs seen in birds infected with NDV vary widely and are dependent on factors such as: the virus, the host species, age of host, and infection with other organisms, environmental stress and immune status. In some circumstances infection with the extremely virulent viruses may result in sudden, high mortality with comparatively few clinical signs. Although none of the variable clinical signs can be regarded as pathognomonic, certain signs do appear to be associated with particular viruses. This has resulted in the grouping of viruses into five pathotypes on the basis of the predominant signs in affected chickens (Beard and Hanson, 1984)

Viscerotropic velogenic: Viruses responsible for disease characterized by acute lethal infection, usually with hemorrhagic lesions in the intestines of dead birds. (Munmun *et al*, 2016)

Neurotropic velogenic: Viruses causing disease characterized by high mortality, which follows respiratory and neurological disease, but in which gut lesions are usually absent. (Munmun *et al*., 2016)

Mesogenic Viruses causing clinical signs consisting of respiratory and neurological signs, with low mortality (Munmun *et al*., 2016)

Lentogenic Viruses causing mild infections of the respiratory tract. (Munmun *et al*., 2016)

Asymptomatic enteric Viruses causing avirulent infections in which replication appear to occur primarily in the gut. (Munmun *et al*., 2016)

These groupings are by no means clear-cut, and even in experimental infections of specific pathogen-free (SPF) chickens, considerable overlapping occurs (Alexander & Allan, 1974). In addition, in the field exacerbating factors may result in the clinical signs induced by the milder strains mimicking those of the more pathogenic viruses. In general terms, ND may consist of signs of depression, diarrhoea, prostration, edema of the head and wattles, nervous signs, such as paralysis and torticollis, and respiratory signs (McFerran & McCracken, 1988). Fall in egg production, perhaps leading to complete cessation of egg laying, may precede more overt signs of disease and deaths in egg-laying birds. Virulent ND strains may still replicate in vaccinated birds, but the clinical signs will be greatly diminished in relationship to the antibody level achieved (Allan *et al*., 1978).

As with clinical signs, no gross or microscopic lesions can be considered pathognomonic for any form of ND (McFerran & McCracken, 1988). Carcasses of birds dying as a result of virulent ND usually have a fevered, dehydrated appearance. Gross lesions vary with the infecting virus. Virulent panoptic ND viruses typically cause hemorrhagic lesions of the intestinal tract. These are most easily seen if the intestine is opened and may vary considerably in size. Some authors have reported lesions most typically in the proventriculus, while others consider them to be most prominent in the duodenum, jejunum and ileum. Even in birds showing neurological signs prior to death, there is usually little evidence of gross lesions in the central

nervous system. Lesions are usually present in the respiratory tract when clinical signs indicate involvement. These generally appear as hemorrhagic lesions and congestion; airsacculitis may be evident. Egg peritonitis is often seen in laying hens infected with virulent NDV. Microscopic lesions are not considered to have any diagnostic significance. In most tissues and organs where changes occur, they consist of hyperemia, necrosis, cellular infiltration and edema. Changes in the central nervous system are those of no purulent encephalomyelitis.

2.5.2 Molecular basis of pathogenicity of ND

During replication, NDV particles are produced with a precursor glycoprotein, F0, which has to be cleaved to F1 and F2 for the virus particles to be infectious (Rott and Klenk, 1988). This post translation cleavage is mediated by host cell proteases (Nagai *et al.* 1976a). Trypsin is capable of cleaving F0 for all NDV strains and *in vitro* treatment of noninfectious virus will induce infectivity (Nagai *et al.*, 1976b). The cleavability of the F0 molecule was shown to be related directly to the virulence of viruses in vivo (Rott, 1979; Rott, 1985). It would appear that the F0 molecules of viruses virulent for chickens can be cleared by a host protease or proteases found in a wide range of cells and tissues. This allows these viruses to spread throughout the host, damaging vital organs. In contrast F0 molecules in viruses of low virulence appear to be restricted in their sensitivity to host proteases resulting in restriction of these viruses to growth only in certain host cell types.

Since the initial studies comparing the deduced amino acid sequences at the cleavage site of the F0 precursor of a number of virulent and avirulent ND strains (Collins *et al*, 1993), a large number of studies have confirmed the presence of multiple basic amino acids at that site in virulent viruses. Usually the sequence has been RQK/RR F in virulent viruses and most have had a basic amino acid at position 112 as well. In contrast, viruses of low virulence usually have the sequence K/RQG/ER L. The major influence on the pathogenicity of NDV is therefore the amino acid motif at the F0 cleavage site, the presence of basic amino acids at positions 113, 115 and 116 and phenylalanine at 117 in virulent strains means that cleavage can be affected by protease or proteases present in a wide range of host tissues and organs. For viruses of low virulence, cleavage can occur only with proteases recognizing a single arginine, i.e. trypsin-like enzymes. Such viruses are therefore restricted in the range of sites

where they are able to replicate to areas with trypsin-like enzymes, such as the respiratory and intestinal tracts, whereas virulent viruses can replicate in a range of tissues and organs resulting in a fatal systemic infection (Rott, 1979).

The genetic material of NDV is RNA rather than DNA. (Csatary *et al*., 1999; Seal *et al*., 2000; Schirrmache, 1998; Schirrmache., 1999; Phuangsab *et al*., 2001; Schirrmacher *et al*., 1986, Schirrmacher *et al*., 1997; Sinkovics et al., 2000 and Gass *et al*., 2000). As with other types of viruses, essentially all of NDV's replication cycle takes place inside infected cells, which are also known as host cells.(Schirrmacher *et all*., 1996; Schirrmacher *et al*., 1998; Sinkovics *et al*.,2000; Haas *et al*.1998). During a replication cycle, new virus proteins and copies of the NDV genetic material (i.e., genome) are made in the host cell's cytoplasm. NDV is also an enveloped virus, which means that progeny virus particles are released from infected cells by budding off from them. (Eaton *et al*., 1973; Sinkovics *et al*., 2000; Schirrmacher *et al*., 1999). In this process, single copies of the NDV genome become wrapped in an outer coat (i.e, an envelope) that is made from a small piece of the host cell's plasma membrane. Generally, the NDV outer coat contains only virus proteins that have been specifically inserted into the host cell's plasma membrane;(Eaton *et al*., 1973; Haas *et al*., 1998; Schirrmacher *et al*., 1986) however, some host cell proteins may be included as well (Webb *et al*., 1970; Beverley *et al*., 1973).Two specific virus proteins, hemagglutinin-neuraminidase and the fusion protein, are the main NDV proteins found in the outer coat of isolated virus particles (Sinkovics *et al*., 2000).

2.5.3 Origins of Virulent ND Viruses

The emergence of ND as a highly pathogenic disease in poultry since 1926, (initially predominantly in South East Asia), suggests that some sudden major change has occurred either in the virus or in its hosts. (Hanson, 1972) considers that the various hypotheses which have been put forward can be grouped into three categories. The virulent virus has always existed in poultry in South East Asia, but it was not until the beginning of the commercialization of the industry in that part of the world that the disease, with its enormous economic impact, was noticed as a significant problem. The virus is enzootic in different species, possibly inhabiting tropical rain forests, and spread to domestic poultry because of the incursion of man into that habitat. There is a major mutation of a precursor virus of low virulence. The first explanation remains a

possibility. Some consider it unlikely that the disease would have gone unreported if it was enzootic in village chickens, but even today village chickens throughout Africa, Asia and the Americas often show high levels of mortality, either regularly or as large die-offs every few years which go largely undiagnosed. Similarly, there have been occasional descriptions of disease outbreaks prior to 1926 that are very similar to ND.

The second explanation has, until recent years, been generally accepted as the most likely. The reason is mainly the discovery that during the 1970-73 panzootic, movement of captive caged birds, particularly psittacines which may be resistant excreters of NDV, was, to some extent, responsible for the introduction and spread in some countries, particularly California (Francis, 1973; Walker *et al.*, 1973). However, as discussed above, viruses isolated from feral birds are usually of low virulence and it has been suggested that caged birds are most probably infected after they have been trapped. Maintenance of the virus in any feral bird species seems unlikely because of the effect that infection is likely to have on the bird's survival. The third explanation has usually been dismissed out of hand as probably representing a mutation too big to be within the bounds of probability, especially without any apparent evolutionary advantage that would result from such selection. However, viruses isolated from ND outbreaks in Ireland and Australia during the 1990s has suggested that this may be how some virulent ND viruses emerge.

In 1990 in Ireland two outbreaks of ND occurred in egg laying birds. The viruses isolated were highly virulent and apparently identical (Alexander *et al.*, 1992). They were very closely related antigenically and genetically (Collins *et al.*, 1998) to viruses of low virulence normally isolated from feral waterfowl but known to have infected chickens in Ireland in 1987 (McNulty *et al.*, 1988). The group formed by these viruses was both antigenically and genetically distant from all other ND viruses. (Collins *et al.*, 1993) has shown that the virulent virus had four nucleotide differences at the site coding for the F cleavage site compared to the related viruses of low virulence (Table 1), which would explain the higher virulence for chickens. However, the distinctiveness of this group of viruses from other ND viruses supports the theory that the virulent viruses arose by mutation from those of low virulence. Phylogenetic studies have shown all the virulent viruses responsible for the outbreaks in Australia from 1998 to 2000 are extremely closely related to each other and to the endemic

The OIE Terrestrial Animal Health Code (2013) states that ND can survive for several weeks in a warm and humid environment and indefinitely in frozen material. When using agents to inactivate virus, it is critical that the manufacturer's directions for the correct concentration of the solution, and the time needed for complete inactivation to occur, be followed. NDV is inactivated by being heated at 56°C (132.8°F) for 3 hours, or 60°C (140°F) for 30 minutes inactivated by acidic pH of ≤ 2. At 55°C the virus can survive 2,521 seconds in whole eggs, 2278 seconds in liquid eggs white and 176 seconds in 10% salted yolk. At 57 °C the virus can survive !596 seconds in whole eggs, 986 seconds in Liquid eggs white and 50.4 hours in Dried eggs white .At 59°C the virus can survive 674 seconds in whole eggs and 301 second for whole eggs white.

Hess *et al*., (1963) stated that Newcastle disease virus was isolated from the carcasses of frozen poultry for over 730 days and from buried carcasses for 121 days.

Pirtle *et al*., (1991) stated that containing approximately 10-15°C CID50/ml was used to contaminate Formica counter tops, cloth gowns, rubber gloves, paper facial tissues and hands . The virus was recovered from counter tops for as long as 6 hours, from rubber gloves for 1.5 hours, from cloth gowns and paper tissues for 30 to 45 min, and from skin for up to 20 min. Self-inoculation by contact with contaminated infant secretions was therefore considered a potential method of nosocomial transmission.

Guan *et al*., (2009) stated that by day 7, temperatures in compost ranged from 50°C to 65°C, and Avian Influenza viruses had been killed in all specimens in bags. In comparison, viruses in sealed vials remained viable to day 10. Viral RNA in mesh-bag specimens had been degraded to no detectable levels by day 10, but it was still detected in sealed vials on day 21. In specimens that were held at ambient temperatures (13°C - 28 °C), the viruses in mesh-bag specimens were inactivated by day 21, but their RNA was still detected. In comparison, the viruses in sealed vials survived to day 21. In an in vitro experiment, the time required for a 1-log10 reduction of viruses was significantly shorter ($P < 0.05$) in water extracts from compost than in phosphate buffers at temperatures of 25°C to 45°C. This study provided evidence that microbial activity during composting contributed to the rapid killing ND viruses and to the degradation of their viral RNA.

Rizwan *et al*., (1999) Stated the effect of physical factors (temperature, pH and ultra violate light) and chemical phenol, formalin, cresol, crystal) factors for the survival of Newcastle disease virus were evaluated. It was observed the virus was endured 56 for 30 minutes but got inactivated within 45 minutes. The virus remained active at pH 4 and 9 up to 24 hours but lost its visibility at pH 1 and pH 13 within six hours. The virus remained active following exposure of ultra violate light for 45 minutes among the chemical factors, formalin at 0.4% concentration inactivated virus in 30 minutes while it's 0.24 and 0.12% concentration did not activate the virus in 45 minutes Phenol crystal at 0.4% and aldekol at 0.5% concentration inactivate the virus within 15 and45 minutes, respectively. Bromosept at 1 and 0.5% concentration inactivate the virus within 15 and 30 minutes, respectively. Iosan at both the concentration i.e.0.5 and 1% inactivate the virus within 15 minutes.

Olivia *et al*., (1972) Stated that Virus mutants (NDVpi) isolated from L cells persistently infected with the Hertz strain of Newcastle disease virus have been previously reported by this laboratory to differ from the wild-type virus (NDVO) in several physical and biological properties. It has now been determined that, in addition to these differences, the NDVpi mutants are also spontaneously selected temperature-sensitive mutants. The temperature sensitivity of 10 NDVpi clones was confirmed by temperature inhibition, plaquing efficiency, and single-cycle yield experiments. The cut-off temperature, at which more than 90% of virus replication is inhibited, was between 41°C and 42°C. All 10 NDVpi clones were also found to be defective in virus-specific ribonucleic acid (RNA) synthesis in infected chick embryo cells at 42°C and are tentatively classified as RNA. The possible relationships of the temperature sensitivity, the other NDVp i properties, infected state are discussed.

Surabhi *et al*., (2014) Stated about the biology of NDV genome and its proteins under different conditions of temperature and pH. Our results indicate that the NDV is non-infective above 42°C and unstable above 72 °C. The study will be useful in defining an optimum storage condition for NDV without causing any deterioration in its viability. The viral RNA showed its dissociation at 72°C suggesting an optimum temperature for its integrity. Slightly lower levels of PCR amplification at 37°C, 42°C and 56°C comparing with the level at RT may be because of different optimization condition.

Irene *et al*., (2013) Found that the thermal stability of the matrix protein (M protein) of Newcastle disease virus (NDV) has been investigated using high-sensitivity differential scanning calorimetric (DSC) at pH 7.4. The thermal folding/unfolding of M protein at this pH value is a reversible process involving a highly cooperative transition between folded and unfolded monomers with a transition temperature (Tm) of 63°C, an unfolding enthalpy, H(Tm), of 340 kcal mol−^{1}, and the difference in heat capacity between the native and denatured states of the protein, Cp, of 5.1 kcal K−1 mol−1 . The heat capacity of the native state of the protein is in good agreement with the values calculated using a structure-based parameterization, whereas the calculated values for the hypothetical fully-unfolded state of the protein is higher than those determined experimentally. This difference between the heat capacity of denatured M protein and the heat capacity expected for an unstructured polypeptide of the same sequence, together with the data derived from the heat-induced changes in the steady-state fluorescence of the protein, indicates that the polypeptide chain maintains a significant amount of residual structure after thermal denaturation.

Huade *et al*., (2003) Stated Several viral transport experiments were conducted in a model aquifer 1 m long, using bacteriophages at various pH (4.6 to 8.3) conditions, to increase our understanding of virus behavior in ground water. The results indicate the existence of a critical pH at which the virus behavior changes abruptly. This is supported by data from field and batch experiments. The critical pH is determined to be 0.5 units below the highest isoelectric point of the virus and porous medium. When water pH is below the critical pH, the virus has an opposite charge to at least one component of the porous medium, and is almost completely and irreversibly removed from the water. This suggests that electrostatic attraction at a subcritical water pH condition is an important factor controlling virus attenuation in ground water.

Knittel *et al*., (1987) Stated that no virus replication or formation of polynuclear inclusion bodies occurred at 37°C. The virus was immediately inactivated upon exposure to pH 2.0 and was inactivated within 1 h at pH 4.0. The virus titer slowly declined, a3-orders of magnitude reduction in virus titer, at pH 5.0 during a 4-h exposure. Virus survival at pH 6.0 was equal to that of the control in cell culture medium 199 MK (pH 7.12).

Guan *et al*., (2015) Stated that The commercial disinfectants Virkon and Accel, supplemented with an antifreeze agent propylene glycol (PG), methanol (MeOH), or calcium chloride ($CaCl_2$), were evaluated for their effectiveness in killing avian influenza virus (AIV) at -20°C or 21°C. An virus suspension was applied to stainless steel disks, air-dried, and covered with a disinfectant or antifreeze agent for 5 to 30 min. Virkon (2%) and Accel (6.25%) with 30% PG, 20% MeOH, or 20% $CaCl_2$ inactivated 6 log_{10} virus within 5 min at -20°C and 21°C. At these temperatures PG and MeOH alone did not kill virus, but the 20% $CaCl_2$ solution alone inactivated 5 log10 virus within 10 min. The results suggested that $CaCl_2$ is potentially useful to enhance the effectiveness of disinfection of poultry facilities after outbreaks of virus infection in warm and cold seasons.

Gerald *et al*., (1976) Stated that virus can survive in incubation at 36° for 48 hr in tissue culture medium adjusted to pH 6.3, 7.0, and 7.8, respectively. The stability of HSV suspended in PBS adjusted to pH 7.0 relative to tissue culture medium at this pH was somewhat enhanced. However, HSV displayed an increased liability at 36°C when suspended in PBS adjusted to pH 6.3 or 7.8. The results indicate that the inactivation of HSV at 36°C is dramatically affected by the pH of the suspending menstrual as well as its composition. These observations are discussed with regard to the contradictory thermal inactivation kinetics of HSV reported in previous studies.

Bean *et al*., (1982) Investigated the transmission of influenza viruses via hands and environmental surfaces, the survival of laboratory-grown viruses on various surfaces was studied. Viruses survived for 24-48 hours on hard, nonporous surfaces such as stainless steel and plastic but survived for less than 8-12 hours on cloth, paper, and tissues. Measurable quantities of virus were transferred from stainless steel surfaces to hands for 24 hours and from tissues to hands for up to 15 min. Virus survived on hands for up to 5 min after transfer from the environmental surfaces. These observations suggest that the transmission of virus from donors who are shedding large amounts could occur for 2-8 hours via stainless steel surfaces and for a few minutes via paper tissues. Thus, under conditions of heavy environmental contamination, the transmission of virus via fomites may be possible.

(Yves *et al.*, 2008) Stated that viruses tested by cell culture survived up to 3 days when they were inoculated at high concentrations. The same inoculums in the presence of respiratory mucus showed a striking increase in survival time (up to 17 days) virus was still infectious after 1 day when it was mixed with respiratory mucus. When nasopharyngeal secretions of naturally infected children were used, influenza virus survived for at least 48 h in one-third of the cases. The unexpected stability of influenza virus in this no biological environment suggests that unusual environmental contamination should be considered in the setting of pandemic preparedness.

Salo *et al.*, (1976) Stated that at 28°C and 30°C enteroviruses are more stable on the acid than on the alkaline side of neutrality. In the range from pH 3 to 9, temperature is so influential that the fastest inactivation rate at 2 degrees C is slower than the slowest inactivation rate at 30 degrees C. Specific ions or salts also affect the rate of inactivation of viruses. NaCl and other chloride salts enhance the inactivation of virus at pH 3. NaCl is considerably less effective against virus in the range of pH 4.5 to 7.0 than at pH less than 4.5. Loss of RNA infectivity of the virus particle proceeds as rapidly as the loss of infectivity of the particle itself, except at pH 3 in the presence of MgCl2. Inactivation results in alterations to the physical integrity of enteroviruses. At pH 5 and 7, RNA hydrolysis of poliovirus particles occurs; and at pH3, 5, 6, and 7 the nucleic acid becomes susceptible to ribonuclease. Only virus particles inactivated at pH 3 show sensitivity to chymotrypsin. The hemagglutinins of echovirus type 7 are destroyed during inactivation at pH 3, 4, 5 and 6, but at pH 6 this alteration precedes the loss of infectivity. The pH of the suspension is a primary determinant of the mechanism of virus destruction and possibly of the loss of infectivity at these temperatures.

Alexander *et al.*, (1975) The growth of Infectious bronchitis viruses at different pH values in the range 6.0-9.0 demonstrated that although the virus was released at a much faster rate at the higher pH values the titre tended to drop more quickly. At the acid pH values the virus was released more slowly but reached a maximum titre similar to that at the higher pH values and showed only minimum reduction in infectivity up to 49 hours post inoculation. The stability of virus in tissue culture medium was shown to be directly related to pH 6.0-8.0, being more stable at the acid pH values. The degree of cytopathogenicity induced in chick kidney cells following

infection with viruses was directly related to the pH at which the cells were incubated, occurring earlier and more extensively in cells at the higher pH values. Cell macromolecule synthesis in chick kidney cells was inhibited following infection with viruses and was apparently due to cell damage and death.

Stallknecht., (1990) Stated that the combined effects of water temperature, salinity, and pH on persistence of avian influenza viruses were tested within the ranges normally associated with surface water. Differences were detected between temperature (17°C and 28°C), pH (6.2, 7.2, 8.2), and salinity (0 ppt and 20 ppt), with a strong interactive effect observed between pH and salinity. Estimated persistence of infectivity for 1 x 10(6) mean tissue-culture infective dose viruses was longest at 17 C/0 ppt/pH 8.2 (100 days) and shortest at 28 C/20 ppt /pH 8.2 (9 days). Differences in the response to these variables were apparent between viruses. Observations were consistent with the model system, with duration of infectivity decreasing with increased salinity and pH. This suggests that experimental results may have application to field conditions.

Scholtisseks *et al*., (1969) Stated that temparature was unstable in vitro as well as in vivo. The synthesis of virus haemagglutinin and At temperatures below 34°C the energy of activation in vitro was 16 kcal./mol, greater than above this temperature. The rate of synthesis of virus haemagglutinin and neuraminidase decreased rapidly below 34°C. At low temperatures the activity of the virus RNA polymerase may be rate limiting for virus multiplication. At 41°C the virus RNA neuraminidase, however, was unimpaired. At 44°C virus subunits were not produced. The host cells with- stood 44°C without any harm during the of the experiment. It is uncertain whether or not at elevated temperatures the activity of the viral RNA polymerase was also rate limiting.

Muhammad Akbar *et al.,* (2009) Stated that virus lost infectivity after 30 min at 56°C, after 1 day at 28°C but remained viable for more than 100 days at 4°C. Acidic pH (1, 3) and basic pH (11, 13) were virucidal after 6 hours contact time; however virus retained infectivity at pH 5 (18 h), 7 and 9 (more than 24 hour). UV light was proved ineffectual in inactivating virus completely even after 60 min. Soap (lifebuoy), detergent (surf excel) and alkali (caustic soda) destroyed infectivity after 5 min at 0.1,

0.2 and 0.3% dilution. All commercially available disinfectants inactivated virus at recommended concentrations.

Mbithi *et al.*, (2010) Stated that virus can be inactivated an air temperature of 5°C, 20°C, or 35 °C. Virus survival was inversely proportional to the level of relative humidity and temperature, and the half-lives of the virus ranged from greater than 7 days at the low relative humidity and 5 °C to about 2 h at the ultrahigh relative humidity and 35 °C.

Lisa *et al.*, (2010) Stated that At 4°C, infectious virus persisted for as long as 28 days, and the lowest level of inactivation occurred at 20% Relative humidity (RH). Inactivation was more rapid at 20°C than at 4°C at all humidity levels; the viruses persisted for 5 to 28 days, and the slowest inactivation occurred at low RH. Both viruses were inactivated more rapidly at 40°C than at 20°C. The relationship between inactivation and RH was not monotonic, and there was greater survival or a greater protective effect at low RH (20%) and high RH (80%) than at moderate RH (50%).

Abad *et al.*, (1994) Stated that the stability of the viruses was generally influenced by environmental factors such as relative humidity (RH), temperature, and the type of surface contaminated. The resistance to the desiccation step appears to be of major significance in determining the survival of a virus dried on fomites. On nonporous surfaces, PV and ADV persisted better in the presence of feces. However, on porous fomites the presence of fecal material had a negative influence on the survival of virus, greater virus survival was observed at 4 degrees than at 20 degrees C. Virus survival was enhanced at high RH; the survival of the latter was enhanced at least for nonporous materials. When dried on porous materials, HRV also exhibited greater persistence at high RH. The survival of ADV was not affected by RH. The validity of using bacteriophages of bactericides fragile as indicators of human viruses dried on fomites was evaluated.

Lu *et al.*, (2003) Stated that the survival or clearance of the virus was evaluated using experimentally infected specific pathogen free (SPF) chickens of different age groups. Birds of different ages were successfully infected with infectious doses ranging between $10^{4.7}$ and $10^{5.7}$ ELD_{50} per bird. In infected birds, the infective virus was

undetectable usually by the third week following exposure. The infectivity or inactivation time of the Newcastle disease virus in various environmental conditions was studied using chicken manure, heat, ethanol, pH, and disinfectants. The virus was effectively inactivated by field chicken manure in less than a week at an ambient temperature of 15 – 20°C. At a pH 2, heating at 56° C, and exposure to 70% ethanol or a specific disinfectant, the viral infectivity was destroyed in less than 30 min.

Guan *et al*., (2009) Stated that Newcastle disease (ND) viruses were killed in compost within 7 days when temperatures had reached at least 50°C. More rapid destruction of the viruses and their RNA was found when infectious specimens were contained in nylon mesh bags with full exposure to compost than when they were contained in plastic vials with exposure only to heat produced by compost. At temperatures ranging from 35–55°C, survival of viruses was similar in the two extracts. However, at 25°C the killing of viruses was significantly more rapid in the suspension that contained microbes from compost than in the one that contained microbes from cage layer manure. In sealed vials buried in compost the viruses survived to day 10. At ambient temperatures that ranged from 13–28°C, viral RNA was degraded more rapidly in used litter than in cage layer manure, suggesting that differences in microbial activity may have been a factor. In the absence of microbial activity, the viral RNA in sealed vials remained stable at ambient temperatures to day 21.

Kinde *et al.,* (2004) Found that The variation in published Newcastle disease virus (NDV) survival times outside the host has been primarily associated with temperature, moisture, environment, and the medium in which the virus was tested and showed that the virus could survive on skin and in bone marrow of plucked and eviscerated chicken carcasses when held at 1.1 °C to 1.7°C for 98 to 134 days, respectively. When the carcasses were unplucked, the survival times increased to 160 and 196 days, respectively, at the same temperature. When the experiment was repeated at 15.5 °C, the survival rate for both sites increased to more than 300 days, and it did not matter whether the carcass was plucked or unplucked. In another experiment, the NDV was tested on filter paper at 36.6 °C and survived for 6 hr but inactivated after 12 hr; on egg shell the virus was active after 24 hr but inactivated after 44 hr; in sterile feces the virus was active after 72 hr but inactive at 90 hr; in fluid suspension at 1.1–1.7 °C C the virus was active after 203 days but inactive after 217 days; in fluid suspension at 0

°C the virus was active after 161 days but inactive after 175 days; and when dried on glass and stored at 1.1–1.7 °C and 0 °C the virus was found to be active after 396 days. In an experimental trial of VVND virus survival in litter at 23–29 °C after housing VVND-infected chickens and turkeys, the virus could not be detected after 10 and 14 days. In a study using the GB Texas strain of NDV, the survival of NDV in some of the substrates that might be utilized by the virus in natural transmission was evaluated. These included sterile and no sterile soil, water, earthworms, and planarian (*Broad et al.*, 1958). NDV-seeded sterile and no sterile soils the ambient temperature, with no heat being generated in the stacked cones of manure at the time of sampling. The mid-winter lower temperatures, higher humidity and moisture, and the presence of organic material to protect the virus are consistent with the literature in favoring a longer NDV survival time.

Stallknecht *et al.*, (2009) Found that virus can survive water at different temperature such as 4°C, 17°C, 28°C.The duration of persistence was inversely relate to water temperature and temperature-related variation was extreme, as some viruses remained infective well over a year at 4°C, but only days at 37°C (Brown *et al.*, 2009)). temperature greatly influences the duration of viral infectivity and the temperature/infectivity relationship can be described with an exponential decay function; variation between viruses is most evident under cold water (4°C) conditions, with little variation observed at temperatures >28°C. pH greatly affects infectivity, with a rapid loss of infectivity below pH 6.5, all viruses were most stable between pH 7.4 and pH 8.2, but variation in pH tolerance was observed between individual viruses. In chicken faeces, inactivation of this virus can be rapid at high temperatures (above 25°C) (Chumpolbanchorn *et al.*, 2006) but is prolonged at low temperatures; for example, at 20°C virus can remain infectious in chicken faeces for 7 days (Castro *et al.*, 2003), but at 4°C the virus can remain infectious for as long as 30 days (Beard *et al.*, 1984). Contaminated poultry litter has a potential role in virus transmission, to date, however, this role remain undefined.

Chapter-3: Materials and Methods

3.1. Study area selection

Upazilas under Chittagong district and one upazila under Dhaka district were selected on the basis of previous history where abundance of migratory birds visited in different natural water reservoirs such as ponds or lakes especially during winter season.

3.2. Selection of water source and collection of water samples

Four Dighi from five upazilas under Chittagong district were selected where migratory birds frequently visit in winter. They were commonly named as Varsity dighi, Padma dighi, Ashroom dighi, Nallikkha dighi located in Hathazari, Ranguinia, Bashkhali, Chandanaish upazila in Chittagong (**figure 2**) and Jahangirnagor Varsity Lake at Savar upazila in Dhaka(**figure 3**). One liter of water per dighi per upazila was obtained and transferred immediately to Microbiology laboratory at CVASU. Water samples were used as a media for exposure to Newcastle disease virus. Water samples were collected in a sterile clean glass bottle.

3.3. Storage of water samples

The water samples were stored in the microbiology laboratory of CVASU at normal temperature until laboratory work started.

3.4. Outline of laboratory work

3.4.1. Grouping of collected water samples

Every water samples were divided into two equal volumes and placed into 500 ml aliquots which were measured by conical flask. They were 10 in numbers. Five of the aliquot were boiled at 100°C for 15 minutes on electric heater followed by filtration with the help of syringe filter (pore size 0.2 μm) to remove microbes. This group was named treated (T) group which contains five water samples. They were marked as T_1, T_2, T_3, T_4 and T_5. Another five aliquots were remaining as they were in non treated condition (unfiltered and no boiled) and they were named as non treated (NT) group which contains five water samples. They were marked as NT_1, NT_2, NT_3, NT_4 and NT_5.

3.4.2. Testing of pH value of water samples

The pH values of both groups of water samples were measured by using AD1030 pH/mV and Temperature Meter (ADWA INSTRUMENTS Kft, Hungary).

3.4.3. Determination of bi-carbonates concentration in different water samples

100ml water sample was taken into a conical flask. Then 2ml normal buffer solution was added into the sample. 10 drops of Methyl orange was added as indicator and the sample became yellow. Then the solution was titrated with 0.02N H_2SO_4 until the color become orange. Titer value was then recorded. (Jagat and Busanta, 2007)

$$Na\,HCO_3 + H_2SO_4 \text{ ----------> } Na_2\,SO_4 + CO_2 + H_2O$$

3.4.4. Determination of water hardness in different water samples

100ml water sample was taken in a conical flask. Then 2 ml of normal buffer solution was added into the sample. Then 4 drop of Erichrome Black-T indicator was added and the mixed solution was turned into blue color according to the color of indicator. The presence or absence of water hardness was determined by color observing. (Jagat and Busanta, 2007)

$$Ca^{2+} + EBT \text{ ----------> } [Ca\text{-}EBT]$$

Unstable complex (Wine red)

$$[Ca\text{-}EBT] + EDTA \text{ ----------> } [Ca\text{-}EDTA] + EBT$$

Stable complex (Blue)

3.4.5. Antibiotic treatment to the water samples

The two groups of both treated and non treated water samples were mixed with 10% solution of Gentamycin for the destruction of biotic organism that hampered our study method.

3.4.6. Collection of embryonated eggs

10 days old embryonated chicken eggs about five in numbers were collected from Regional Poultry Farm, Pahartoli, Chittagong. Then the eggs were transferred into Microbiology laboratory. Eggs were candled properly with the help of Candler. The candling was done to mark the circular air sac area and marked the air sac. The blood

vessels were also marked for identifying the drilling point on the circular mark by the help of a pencil.

3.4.7. Newcastle Disease virus collection and propagation

Previously characterized Velogenic strain of Newcastle Disease Virus was taken from the microbiology lab at CVASU. As soon as collected the virus placed in deep freezed condition (-80) ˚C Temperature. Then, he drilling points were properly swabbed with 70% alcohol by cotton to destroy extraneous microbes on the egg shell. The virus suspension was prepared by mixing 2 ml of saline solution with one vial vaccine. Then the virus suspension was stored in -80˚C. After that the virus suspension was kept in room temperature (26˚C) for few minutes for melting before started working. By that time, the marked points of eggs were drilled properly. The Newcastle disease virus was propagated in those chicken eggs via allantoic route for further use. At first, melted viral suspensions were inoculated @ 0.1 ml to the allantoic route of those embryonated chicken eggs. After completion of inoculation in all the eggs, those were transferred into incubator for 48 hours at 37˚C without any disturbances. Allantoic fluids were collected via 10 ml sterile syringe and stored at - 80˚C temperature in 15 ml tubes for further use.

3.4.8. Haemagglutination test

3.4.8.1. Collection of chicken blood and preparation of 1 percent chicken red blood cells (RBC's)

A disease free broiler chicken was collected from market. 1 ml of blood was drawn from wing vein by using a 10 ml syringe where anticoagulant were previously added and kept it after gentle mixture. This mixture was kept in a large, conical centrifuge tube where equal volume of 1×phosphate-buffered saline (PBS) was added and mixed properly. The suspension was centrifuged at 1000 rpm for 10 minutes. The supernatant was poured off and 20 to 30 volumes of 1×PBS were added to the packed cells. The cells were re-suspended gently and the centrifugation step was repeated two to three times. The cells can then be used to prepare a 1 percent cell suspension in isotonic 1×PBS. 1 ml of chicken RBC's suspension was mixed with 99 ml of 1×PBS in a conical flask to prepare 1percent chicken RBC. This suspension was stored at 4°C for 48 hours for further purposes.

3.4.8.2. Preparation of 8 HA unit virus

Fifty µl of PBS was transferred in all well of microtitre plate. Fifty µl of collected allantoic fluid was transferred into first well. It was mixed well and fifty µl was successively transferred into next until 9th well.10th, 11th and 12th wells were kept as virus, RBC and Saline control. The end point was determined by button or no HA. The well next to end point was considered as 1 HA unit.

3.4.9 Viral culture in embryonated chicken eggs

Stored viral suspensions were kept in room temperature for thawing and then 4 ml Newcastle disease virus suspension was mixed with 4ml both of the filtered and unfiltered water samples and incubated for at 6, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66, 72, 78, 84, 90, 96 hours in the temperature of 22°C in air condition (AC), 30°C in room temperature and 37°C in incubator temperature. Then, 2 ml from each mixture or suspension was removed after being incubated for each hour both treated (T) and non treated (NT) water samples which were marked as T_1, T_2, T_3, T_4, T_5 and NT_1, NT_2, NT_3, NT_4 and NT_5 at 22°C, 30°C and 37°C. After exposing the virus at different times and temperatures it was stored at - 80°C for further use.

0.1ml of viral suspension was inoculated into the allantoic route of 10 days old embryonated chicken eggs for each temperature, time, categories and water samples. Incubated at 37°C for 48 hours. After 48 hours of incubation at 37°C, the allantoic fluids were harvested by 10 ml syringe. 1ml of allantoic fluid was harvested from every inoculated embryonated egg and the haemagglutinating activity of Newcastle disease viruses was observed with the help of Slide Haemagglutination test for each and every temperature (22°C, 30°C and 37°C), time (6, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66, 72, 78, 84, 90 and 96 hours), categories (Non treated and Treated) and water samples sources (five dighi and lakes) of the study. The collected allantoic fluid which showed positive in spot HA test was checked further for determining HA titre by plate HA test.

3.4.10. Slide haemagglutination test

It is very easy and rapid technique to detect the survival of haemagglutinating viruses. In case of slide haemagglutination test, 5% chicken RBC's (5 ml of RBC's was mixed with 95 ml of PBS) was prepared for clear detection of haemagglutination instead of 1percent chicken RBC. Otherwise, the same procedure was followed as like as preparation of 1 percent chicken RBC (Topic no. 3.4.8.1).

An equal volume of 5 percent chicken RBC and virus containing allantoic fluid on a glass slide were poured. Then gentle mixture was done and waits (5-10) minutes. In case of positive results, agglutinated RBC's on the glass slides were observed with naked eyes within 5-10 minutes. On the other hand, blood was as usual in negative results.

3.4.11. Plate haemagglutination test

Fifty µl of PBS was kept into each well of V plastic microwell plate. Then fifty µl virus suspensions was added in first well and mixed properly. Then two fold Dilution of fifty µl volume of virus suspension across the plate until column 11. Then added fifty µl of PBS to each well. Then added 50 µl of 1 percent chicken RBC's to each well including column 12. The wells in this column were control wells that contain only PBS and red blood cells. The plate was allowed to stand for 45 minutes at room temperature. The results were recorded for each sample.

3.5. Statistical analysis

The estimated data were entered into spread sheet program Microsoft Office Excel-2007 and was analyzed by STATA software to observe the differential significance of two groups of data (raw water and treated water) for several chemical parameters. Differences were considered significant when those had P value < 0.05.

Figure 2. Map showing the study areas in Chittagong district with round marks

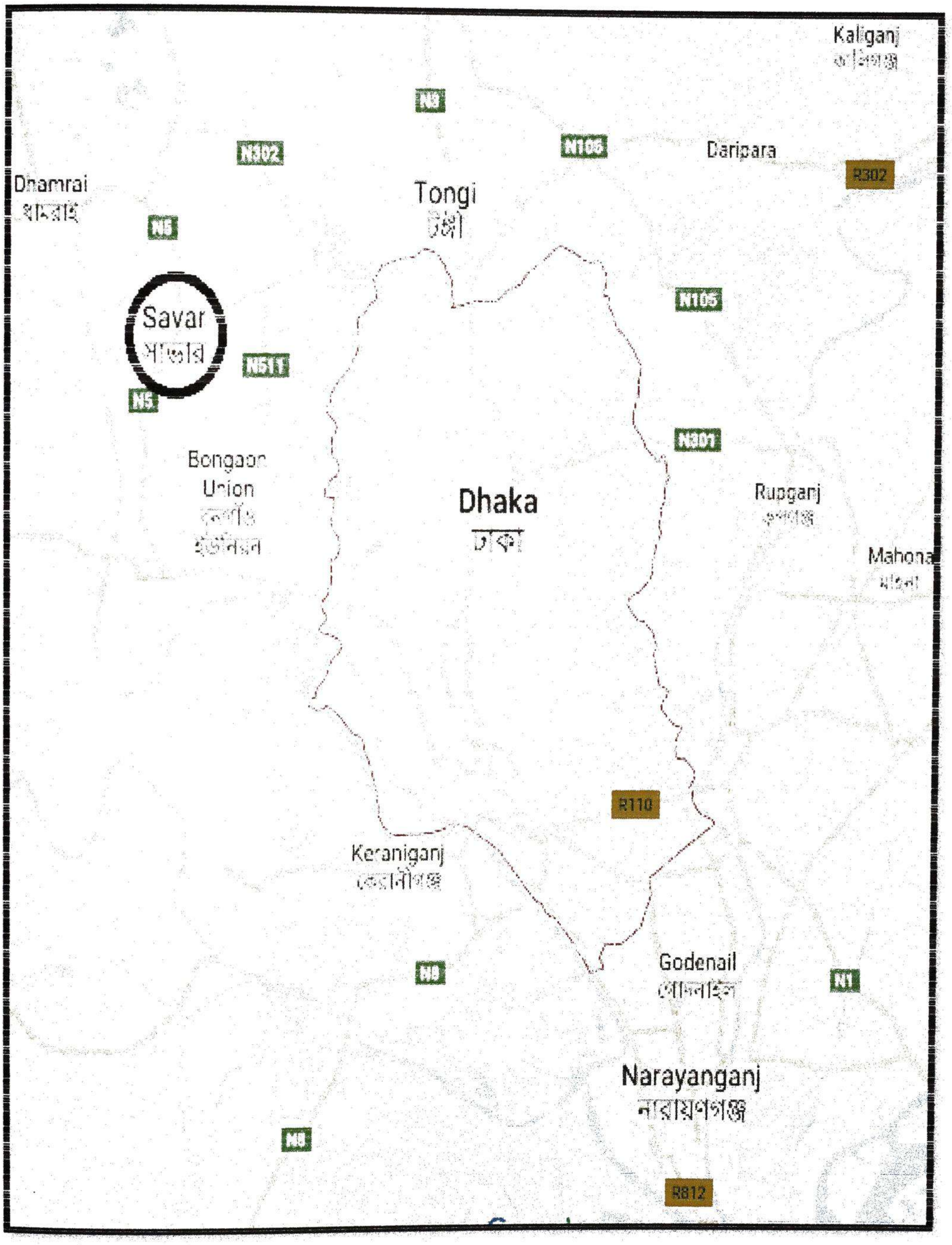


Figure 3. Map showing the study area in Dhaka district with round marks

Chapter-4: Results

Among all the water samples lowest pH value for non-treated (NT) water sample was 7.21 and the highest 7.98. The lowest pH value of treated (T) water sample was 7.10 and the highest pH 7.86. The average pH value of treated (T) water samples (7.50) was less than the average pH value (7.65) of non-treated water samples and there is no significant difference (p value is 0.22) between the pH value of given water samples. (Table 3).

Table 3. pH value of non-treated (un-filtered and un-boiled) and treated (filtered and boiled) water samples

Water sample	Non-Treated (NT)	Treated (T)	Difference
1	7.73	7.62	0.11
2	7.98	7.86	0.12
3	7.68	7.53	0.15
4	7.21	7.10	0.11
5	7.66	7.52	0.14
Average	**7.65**	**7.50**	**0.12**

So, it is clear that the non-treated water samples are more alkaline than the treated water samples. Water samples 1,2,3 and 5 were collected four upazilla under Chittagong district where the water sample 4 sample was collected from Jahangirnagar University Lake at Saver upazila under Dhaka district. Here, pH value of water samples in Chittagong are more than pH value of water samples in Dhaka. It is clear that among the five samples the pH value of fourth sample is less than that of other samples. (Table 3).

The lowest bicarbonate value of non-treated (NT) water sample was 18 (PPM) and the highest value was 26 (PPM). The lowest value of treated (T) water sample was 17 (PPM) and the highest 23 (PPM). (**Table 4**).

Table 4. Bicarbonate value of both non-treated (un-filtered and un-boiled) and treated (filtered and boiled) water samples

Sample no	Non-Treated(NT) (PPM)	Treated (T) (PPM)	Difference (PPM)
1	23	22	1
2	26	23	3
3	24	23	1
4	18	17	1
5	23	20	3
Average	**22.8**	**21**	**1.8**

From the above data it is clear that among the five samples the bicarbonate value of 4th sample both treated and non-treated was less than that of other samples. Bicarbonate value of water samples in Chittagong are higher than (i.e. more salinity) bicarbonate value of water samples in Dhaka. Fresh water is generally characterized by having low concentrations of dissolved salts and other total dissolved solids.

The lowest water hardness of non-treated (NT) water sample is 40 (mg/l) and the highest value is 49 (mg/l). The lowest value of treated (T) water sample is 36 (mg/l) and the highest value is 43(mg/l). (**Table 5**).

Table 5. Water hardness of non-treated (un-filtered and un-boiled) and treated (filtered and boiled) water samples

Sample no	Non-Treated (NT) (mg/l)	Treated (T) (mg/l)	Difference
1	44	40	4
2	47	43	4
3	46	40	6
4	40	36	4
5	49	43	6
Average	**45.2**	**40.4**	**5**

It is clear that among the five samples, the water hardness of 4th sample of both treated and non-treated is less than that of other samples. The average water hardness of treated water sample is 40.4 which is less than bicarbonate value of non-treated water samples (45.2).

Table 6. Distribution of different categorical variables

Variable	Categories	Frequency	Mean	SD	Mean±SD	95% CI	P-value
pH	Non-treated	5	7.7	0.28	7.7±0.28	7.3-8.0	0.49
	Treated	5	7.5	0.27	7.5±0.27	7.2-7.9	
Bicarbonate	Non-treated	5	22.8	2.9	22.8±2.9	19.1-26.5	0.33
	Treated	5	21	2.5	21±2.5	17.8-24.2	
Water Hardness	Non-treated	5	45.2	3.4	45.2±3.4	40.9-49.4	0.04
	Treated	5	40.4	2.9	40.4±3.4	36.8-43.9	

SD= Standard deviation; CI=Confidence interval

Water hardness of both non-treated (NT) and treated (T) water samples are also shown in this **table 6**.The average bicarbonate value of treated water sample is 40.4±2.9 which is less than bicarbonate value of non-treated water samples (45.2±3.4) and difference is significant ($P<0.05$).

Table 7. Slide Haemagglutination (HA) test for the survival of Newcastle Disease virus(NDV)at 22˚Cfor both non-treated (NT_1-NT_5) and treated (T_1-T_5) water samples

	6 hrs	12 hrs	18 hrs	24 hrs	30 hrs	36 hrs	42 hrs	48 hrs	54 hrs	60 hrs	66 hrs	72 hrs	78 hrs	84 hrs	90 hrs	96 hrs
NT_1	+	+	+	+	+	+	+	+	+	+	+	-	NA			
T_1	+	+	+	+	+	+	+	+	+	+	+	+	-	NA		
NT_2	+	+	+	+	+	+	+	+	+	+	+	-	NA			
T_2	+	+	+	+	+	+	+	+	+	+	+	+	-	NA		
NT_3	+	+	+	+	+	+	+	+	+	+	+	-	NA			
T_3	+	+	+	+	+	+	+	+	+	+	+	+	-	NA		
NT_4	+	+	+	+	+	+	+	+	+	+	+	+	+	-	NA	
T_4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	NA
NT_5	+	+	+	+	+	+	+	+	+	+	+	-	NA			
T_5	+	+	+	+	+	+	+	+	+	+	+	+	-	NA		

NA- Not applicable, NT-NonTreated, T- Treated, hrs-Hours

+Ve= Positive and –Ve= Negative

In **Table 7.** it is found that at 22˚Cthe virus of NT_1 can survive upto 66 hours where it's 72 hours for T_1. At the same temperature viral survival in NT_2, NT_3 and NT_5 samples were 66 hours for all treated samples (T_2, T_3 and T_5) it was 72hours. But the virus in NT_4 can survive about 78 hours and in treated (T_4) upto 84 hours. From above observation it can be said, the virus present in treated water sample can survive longer compared to the virus present in non treated water sample. It is also found that, the virus in water sample-4 can survive more times compared other four water samples.

Table 8. Slide Haemagglutination (HA) test for the survival of Newcastle Disease virus (NDV) at 30°C both non-treated (NT_1-NT_5) and treated (T_1-T_5) water samples

	6 hrs	12 hrs	18 hrs	24 hrs	30 hrs	36 hrs	42 hrs	48 hrs	54 hrs	60 hrs	66 hrs	72 hrs	78 hrs	84 hrs	90 hrs	96 hrs
NT_1	+	+	+	+	+	+	+	+	-	NA						
T_1	+	+	+	+	+	+	+	+	+	-	NA					
NT_2	+	+	+	+	+	+	+	+	+	-	NA					
T_2	+	+	+	+	+	+	+	+	+	+	-	NA				
NT_3	+	+	+	+	+	+	+	+	+	-	NA					
T_3	+	+	+	+	+	+	+	+	+	+	-	NA				
NT_4	+	+	+	+	+	+	+	+	+	+	+	+	-	NA		
T_4	+	+	+	+	+	+	+	+	+	+	+	+	+	-	NA	
NT_5	+	+	+	+	+	+	+	+	+	-	NA					
T_5	+	+	+	+	+	+	+	+	+	+	-	NA				

NA- Not applicable, NT-NonTreated, T- Treated, hrs-Hours

+Ve= Positive and –Ve= Negative

In **Table 8.** it is found that at 30°C, the virus in NT_1 water sample survived upto 48 hours where it's 54 hours for T_1. At the same temperature virus survived 54 hours in NT_2, 72 hours in NT_3 and 54 hours in NT_5. But the survival periods were 60 hours in T_2, 78 hours in T_3 and 60 hours in T_5. Virus in NT_4 survived about 72hours and 78 hours inT_4. Again, it's seen that the virus in treated water sample can survive longer compared to the virus in non-treated water sample. It is also found that, the virus in water sample-4 can survive more times as compared with the other viruses in other four water samples.

Table 9. Slide Haemagglutination (HA) test for the survival of Newcastle Disease virus (NDV) at 37°C in both non-treated (NT_1-NT_5) and treated (T_1-T_5) water samples

	6 hrs	12 hrs	18 hrs	24 hrs	30 hrs	36 hrs	42 hrs	48 hrs	54 hrs	60 hrs	66 hrs	72 hrs	78 hrs	84 hrs	90 hrs	96 hrs
NT_1	+	+	+	+	+	-	NA									
T_1	+	+	+	+	+	+	-	NA								
NT_2	+	+	+	+	+	-	NA									
T_2	+	+	+	+	+	+	-	NA								
NT_3	+	+	+	+	-	NA										
T_3	+	+	+	+	+	-	NA									
NT_4	+	+	+	+	+	+	+	-	NA							
T_4	+	+	+	+	+	+	+	+	-	NA						
NT_5	+	+	+	+	+	-	NA									
T_5	+	+	+	+	+	+	-	NA								

NA- Not applicable, NT-NonTreated, T- Treated, hrs-Hours

+Ve= Positive and –Ve= Negative

In **Table 9.** it is found that at 37°C the virus of NT_1survived up to 30 hours where the virus in T_1 for 36 hours. The virus in NT_2 survived about 30 hours and in T_2 up to 36 hours. For NT_3 and T_3 the survival periods were 24 hours and 30 hours respectively. The virus in NT_5 survived about 30 hours in T_5 up to 36 hours. But the virus in NT_4 survived about 42hours and 48 hours in T_4 water sample. So, the virus present in treated water sample can survive more time as compare to the virus present in non treated water sample. It is also found that, the virus in water sample-4 can survive more times as compared with the other viruses in other four water samples.

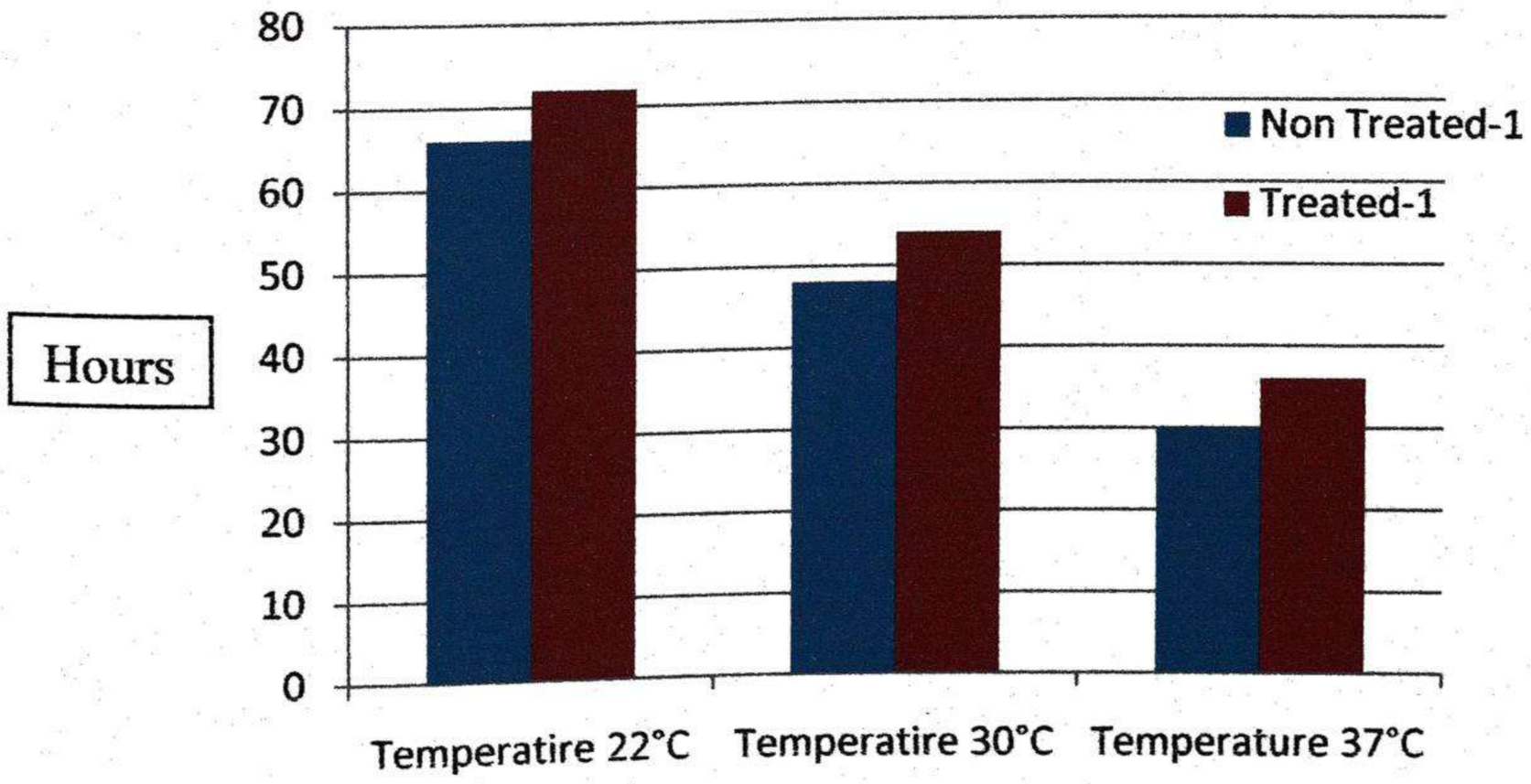


Figure 4. Survivability of virus in water sample-1 (NT_1 and T_1).

In **Figure 4.** the "X" axis indicates the survivability of virus in water sample-1 both treated and untreated (NT_1 and T_1) at different temperature such as 22°C, 30°C and 37°C.The "Y" axis indicates several hours of viral survivability. We found that as temperature gradually increases the gradually viral survivability decreases. The virus survivability is highest at 22°C for both NT_1 and T_1 whereas virus survivability is lowest of both NT_1 and T_1 at 37°C.

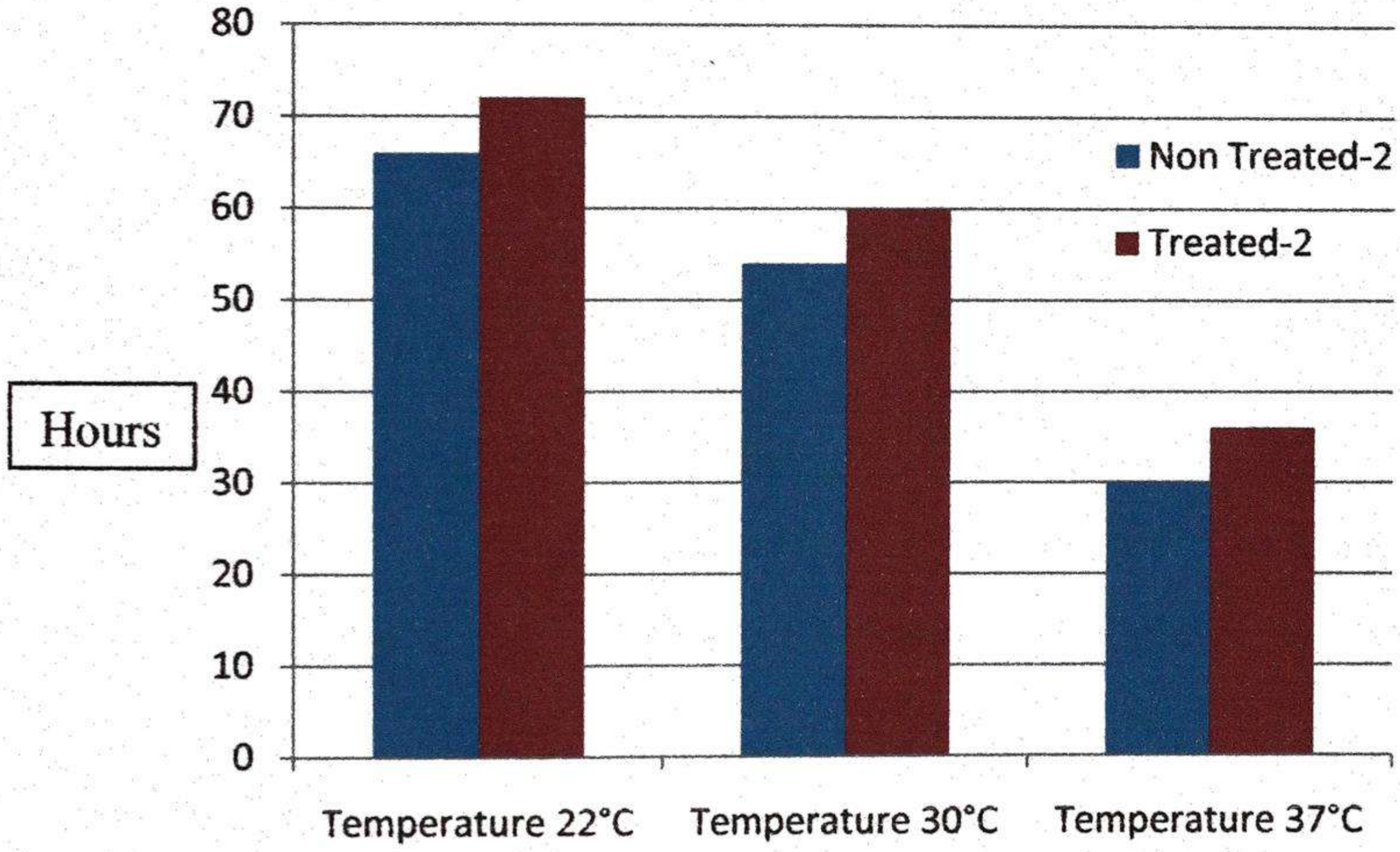


Figure 5. Survivability of virus in water sample-2 (NT_2 and T_2).

In **Figure 5.** the "X" axis and ''Y" axis indicate the survivability of virus indifferent temperature (22°C, 30°C and 37°C) for both NT_2 and T_2 . We found that as gradually increases the temperature causes gradually decreases the viral survivability. The virus survivability is highest in 22°C for both NT_2 and T_2 where as virus the lowest at 37°C.

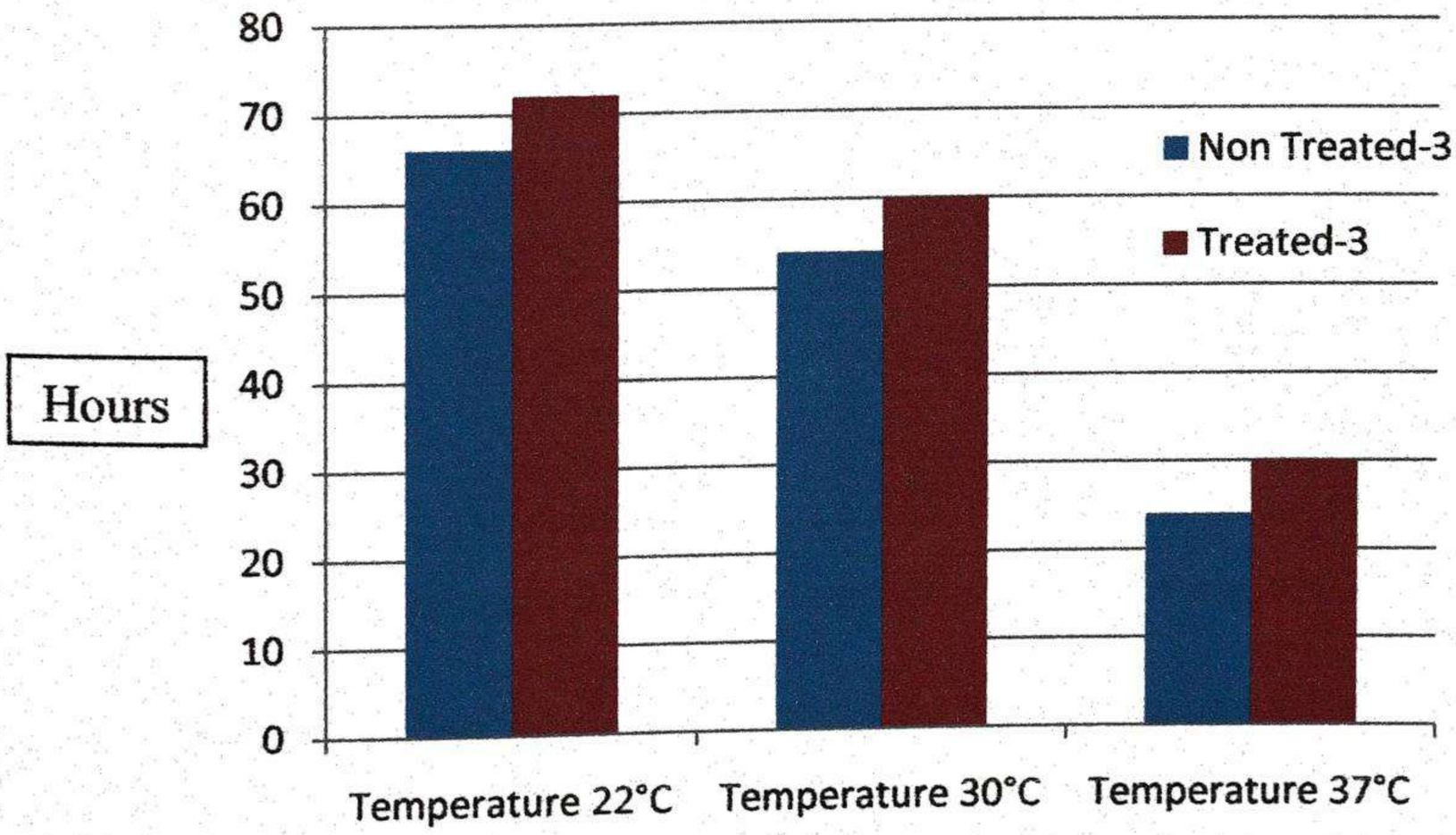


Figure 6. Survivability of virus in water sample-3 (NT_3 and T_3).

In **Figure 6.** the "X" axis and "Y" axis indicate the survivability of virus indifferent temperature (22˚C, 30˚C and 37˚C) for both NT_3 and T_3 sample. It's observed that as gradually increases the temperature causes gradually decreases the viral survivability. The virus survivability is highest in 22˚C of both NT_3 and T_3 whereas lowest at 37˚C.

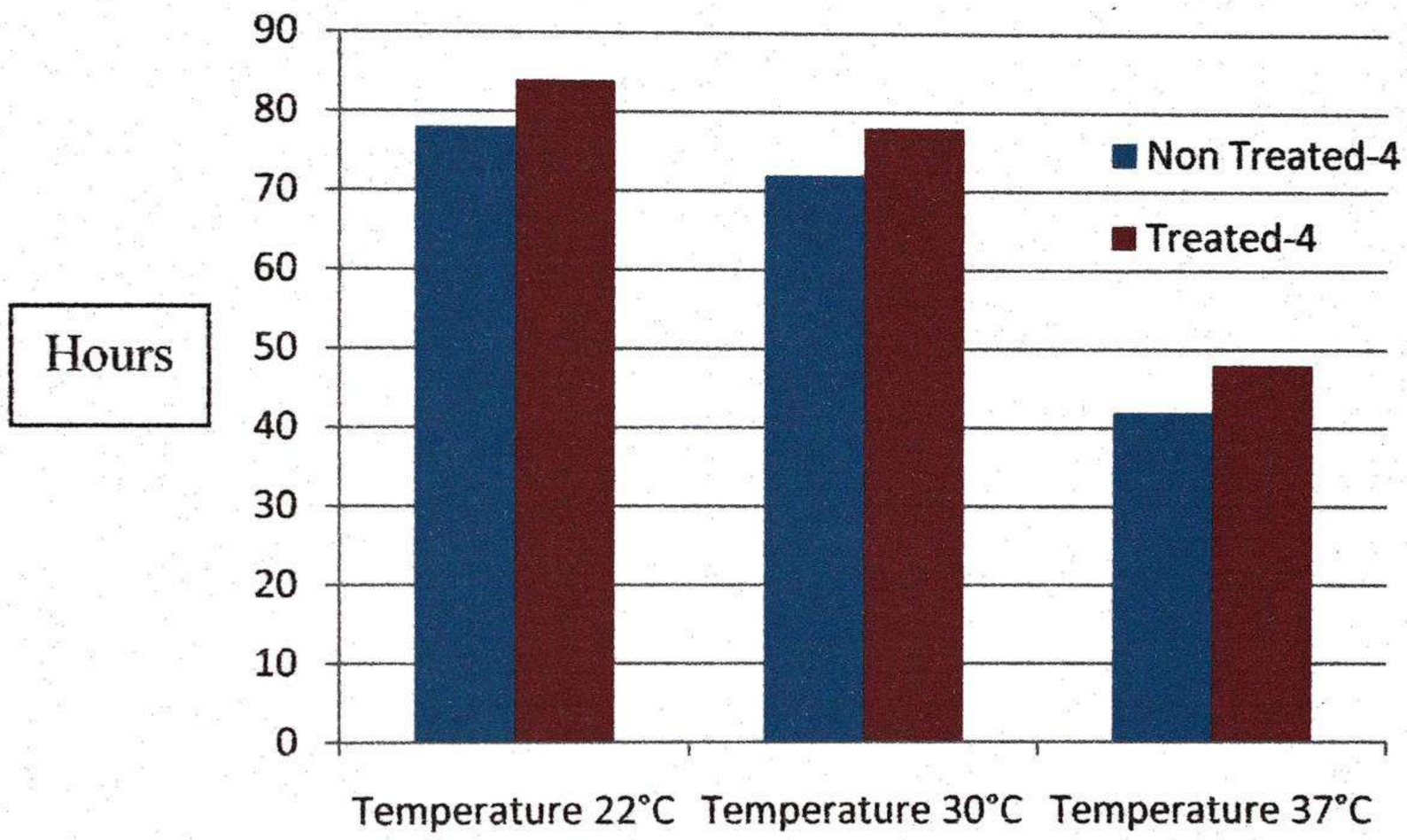


Figure 7. Survivability of virus in water sample-4 (NT_4 and T_4).

In **Figure 7**, the "X" axis and "Y" axis indicate the survivability of virus indifferent temperature (22˚C, 30˚C and 37˚C) for both NT_4 and T_4 sample. Viral survivability decreased with increased temperature. The survivability was highest at 22˚C for both NT_4 and T_4 where as lowest at 37˚C.

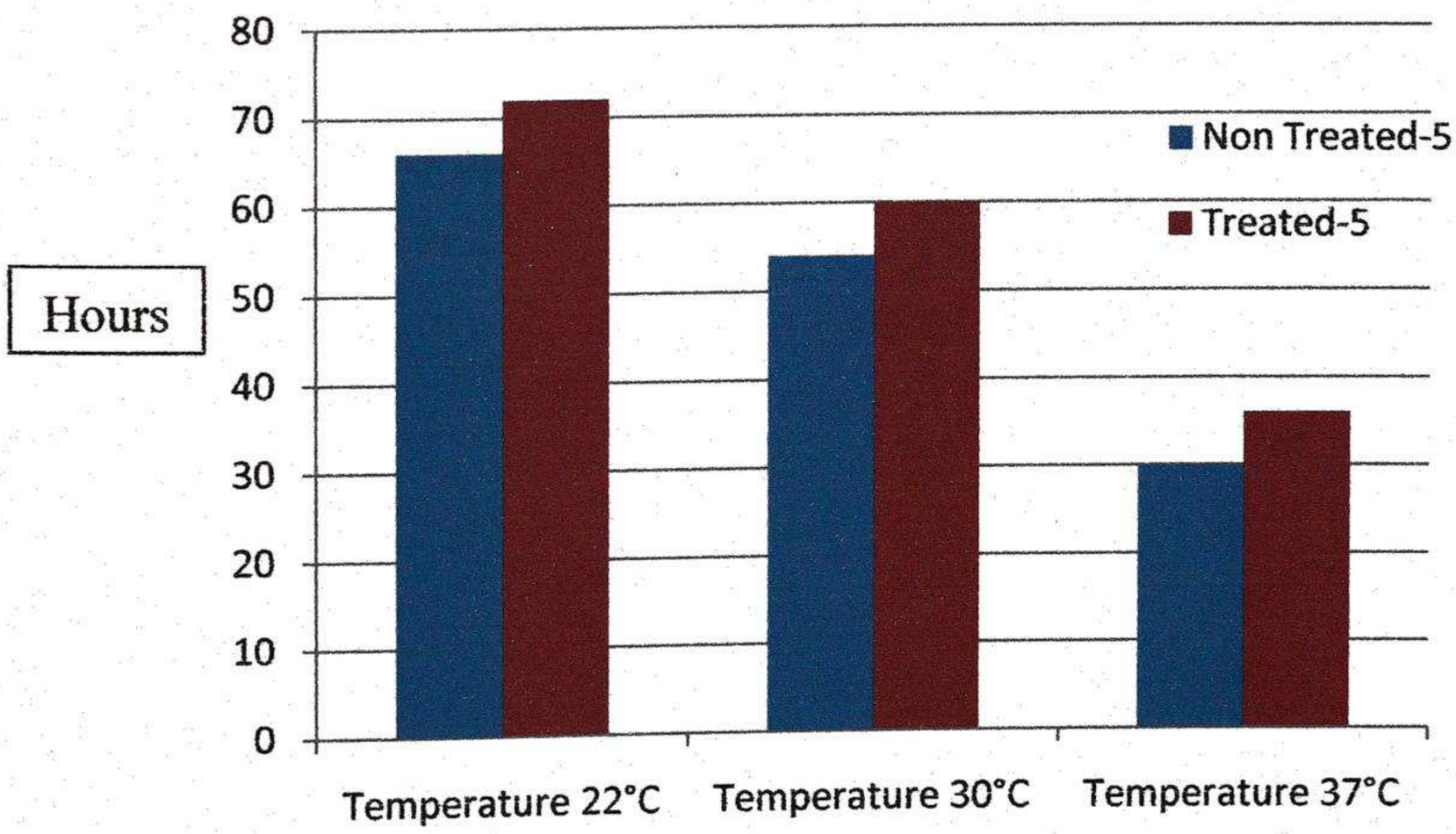


Figure 8. Survivability of virus in water sample-5 (NT5 and T5).

In **Figure 8.** the "X" axis and ''Y" axis indicate the survivability of virus indifferent temperature (22°C, 30°C and 37°C) for both NT_5 and T_5. Viral survivability gradually increases in temperature decreased. The virus survived highest at 22°C for both NT_5 and T_5 whereas lowest survivability found at 37°C.

Table 10. HA titre of Newcastle Disease virus (NDV) at 22°C both for non-treated (NT_1 -NT_5) and treated (T_1-T_5) water samples by plate agglutination test

	6 hrs	12 hrs	18 hrs	24 hrs	30 hrs	36 hrs	42 hrs	48 hrs	54 hrs	60 hrs	66 hrs	72 hrs	78 hrs	84 hrs	90 hrs	96 hrs
NT_1	2^4	2^4	2^4	2^4	2^4	2^3	2^3	2^3	2^3	2^2	2^2	NA				
T_1	2^4	2^4	2^4	2^4	2^4	2^3	2^3	2^3	2^3	2^3	2^2	2^2	NA			
NT_2	2^4	2^4	2^4	2^4	2^4	2^3	2^3	2^3	2^2	2^2	2^2	NA				
T_2	2^4	2^4	2^4	2^4	2^4	2^3	2^3	2^3	2^3	2^2	2^2	2^2	NA			
NT_3	2^4	2^4	2^4	2^4	2^4	2^3	2^3	2^3	2^3	2^2	2^2	NA				
T_3	2^4	2^4	2^4	2^4	2^4	2^3	2^3	2^3	2^3	2^2	2^2	2^2	NA			
NT_4	2^4	2^4	2^4	2^4	2^4	2^4	2^4	2^3	2^3	2^3	2^3	2^3	2^2	NA		
T_4	2^4	2^4	2^4	2^4	2^4	2^4	2^4	2^3	2^3	2^3	2^3	2^3	2^2	2^2	NA	
NT_5	2^4	2^4	2^4	2^4	2^3	2^3	2^3	2^3	2^2	2^2	2^2	NA				
T_5	2^4	2^4	2^4	2^4	2^3	2^3	2^3	2^3	2^2	2^2	2^2	2^2	NA			

NA-Not Applicable, hrs-Hours

In **Table 10**, It's shown that the HA titre of viruses incase of NT_1 is 2^2 at 66 hours where the viruses incase of T_1 the HA titre is 2^2 at 72 hours. Incase of NT_2 is 2^2 at 66 hours where the viruses incase of T_2 the HA titre is 2^2 at 72 hours. In case of NT_3 the HA titre of viruses is 2^2 at 66 hours where in T_3 the HA titre is 2^2 at 72 hours. In case of NT_4 the HA titre of viruses is 2^2 at 78 hours where in T_4 the HA titre is 2^2 is at 84 hours. In case of NT_5 the HA titre of viruses is 2^2 at 66 hours where in T_5 the HA titre is 2^2 at 72 hours. So it is clear that the HA titre of viruses was higher in treated (T) samples compared to non-treated (NT) samples.

Table 11. HA titre of Newcastle Disease virus (NDV) at 30°C in both non-treated (NT_1-NT_5) and treated (T_1-T_5) water samples by plate agglutination test

	6 hrs	12 hrs	18 hrs	24 hrs	30 hrs	36 hrs	42 hrs	48 hrs	54 hrs	60 hrs	66 hrs	72 hrs	78 hrs	84 hrs	90 hrs	96 hrs
NT_1	2^4	2^4	2^4	2^3	2^3	2^3	2^2	2^2	NA							
T_1	2^4	2^4	2^4	2^3	2^3	2^3	2^3	2^2	2^2	NA						
NT_2	2^4	2^4	2^4	2^3	2^3	2^3	2^2	2^2	2^2	NA						
T_2	2^4	2^4	2^4	2^3	2^3	2^3	2^2	2^2	2^2	2^2	NA					
NT_3	2^4	2^4	2^4	2^3	2^3	2^3	2^2	2^2	2^2	NA						
T_3	2^4	2^4	2^4	2^3	2^3	2^3	2^2	2^2	2^2	2^2	NA					
NT_4	2^4	2^4	2^4	2^3	2^3	2^3	2^3	2^2	2^2	2^2	2^2	2^2	NA			
T_4	2^4	2^4	2^4	2^3	2^3	2^3	2^3	2^2	2^2	2^2	2^2	2^2	2^2	NA		
NT_5	2^4	2^4	2^4	2^3	2^3	2^3	2^2	2^2	2^2	NA						
T_5	2^4	2^4	2^4	2^3	2^3	2^3	2^2	2^2	2^2	2^2	NA					

NA-Not Applicable, hrs-Hours

In **Table 11**, It is shown that the HA titre of viruses incase of NT_1 is 2^2 at 48 hours where the viruses incase of T_1 the HA titre is 2^2 at 54 hours. In case of NT_2 titre is 2^2 at 54 hours where the viruses in case of T_2 the HA titre is 2^2 at 60 hours. In case of NT_3 the HA titre of viruses is 2^2 at 54 hours where in T_3 the HA titre is 2^2 at 60 hours. In case of NT_4 the HA titre of viruses is 2^2 at 72 hours where in T_4 it's 2^2 at 78 hours. In case of NT_5 the HA titre of viruses is 2^2 at 54 hours where in T_5 the HA titre is 2^2 at 60 hours. So it is clear that the HA titre of viruses was found higher in treated (T) water samples as comparedto non-treated water (NT) samples.

Table 12. HA titre of Newcastle Disease virus (NDV) at 37°C both non treated (NT_1-NT_5) and treated (T_1-T_5) water samples by plate agglutination test

	6 hrs	12 hrs	18 hrs	24 hrs	30 hrs	36 hrs	42 hrs	48 hrs	54 hrs	60 hrs	66 hrs	72 hrs	78 hrs	84 hrs	90 hrs	96 hrs
NT_1	2^4	2^4	2^3	2^3	2^2	NA										
T_1	2^4	2^4	2^3	2^3	2^2	2^2	NA									
NT_2	2^4	2^4	2^3	2^3	2^2	NA										
T_2	2^4	2^4	2^3	2^3	2^2	2^2	NA									
NT_3	2^2	2^4	2^3	2^2	NA											
T_3	2^2	2^4	2^3	2^2	2^2	NA										
NT_4	2^4	2^4	2^3	2^3	2^3	2^2	2^2	NA								
T_4	2^4	2^4	2^3	2^3	2^3	2^2	2^2	2^2	NA							
NT_5	2^4	2^4	2^3	2^3	2^2	NA										
T_5	2^4	2^4	2^3	2^3	2^2	2^2	NA									

NA-Not Applicable, hrs-Hours

In **Table 12**, It's shown that the HA titre of viruses in case of NT_1 and T_1 is 2^2 at 30 hours and 2^2 at 36 hours. Titre for NT_2 and T_2 were 2^2 at 30 hours and 2^2 at 36 hours. In case of NT_3 the HA titre of viruses is 2^2 at 24 hours where it's 2^2 at 30 hours for T_3. In case of NT_4 the HA titre of viruses is 2^2 at 42 hours and in T_4 the HA titre is 2^2 at 48 hours. In case of NT_5 the titre is 2^2 at 30 hours and for T_5 the titre is 2^2 at 36 hours. So it is clear that the HA titre of viruses found higher in treated (T) water samples as compared to non-treated water (NT) samples. Again, water sample-4 (both NT_4 and T_4) showed highest HA titre and longer survival period in comparison to other water samples.

Chapter-5: Discussion

Our study revealed that temperature is an important factor for the survival of virus. As the temperature increased the perpetuation of Newcastle Disease virus was decreased which support to the study (Rizwan *et al.*, 1999).

At 22°C, the lowest time for the survival of virus among the non treated (unboiled and unfiltered) and treated (boiled and filtered) water samples was found 66 hours (HA titre $2^{2)}$ and 72 hours (HA titre 2^2) respectively where the highest time for survival of virus among the non treated (unboiled and unfiltered) and treated (boiled and filtered) water samples was found 78 hours (HA titre 2^2) and 84 hours (HA titre 2^2) respectively.

At 30°C, the lowest time for the survival of virus among the non treated (unboiled and unfiltered) and treated (boiled and filtered) water samples was found 48 hours (HA titre 2^2) and 54 hours (HA titre 2^2) respectively where the highest time for survival of virus among the non treated (unboiled and unfiltered) and treated (boiled and filtered) water samples was found 72 hours (HA titre 2^2) and 78 hours (HA titre 2^2) respectively.

At 37°C, the lowest time for the survival of virus among the non treated (unboiled and unfiltered) and treated (boiled and filtered) water samples was found 24 hours (HA titre 2^2) and 30 hours (HA titre 2^2) respectively where the highest time for survival of virus among the non treated (unboiled and unfiltered) and treated (boiled and filtered) water samples was found 42 (HA titre 2^2) hours and 48 hours (HA titre 2^2) respectively.

But there is variation in thermo stability of Newcastle Disease virus followed (John et al., 2016 and Qayyum *et al.,* 1999). This variation of thermo stability is found due to variation of strains of Newcastle Disease virus. Therefore, quite controversial results from different parts of the world have been reported. For instance, (Kim *et al.*, 1978; Lomniczi, 1975 and Hanson & Spalatin. 1978) observed that the V_4 strain of Newcastle Disease virus retained HA at 56°C for longer than 6o minutes. Likewise,

(Sing and Sing. 1969) reported that 22 virulent virus strains retained their hemagglutinating activity when exposed 56°C. As opposed to it, (Khadzhiev and Hadjiev. 1974; Buxton and Fraser. 1977) inactivated the Newcastle Disease virus at 56°C within 1 hour. The exact mechanism of heat mediated virus inactivation is not known. It is however expected that the temperature is responsible for decreasing the polymerase activity of the induced inactivation of Newcastle Disease virus which ultimately affects its replication activity (Stanwich and Hallum. 1976). However, the extent of virus survival depends upon quantity of virus, time of exposure and interaction between the treatments (Beared and Hanson. 1984).

The pH values of plays an important role for the survival of virus. Our calculated pH value of both No treated and treated were measured by using AD1030 pH/mV & Temperature Meter were ranged from 7.5-7.9 where the viral survivality is high which is supports (Stallknecht *et al*., 2009) where the optimal pH value for survival of virus ranging from 7.4-7.8. There were found that, all the samples containing the pH values higher than the neutral level (pH-7). That means there all the collected pond water samples were slightly alkaline in nature. As the pH value increased the survival time of virus is decreased. . The highest value was found in sample No. 2 (Chandanaish) (pH 7.98 and 7.86) and lowest value in sample No. 4 (Jahangirnagar varsity lake) (7.21 and 7.1). In water sample-4 (pH 7.1-7.2) virus survival time is more as compare to others water samples. It was due to optimum pH value for newcastle disease virus survival which followed (Irene *et al*., 2013). Lower pH causes a conformational change of viral haemaglutinine glycoprotein which support to (Doms *et al*., 1985). This is often due to a salt content (though not every salt lake has a high pH). These lakes have high concentrations of minerals, particularly dissolved salts: sodium, calcium, magnesium carbonates and bicarbonates (Boros *et al*., 2003).

Zhang *et al*., (2012) were studied four lake water samples in China and found that the pH of those water ranges from 6.93 to 8.89. They found both acidic and alkaline waters.

The variation of pH values in lake or pond water samples may depends upon the soil constituents of water reservoirs, utilization of water by local people, presence of crop lands and industries besides the water reservoirs. Chemical wastage and pesticides might have possibilities to mix up to the natural water via rain or drainage systems. (Olivia *et al.*, 1972)

Another finding is that, after filtration and boiling of water pH values are reduced in all the cases. Raw water pH value ranges from 7.21 to 7.98 and treated water samples pH values ranges from 7.10 to 7.86. This might be due to entrapment of little quantity of bi-carbonates by the syringe filter, evaporation and deactivation via boiling.

In present study, the bi-carbonate and hardness (Ca++ and Mg++) concentrations of collected water samples were measured by titration method and found that, concentration of chemical ingredients were reduced due to filtration and boiling of water samples. Entrapment and evaporation of alkaline bi-carbonates might be cause of reduction of pH values in treated water samples than those of raw samples (Table 6). Bi-carbonates concentration in case of non treated water it ranges from 18 to 26 PPM, where it ranges from 17 to 23 PPM in case of treated water samples.

The highest value of bi-carbonate concentration was found in sample no. 2 (Chandanaish) (26 and 25) and lowest value in sample no. 4 (Jahangirnagar varsity lake) (18 and 17). The virus survives more time in water sample-4.Increase bicarbonate may decrease survival of virus.

Salinity is an ecological factor of considerable importance, influencing the types of organisms that live in a body of water. Organisms (mostly bacteria) that can live in salty conditions are classified as extremophiles, or halophiles specifically. An organism that can withstand a wide range of salinities is euryhaline. Seawater pH is typically limited to a range between 7.5 and 8.4 (*Chester et al., 2012).*

On the other hand, it was not possible to observe the actual effect of hardness on the survival of virus due to the dominant effects of pH & bicarbonates. For studying the effects of hardness on viral persistence, further investigation is required by using the water samples from different types of water reservoirs like lake, river, sea, rain,

distilled water etc. It was established previously that, any water containing hardness (Ca++ and Mg++) concentration < 60 PPM, it is a soft water. In this study, only the pond water samples were used as viral survival media which contain hardness (Ca++ and Mg++) level < 50 PPM (Table 6).

The result will be more acceptable if permanent water hardness can be removed. Permanent hardness of water cannot be removed by boiling because the presence of calcium sulfate or magnesium sulfate in water does not precipitate out as the temperature increases. It can be permanently removed by using soda, other softening agents such as soap, caustic soda, solution of ammonia, borax etc.

Chapter-6: Conclusion

NDV can infect more than 210 species of birds where in web footed birds it causes less problem. But it can shed the virus up to considerable amount of time. Water acts as a vehicle for the transmission of virus from one place to another. It is shown in summer season where the virus cannot survive more than 36 hours. However at 22°C it can survive up to 72 hours.

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Appendix

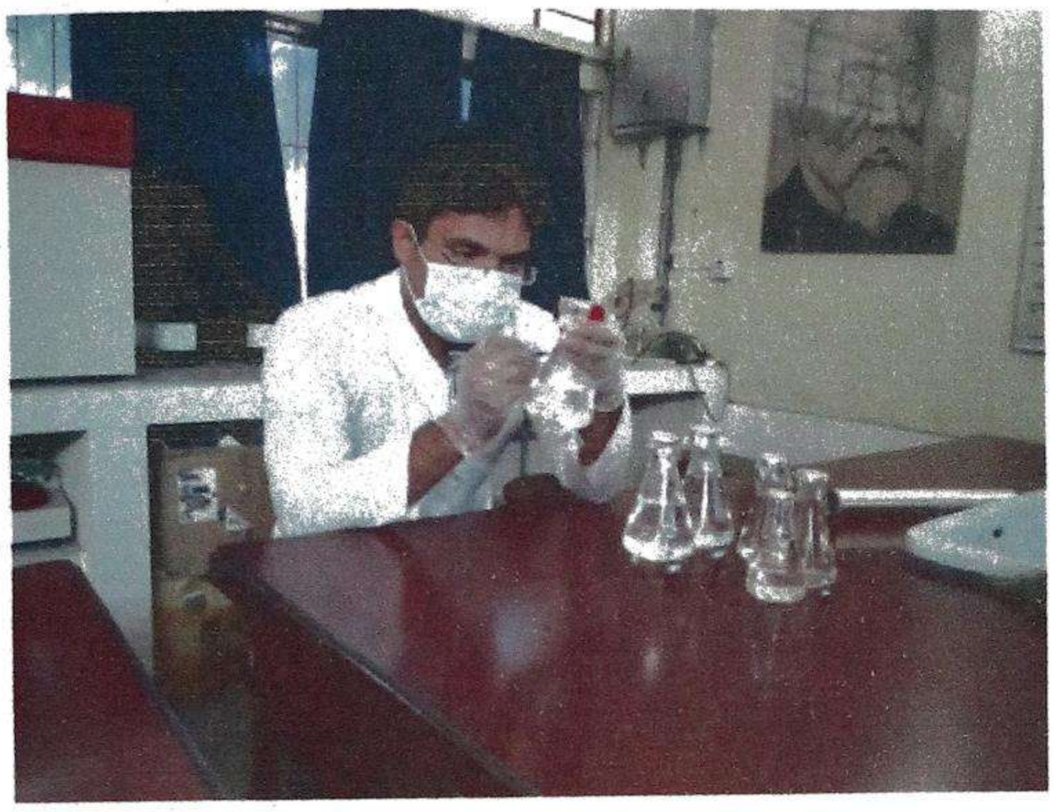

Figure 9. Marking of water samples

Figure 10. Boiling of water

Figure 11. Bicarbonate test

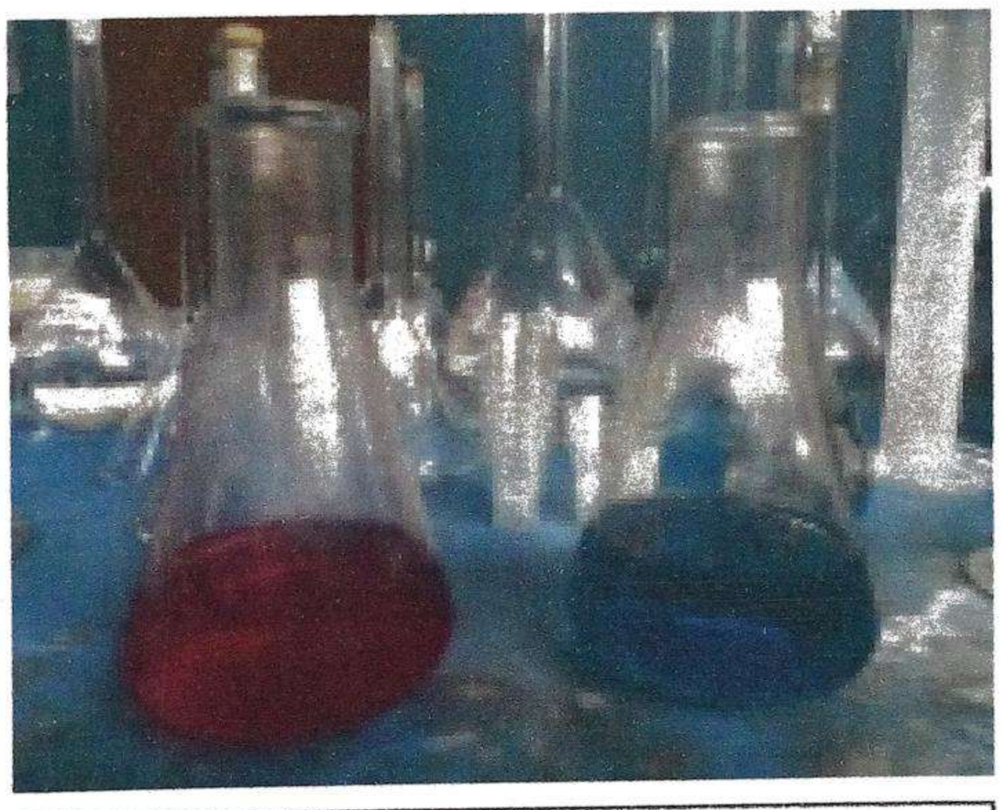

Figure 12. Water hardness test

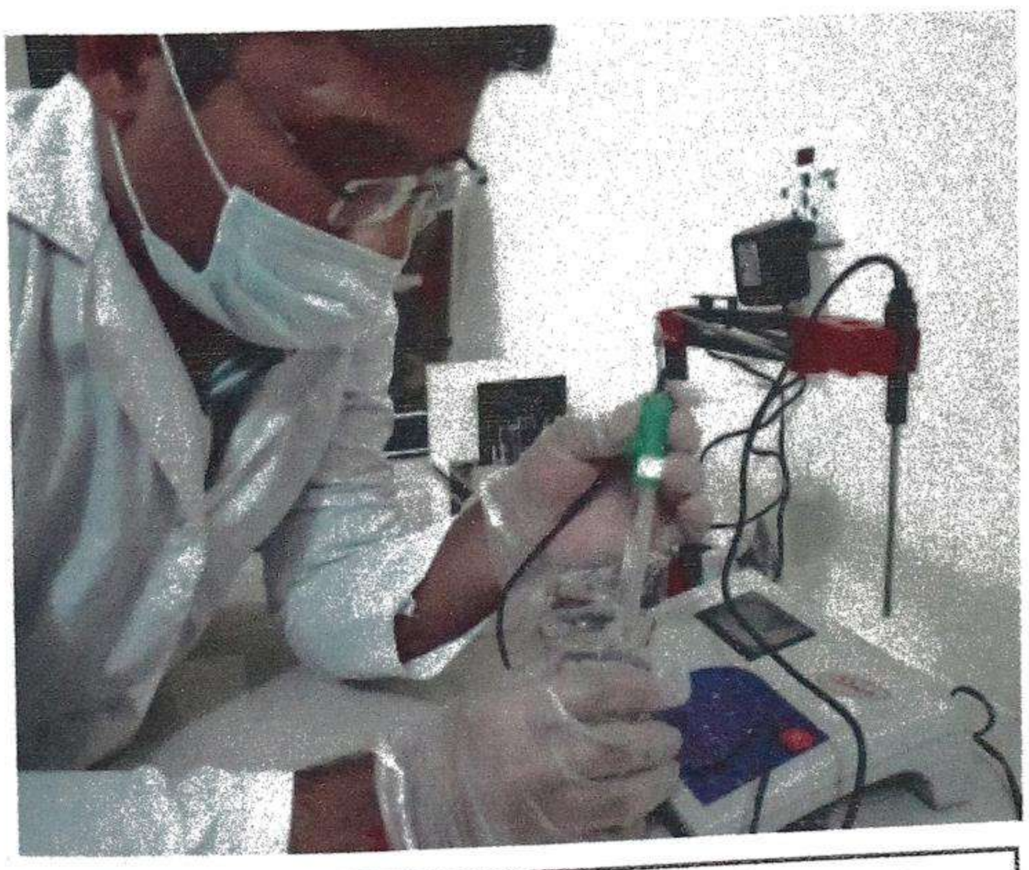

Figure 13. pH test of water samples

Figure 14. Preparation of 1% RBC

Figure 15. Inoculation of virus

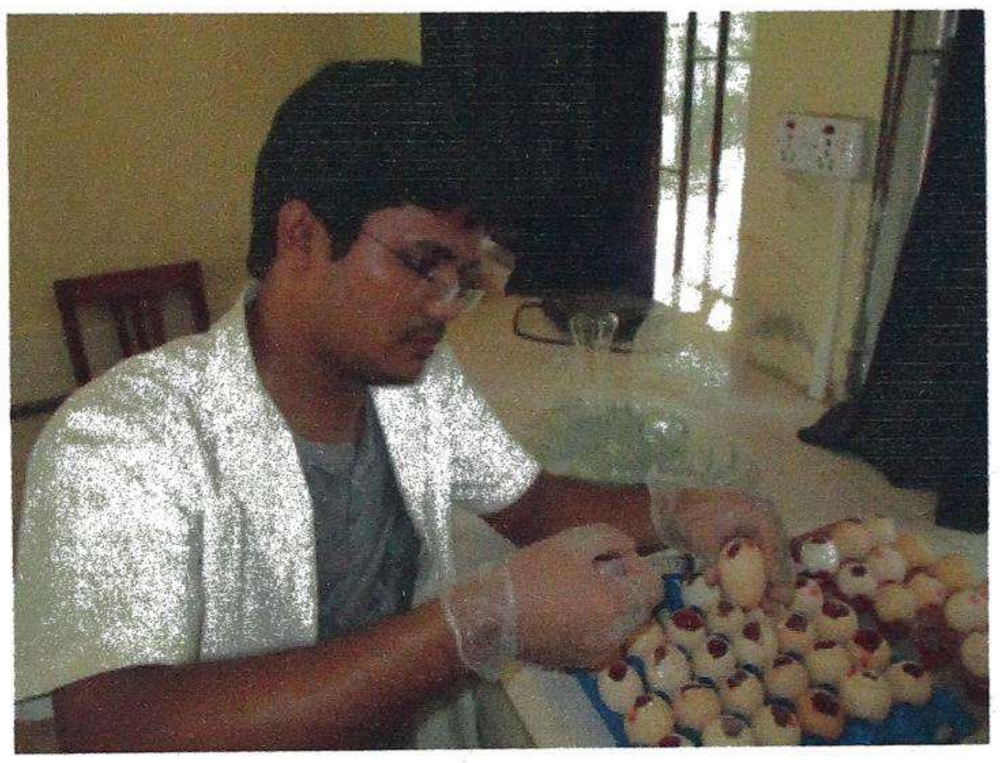

Figure 16. Collection of allantoic fluid

Figure 17. Fluid collected in eppendorf tube

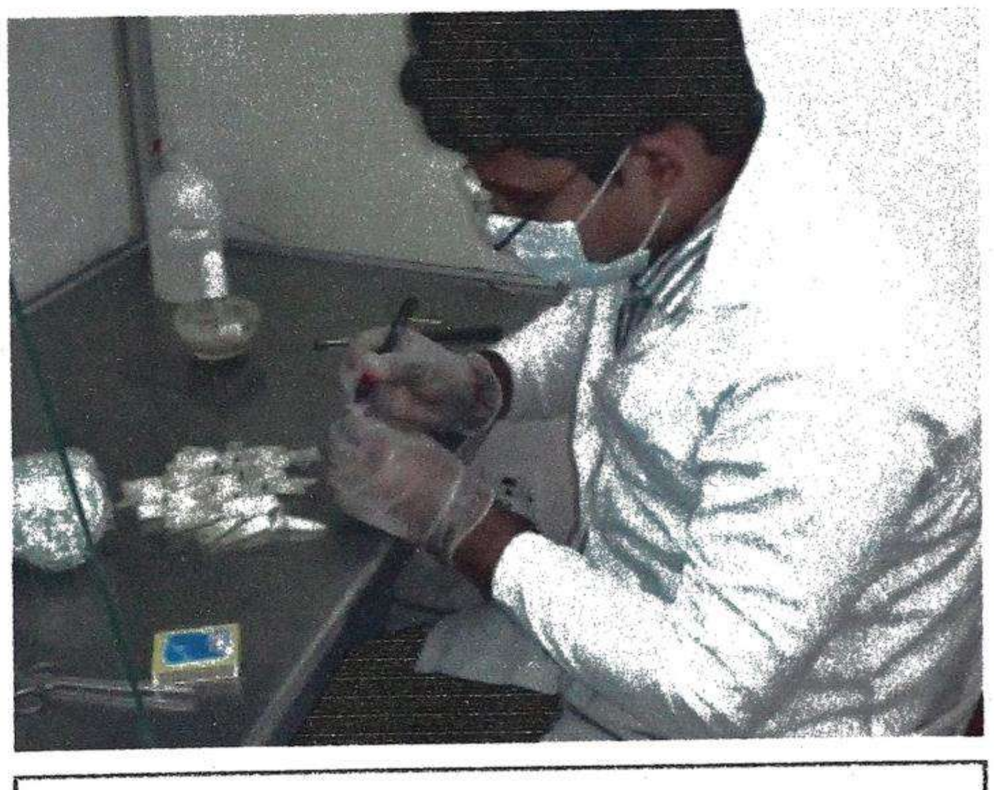

Figure 18. Marking of tubes

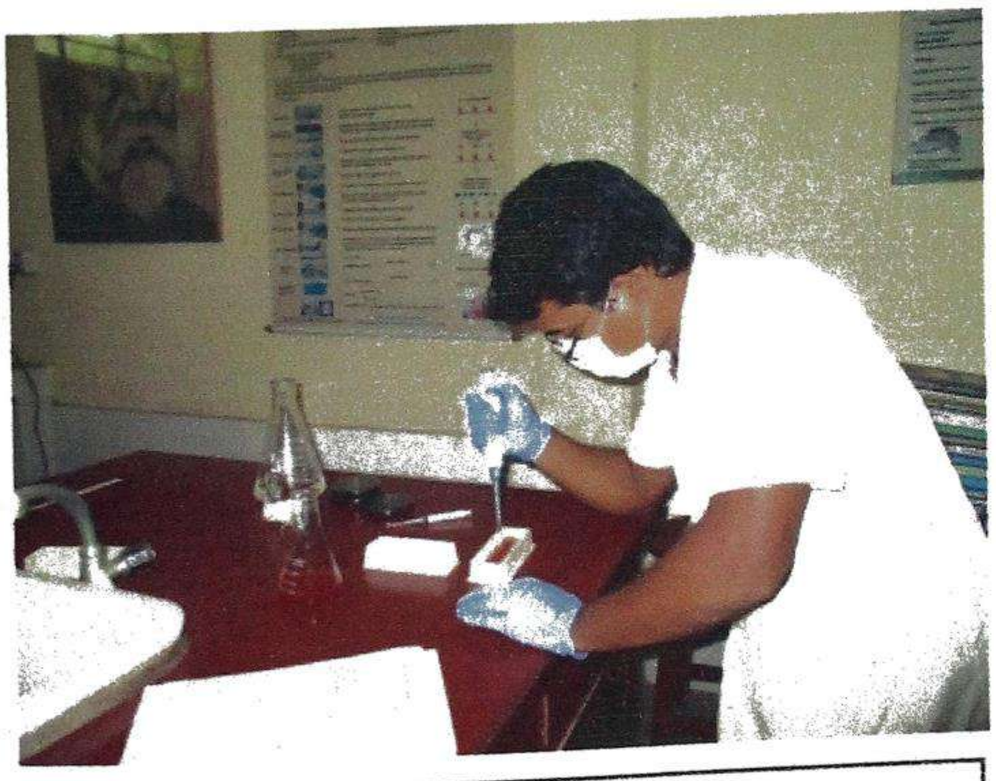

Figure 19. HA test

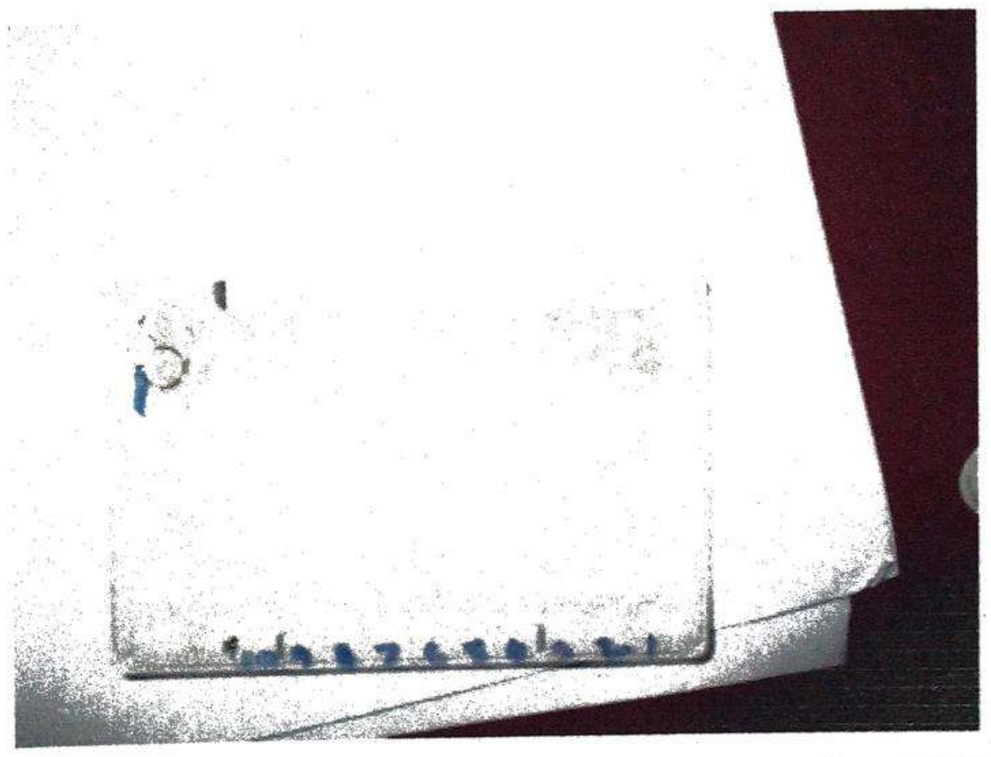

Figure 20. Result of HA test