ISOLATION, IDENTIFICATION, BIOCHEMICAL AND ANTIBIOTIC SUSCEPTIBILITY OF SALMONELLA FROM THE RIVER, TAP WATER AND SEWAGE SAMPLE OF DHARAN, ITAHARI AND BIRATNAGAR

A

Dissertation

Submitted to the **Department of Microbiology**,

Central Campus of Technology, Tribhuvan University, Dharan, Nepal,

in the partial fulfillment of the Requirements for the Award

of Degree of Masters of Science

(Public Health)

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CERTIFICATE OF APPROVAL

On the recommendation of **Mr. Hemanta Khanal,** this dissertation work by **Ms. Radha Pandit entitled ISOLATION, IDENTIFICATION, BIOCHEMICAL AND ANTIBIOTIC SUSCEPTIBILITY OF SALMONELLA FROM THE RIVER, TAP WATER AND SEWAGE SAMPLE OF DHARAN, ITAHARI AND BIRATNAGA has been approved for the examination and is submitted to** the Tribhuvan University in partial fulfillment of the requirements for M.Sc. Degree in Microbiology (**Public Health**).

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ABSTRACT

Salmonella is a genus of rod -shaped (bacillus) gram negative bacteria of Enterobacteriaceae. *Salmonella* is one of the leading causes of intestinal illness all over the world. as well as the etiological agent of more severe systemic diseases such as typhoid and paratyphoid fevers. *Salmonella*e are mainly known as water and food borne pathogens to humans, and animals. *Salmonella* is commonly reported in water-borne outbreaks despite it being frequently detected in surface waters including recreational waters and waters used for irrigation or as a drinking water source the main sample to isolate *salmonella* is water, which is the main component of environment.

In our research work we focused on collection, transportation, culture, gram staining, biochemical and antibiotic susceptibility

The water samples were from different places of Dharan, Itahari and Biratnagar. Water was collected aseptically in the pre-sterilized bottle, and transported to the laboratory, following standard methods with APHA, American Public Health Association, 1995. The collected water samples were analyzed on the same day immediately after its delivery and always within 6 hours of collection. *Salmonella* detection was done by enrichment of water sample in Selenite Cysteine broth then spread plate on *Salmonella Shigella* Agar (SSA) and identified by biochemical tests according to Bergey's Manual of Determinative Bacteriology and antibiotic susceptibility was done.

Total 100 samples were collected 76 (76%) of the samples shows the presence of *Salmonella*. Based on place, Dharan (81.30%) showed highest prevalence followed by Itahari (73.68%) and Biratnagar (63.63%). Similarly, the prevalence rate of sewage is 81.81% whereas the prevalence of river and tap water are 74.07% and 68.62% respectively. After isolation and identification *Salmonella* were tested against different antibiotics among them Ampicillin was found to be highly resistant whereas azithromycin was found to be highly sensitive.

Key words: *Salmonella*, Water, Antibiotics

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LIST OF ABBREVIATIONS

S.N.	Abbreviations	Full Form
1.	CDC	Center of Disease Control
2.	MR	Methyl Red
3.	VP	Voges Proskauer
4.	TSI	Triple Sugar Iron
5.	AZM	Azithromycin
6.	AK	Amikicin
7.	CTX	Cefoxitime
8.	NA	Nalidixick Acid
9.	AMP	Ampicillin
10.	C	Chloromphenicol
11.	CIP	Ciprofloxacin
12.	APHA	American Public Health Association
13.	BOD	Biological Oxygen Demand
14.	CLSI	Clinical Laboratory Standard Institute

CHAPTER-I

INTRODUCTION

1.1 Background:

Salmonella are Gram negative, small rod shaped, non-spore forming, non-capsulated, aerobic and facultative anaerobic organisms and classified under the family Enterobacteriaceae. Members of *Salmonella* species can cause mild to life threatening *salmonellosis* in human, so it is very necessary to isolate *Salmonella* from water sample (Song et al., 2018).

Salmonella is a leading cause of food borne illness worldwide, with an estimated million cases each year in the world. Salmonella infections are typically due to consumption of food products of animal origin. (Parbati et al 2017). Since *salmonellosis* is water borne diseases the quality of drinking water has an important role in human health. Water is life, it is one of the most precious and important resources to sustains life. The earth is made lively, green, happy and growing because of water. In fact, it is true that no water no life on this planet. Around 71% or more area of earth is covered with water. Water is the primary source of living on which all the creature is dependent. Water comes to us through different sources like oceans, rivers, lakes, ground water, rains etc. Water is important for our domestic, industrial and agricultural purposes. (Bonyadian et al. 2018). Here in my thesis mainly I took the samples of three major sources tap water, river and sewage

Tap water is the pure and safe to drink than river. Quality wise tap water has highest quality of assurance. In many remote areas of our country Tap water has become the main sources of drinking water. (John J et al 2017) Many rural residents get their water from private wells where treatment is the responsibility of the homeowner. (Credit: Victoria Christensen, USGS Upper Midwest Water Science Center. Public domain) Water chemistry can change, however, from the point of distribution at a drinking water facility to the taps in homes and businesses owing to leaks, cross-connections, onsite plumbing, and back-siphonage. Moreover, very few chemicals (apart from disinfection byproducts, disinfectant residuals, lead, and copper) are monitored at the tap in homes or businesses and novel pathogens continue to emerge.

Similarly, river water is another source of water, this is also one of the sources from where we took sample to check whether the different river source shows the presence of *salmonella* or not. These days river water has become dumping sites for all the people which is the main cause of pollution of river water. Water pollutant can be defined as a physical, chemical or biological factor causing aesthetic or detrimental effects on aquatic life and on those who consume the water. (Bhetawal et al 2017) Majority of water pollutants are in the form of chemicals which

remain dissolved or suspended in water and give an environmental response which is often objectionable. Sometime physical and biological factor also act as pollutant. Certain microorganism present in water especially pathogenic organism cause diseases to human beings they are called as bio pollutants. (Tajud et al 1998) A rapid interpretation of river water quality is a compulsory since river is a dynamic ecosystem. (Effend et al 2016).

Similarly, another sample taken in our research is sewage. Sewage is a mixture of water and whatever waste that contains some ions solid wastes and or harmful bacteria from domestic and industrial waste. Presence of all these organisms along with other solid waste makes sewage which is the source of another polluted water. The term sewage is used to indicate the liquid waste from the community, and it includes the following- sullage, discharge from latrines, urinals, industrial waste and storm water. Sewage is a complex mixture of chemicals, with many distinctive chemical characteristics. These include high concentrations of ammonium, nitrate, nitrogen, phosphorus, high conductivity, high alkalinity. (Adhikari et al 2008) The main composition of sewage is 99% water and 1% solid with offensive order with millions of pathogens. Since the sewage is also regarded as one of the health problems in the environment, there should be proper management system for sewage from personal level to government level. (Ngui et al 2011). Different types of sewages are:

- **Domestic sewage:** includes sludge and animal discharges.
- **Industrial wastewater** includes commercial and industrial wastes.
- **Surface run-off:** Includes suspended matter from lands and debris from streets (Punmia et at, 1998).

1.1.1 Prevention of Salmonella Transmission from sewage

The Centers for Disease Control and Prevention (CDC) and the Pan American Health Organization (PAHO) have developed an inexpensive, rapidly implementable alternative for water quality improvement (Gerardia et al 1999). This intervention consists of three elements: (1) point-of-use treatment of contaminated source water with disinfectant produced locally using appropriate technology; (2) safe storage of treated water; (3) community education. (Maier et al 2009) UV light disinfection showed inefficient removal of fecal bacteria compared to chlorination. (Reemtsma et al 2008).

Water available in the environment may be at any stages but, we can bring them in use after undergoing the water through different phases of purification. There are different phases of purification of water they are listed below

- Physical purification: Filtration, Sedimentation
- Biological Process: Slow sand filters, Activated sludge
- Chemical process: Flocculation, Chlorination
- Radiation: Ultraviolet light (Tripathi et al 2015)

Since water and sewage, the main targeted sample, and the main organism to be isolated is *salmonella* we discussed in above few paragraphs about organism and sources of samples.

In due course of research work there were some limitations, like we used only Salmonella shigella agar for the isolation of salmonella since there were other culture media also for the identification of Enterobacteriaceae family. We used limited antibiotics for antibiotic susceptibility test. Research study was completed up to antibiotic susceptibility test. And also due to covid crisis the study was not able to complete on time.

1.2 Statement of Problem

Water is one of the basic requirements for all the living things. Without water no life is possible. Among many cities of Nepal, Dharan is also one of the cities where there is scarcity of water. From the history, it is a well-known fact that all the great civilizations around the world evolved around rivers waters. Global freshwater scarcity due to the pollution of water demands for integrating water management and monitoring all over the

world (Ayandele, et al., 2014). Water is one of the main vectors for feco-oral route of transmission of many waters borne diseases and food borne diseases. Among many diseases some diseases like typhoid, diarrhea, enteric.

1.3 Rationale

Water is an essential natural resource in the world on which existence of life depends.

All living things need to consume water, an individual may be able to live up to 30days without food but would perish in a few short days without water. Rivers and wetlands are considered as one of the most diverse ecosystems of the world, providing irreplaceable ecological functions, unique role in maintaining the food chain and economic values.

They are important economic resources for any country as they provide water for agricultural, industrial and human activities. They have ecological, socio-economic and cultural values and many indigenous communities depend on rivers and wetlands resources for food and water. Water sources and rivers are also habitats for several species of wildlife, from aquatic animals to migratory birds, which lie within various ecosystems of the high mountains and lowland plains. Hence, they function as a corridor connecting marine, aquatic and terrestrial ecosystems.

They can provide us with all these services only if they are reasonably healthy. If these sources are polluted, then animals and humans may suffer from different waterborne infections. Water serves one of the most common routes of transmission of no. of infectious diseases like diarrhea, typhoid, dysentery and cholera. According to WHO, 2003, 80% of ill-heath are due to lack of safe drinking water so the physicochemical and bacteriological parameters should be analyzed, which plays vital role for the conservation and improving water quality of the rivers and wetlands and for the existence of flora, fauna and humans and for maintaining of the ecosystem.

1.4 Objective:

1.4.1 General objectives:

- To isolate, identify, biochemical and antibiotic susceptibility test of *Salmonella* from river, tap water and sewage of Dharan, Itahari and Biratnagar

1.4.2 Specific objectives:

- To isolate and identify the *Salmonella* from the collected samples
- To perform the biochemical tests of isolated organism.
- To perform the antibiotic susceptibility isolated organism.

CHAPTER-II

LITERATURE REVIEW

In this chapter we are going to discuss about the findings of organism from other research articles, and we are going to discuss about the organism and sample in details.

2.1 Review from Related Research Articles

Salmonella is a ubiquitous enteric pathogen with a worldwide distribution that comprises many serovars characterized by different host specificity and distribution. This microorganism is one of the leading causes of intestinal illness through the world as well as the etiological agent of more severe systemic diseases such as typhoid and paratyphoid fever (Pond, 2005). Zoonotic *salmonella* is commonly described as foodborne pathogens, however, drinking water as well as natural waters are known to be an important source for the transmission of these enteric microorganisms (Ashbolt et al 2004). Salmonella, just like other enteric bacteria, is spread by the fecal–oral route of contamination. This microorganism can enter the aquatic environment directly with feces of infected humans or animals or indirectly, e.g., via sewage discharge or agricultural land run off. Overall Salmonella spp. and subspecies can be found in a large variety of vertebrates. Beside humans, animal sources of Salmonella include pets, farm animals and wild animals; calves, poultry, pigs, sheep as well as wild birds (Ningho et al., 2018).

From the study of Gosa Garima shows that an estimated 70% of cases of diarrheal diseases are associated with the consumption of foods contaminated with pathogenic microorganisms. Among these microorganisms Salmonella and Shigella are the major ones. (Gosa et al 2015).

From the study of Ningho and all his co-worker they examine the occurrence of *Salmonella* in different rivers and distribution of their genotypes. The result obtained from this research that *Salmonella* detected in the upstream was 45.5% similarly from midstream and downstream 30.4% and 11.4% respectively which concludes that for the determination of water quality there is influences of occurrence of *Salmonella* in aquatic environment *(*Ninghoet al. 2018*).*

Similarly, from the study (Bhatta et al 2007) out of 300 water samples *Salmonella* was detected in 42 samples by enrichment culture in selenite F broth. This study conclude that microbiological quality of urban water supply is poor and indicates possibility of fatal outbreak of enteric fever and related infection in Nepal. (Bhatta et al., 2007)

Similarly, from the study, which was done in Indonesia Jarkata, I which a total of Beverages and 50 ice were collected MPN result ranged from <30 to >11,000 Mpn/ml. This result indicates that the presence of *Salmonella* enterica serovar becomes a concern in relation to the safety of drinking water and ice. (Waturangi et al., 2019) Besides river water there are research done in

drinking water also like in the topic “Drinking water from dug wells in rural Ghana – *Salmonella* contamination environmental factors and genotypes”. Here out of total sample 398, water samples 99.2% i.e., 395 were contaminated with *Salmonella*. This study provides an overview of the level of contamination in wells with gram negative rods bacteria like *Salmonella*. (Enver et al 2015).

From the study which was done in India on the topic “Genomic characterization of *Salmonella* bacteriophage isolates” 2016, this study explains that *Salmonella* is medically important foodborne pathogen including water borne pathogen. This study also explains that bacteriophage are most numerous and ancient biological entities. (Hyatri et al 2016). This was the is’t report of *Salmonella* bacteriophage fill genomic sequence from India. Including India, the research about *Salmonella* species were also done in China in flooded man-made rivers. This study explains that out of total 95 sample 67 were completely serotyped representing 13 different serotypes in *Salmonella* serogroups. This result conclude that heavy flood greatly increased in *Salmonella* contamination in manmade rivers and there after putting the public health in great risk (Song et al., 2018).

Out of total 60 samples 32 (53.33%) were found positive for Salmonella. Highest number of *Salmonella* was isolated from egg surface washing (90%), followed by cloacal swab (80%), intestinal content (60%), hand wash of poultry handler (50%) and in fecal materials (40%) but no isolate was found on environment (air) samples (Rhman et al 2017) From this study says that Salmonella causes only water borne diseases it is not responsible for any kind of air borne diseases.

Here most of the research related to *Salmonella* are isolated from water sample since our targeted sample is different sources of water. Besides water there are also some research which are done in edible betel leaf sample to isolate *Salmonella*. This study proves that not only water is main cause of salmonellosis, but public health risk is also associated with consumption of food on nonanimal origin on betel leaves. These leaves were most commonly *Salmonella* contaminated. (Mclauchlin et al 2018)

Salmonella is an important zoonotic pathogen and its prevalence in the animals acts as a continuous threat to man. The present study was carried out to report the isolation along with the serotypes, phage types and antibiogram pattern of Salmonella among man, livestock and poultry in the northeastern India. (Schwartz et al 2002), (Rahman et al 2004) Findings of H Murgukar from animals and man shows that that Ninety-five isolates of Salmonella enterica belonging to 5 serotypes- S. Typhimurium, S. Enteritidis, S. Gallinarum, S. Paratyphi B and S. Bareilly were obtained with an overall prevalence rate of 14.40 per cent. S. Typhimurium

isolates were distributed among four phages DT003, DT004, DT096 and DT193 and all the S. Enteritidis isolates belonged to a single phage type, PT13a/7. Interspecies sharing of the phages was observed. (Murgukar et al 2018)

Similarly, regarding their antibiotic sensitivity test also shows that Norfloxacin, enrofloxacin, gentamycin and ciprofloxacin were most effective, whereas, doxycycline, ampicillin, amoxycillin and tetracycline were relatively less effective.

Their findings showed that three of the five serovars as well as some of the phage types of these serovars were shared by animals and humans indicating the zoonotic potential of the organism. Thus, it is imperative that salmonellosis control measures adopted for humans should give adequate importance to its control in the animals particularly. (Murgukar et al 2018).

From above we came to know that many researches have been done related to *Salmonella* from different countries of world. Including all these countries Nepal is also involved in research study of *Salmonella*. Though this research was done in 2007 in which *Salmonella* were isolated from urban drinking water supply system of Nepal here, total 300 samples isolated in which only 14% were positive *Salmonella* were isolated by conventional biochemical tests. This was the first report in Nepal on predominant phage type of drinking water isolates of *Salmonella* from urban drinking water supply system. (Bhatta et al2007) In the year 2016 from the municipal Kathmandu drinking water *Salmonella* were isolated in which 432 samples were collected in which 50% of sample were positive. From which it was concluded that Kathmandu drinking water exhibits longitudinal fecal contamination excess of WHO guideline (Karkey et al.2016)

A total of 102 isolates belonging to genus *Vibrio*, *Salmonella* and *E. coli* were subjected to antibiotic sensitivity test by disc diffusion method by using nine different types of antibiotic discs. *Salmonella* spp. from salad and water showed resistance against Amoxicillin (75%), Cephradine and Cephalexin (68.75%). 85.71% *Vibrio* spp. isolated from salad and water were resistant to Amoxicillin respectively. Multiple drug resistance was seen in 39 and 51 isolates of *Salmonella* and *Vibrio* isolates, respectively. (T Niwas et al2012) The results suggest the necessity to follow the hygienic practices in salad preparation and salad might have an important role as a source of multiple antibiotic resistant bacteria. (Olovo et al 2019)

An antibiotic sensitivity (or susceptibility) test is done to help choose the antibiotic that will be most effective against the specific types of bacteria or fungus infecting an individual person.

Some types of bacteria or fungus are resistant to certain antibiotics because of differences in their genetic material (genes). Infections caused by resistant bacteria or fungi are not cured by treatment with those antibiotics. Since antibiotic sensitivity test is also one of my parts of

research here are some of the findings related to the Antibiogram of *Salmonella* From the research study of K. Minara on the topic "Isolation and Identification and Antibiogram of Salmonella species from Poultry Farm Environment "they find that Norfloxacin, enrofloxacin, gentamycin and ciprofloxacin were most effective, whereas, doxycycline, ampicillin, amoxycillin and tetracycline were relatively less effective. (Minar et al 2017)

A total of 298 *Salmonella* isolates showed an overall per cent positivity of 5.58. Multidrug resistance was found in 11.96 per cent and 15.62 per cent isolates of S. Typhi and S. Paratyphi A respectively. Less than 2 per cent isolates of *Salmonella* showed resistance to ciprofloxacin. A resistance of 3.0 to 6.25 per cent against third generation cephalosporins was observed among the salmonella isolates. (S Bhattacharya et al 2011).

(Raman et al 2017) From this study it shows that 100% of the tested Salmonella isolates from poultry sources were found to be resistant to Erythromycin, Tetracycline and Nalidixic Acid and 80% strains showed resistance to Ciprofloxacin whereas, Ampicillin, Amoxicillin, Sulphamethoxazole and Kanamycin showed 60% resistance. No isolate showed resistance to Gentamicin. Sensitivity was recorded in case of 40% strains to Chloramphenicol, Ampicillin, Amoxicillin, Sulphamethoxazole, Kanamycin and 20% isolate against Ciprofloxacin. Both, resistance and susceptibility were exhibited against Chloramphenicol, Ampicillin, Amoxicillin, Sulphamethoxazole, Kanamycin, and Ciprofloxacin. Which concludes that Attention is to be paid for personnel hygiene in processing and handling of poultry and poultry products and excess use or abuse of antibiotics should be reduced or stopped by the judicious application of antibiotics for the safety of public health in Bangladesh. (S Bhattacharya et al 2011) Their findings showed that three of the five serovars as well as some of the phage types of these serovars were shared by animals and humans indicating the zoonotic potential of the organism. A total of 298 *Salmonella* isolates showed an overall per cent positivity of 5.58. Multidrug resistance was found in 11.96 per cent and 15.62 per cent isolates of S. Typhi and S. Para typhi A respectively. Less than 2 per cent isolates of *Salmonella* showed resistance to ciprofloxacin. A resistance of 3.0 to 6.25 per cent against third generation cephalosporins was observed among the salmonella isolates. (Bhattacharya et al 2011).

In most studies of Antibiograms tests, Salmonella and Shigella spp. showed high resistance to the commonly used antibiotics which indicate serious problems in antimicrobial therapy globally, especially in developing countries. In challenge studies, Salmonella and Shigella spp. reached the infective dose within 4 to 24 hours of inoculation, respectively in various food samples. In this review, it is noted that these potentially pathogens are still public health problems. Therefore, there needs health education, frequent monitory and evaluation system of microbiological and antimicrobial surveillance to plan intervention strategies for at risk

population in the area of water sanitation and hygienic food handling practice to minimize the burden posed by the diseases Salmonellosis and Shigellosis. (Gosa et al 2015).

enterica serovar *typhimurium* (*S. typhimurium*) can cause diseases ranging from gastroenteritis to life-threatening systemic infection. (M lencho et al 2017). (Yusuke et al 2012). *Salmonella* is estimated to cause 93.8 million human gastroenteritis infections and 155, 000 deaths each year worldwide (Ashok et al 2016) Although most of these infections cause mild gastroenteritis, life-threatening dispersed infections are common among children, the elderly, and immunocompromised patients' Various animals, including poultry, pigs, cattle, and reptiles, are reservoirs of *Salmonella* species. Most of human *Salmonella* infections are developed after the ingestion of undercooked food of animal origin or contaminated water and vegetables (Gezahegn et al 2021).

2.2 Water

Water is the most vital resource for the existence of all life and ecosystems in Earth. Certain standards in terms of its physical, chemical and biological parameters determine its suitability for intended purposes. Water is considered polluted when these parameters shift from the acceptable range of quality standards (KC et al 2013).

The main sample for the isolation of *salmonella* is water. Water is life without water no life and living are possible. Since water is major integrated part of life it is compulsory to be the water safe and clean. But these days pollution of water has become a main factor to degrade the quality of water. Water pollution is any chemical, biological or physical change in water quality that has a harmful effect on living organism or makes water unsuitable for desired uses. It has been suggested that it is the leading worldwide cause of deaths and diseases and that accounts for the death of more than 14,000 people daily. Water is typically referred to as polluted when it is impaired by anthropogenic contaminants. (Gowtham2012). Natural phenomena such as volcanoes, alga blooms, storms and earthquake also cause major changes in water equality. There are many and different causes of water pollution like some of them are:

- Eutrophication
- Hypoxia
- Marine pollution
- Ocean acidification
- Thermal pollution
- Urbanization
- Water Stagnation

Beside these the major sources of water pollution are agricultural activities, industrial activities, and housing developments When water get polluted, the use of this polluted water may lead to different types of infections like.
Protozoan Infection (presence of Protozoas like Giardia, Ascaris, hookworm) Bacterial infection (presence of bacteria like Salmonella, shigella, clostridium botulism)

Viral infection (Presence of Adeno virus, polio virus, hepatitis virus etc.) (Shubam.et al2015)

2.3 Sewage

Presence of all these organisms along with other solid waste makes sewage which is the source of another polluted water. The term sewage is used to indicate the liquid waste from the community, and it includes the following- sullage, discharge from latrines, urinals, industrial waste and storm water. Sewage is a complex mixture of chemicals, with many distinctive

chemical characteristics. These include high concentrations of ammonium, nitrate, nitrogen, phosphorus, high conductivity, high alkalinity with pH typically ranging between 7 and 8. Sewage often contains pathogens like bacteria, viruses, parasites, and protozoans. Sewage water is classified into following types namely:

- **Domestic sewage:** includes sludge and animal discharges.
- **Industrial wastewater** includes commercial and industrial wastes.
- **Surface run-off:** includes suspended matter from lands and debris from streets (Punmia et al, 1998).
- Sewage water contains the huge diversity of microorganisms: viruses, bacteria, protozoa parasites and helminths eggs. The presence and concentration of pathogens are mainly determined by two factors, namely, the prevalence of pathogens among the population connected to sewage network and ability of these organisms to survive the sewage and sewage treatment processes. Sewage may become contaminated by approximately 300 species of bacteria, and the number in 1 g of a dry weight reaches them from 109 into 1012 cells. The survival time of bacteria in the environment is a few months to several months (Gerardi & Zimmerman, 2004). Several pathogenic microorganisms and parasites are commonly found in domestic wastewater, sewage sludge as well as in effluents from wastewater treatment plants. Sewage water bacteria belong to the following groups:
- **Gram-negative facultative anaerobic bacteria:** e.g., *Aeromonas, Plesiomonas, Vibrio, Enterobacter, Escherichia, Klebsiella, Salmonella* and *Shigella*,
- **Gram-negative aerobic bacteria:** e.g., *Pseudomonas, Alcaligenes, Flavobacterium, Acinetobacter*,
- **Gram-positive spore-forming bacteria:** e.g., *Bacillus* spp.,
- **Nonspore-forming Gram-positive bacteria:** e.g., *Arthrobacter, Corynebacterium, Rhodococcus* (Maier et al, 2009).

Finally, we can conclude the literature review in transmission of *salmonellosis* to human there are two factors making the *Salmonella* contaminated river water one is greater diversity of *Salmonella* and the next is increased number of humans. (Songe et al 2018).

CHAPTER-III

MATERIALS AND METHODS

3.1 Materials

A complete list of materials, types of equipment, chemicals, reagent, antibiotics and media used for the study are listed in Appendix 1.

3.2 Methods

3.2.1 Study Design and study area

The research was laboratory based cross sectional study conducted in the microbiology laboratory of the central campus of technology in Hattisar, Dharan, using water and sewage samples from Dharan, Biratnagar and Itahari.

3.2.2 Sample size and types

During the study, 100 samples were analyzed. And the sample type was river, tap water and sewage.

Sample size (N) was calculated using the formula

$N = 4pq/e^2$

Where,

N = Sample size
P = Prevalence of study from research study
q = 1-p
e =0.05

3.2.3 Sample collection and transportation

Water sample from different places of Dharan, Biratnagar and and Itahari were collected following the aseptic manner. For the collection of samples, 250ml Biological Oxygen Demand bottles were used. For self-protection, gloves and a face mask were worn during the sample collection. The samples were collected using sterile Biological Oxygen Demand bottles that had been dipped in water source, but in case of tap water the water was directly collected from tap.

The samples were subsequently transported aseptically from the collection site to the laboratory. The samples were preserved in Biological Oxygen Demand bottles that were kept in an icebox of sigma company from Germany to maintain optimal microbiological conditions.

3.2.4 Enrichment of organism

After transportation the sample was mixed with F selenite broth (in 1:5 ratio) for enrichment in test tube and they were allowed to enrich for 6 hours in refrigerator.

3.2.5 Isolation of *Salmonella*

In laboratory the collected water sample was enriched in Broth for 6 hours. Then Salmonella was isolated by inoculating 0.1ML of broth solution sample on Salmonella shigella agar medium followed by spread plate technique using sterile dolly rod. The media plates were incubated at 37°C for 24 hours. After incubation, the culture was studied by its colony characteristics, observation of black head colony on the plate was supposed to represent *salmonella* further identification and conformation gram staining and biochemical were performed before that, it was sub-cultured on Nutrient Agar (Hi-Media, India) for further (Chee's Brough 2006).

3.2.6 Storage of isolated organisms

Nutrient broth medium was freshly prepared, and 10% glycerol was added on it to preserve isolated organisms for a longer duration and future use.

3.2.7 Identification of organism

The organisms were identified by following gram staining biochemical tests. Biochemical tests such as MR VP, Citrate and TSI, test, were performed.

3.2.7.1 Gram Staining Technique

Gram staining involves the staining fixing the color with mordant, decolorizing the cells and appling the counterstain. Steps of gram staining are

- As staining crystal violet was used which binds to peptidoglycan and colors the cells in purple.
- Gram's iodine was applied as mordant which fixes the stain.
- Then it was washed with 95% alcohol or acetone.
- Lastly counterstain with Safranin, they take the stain and appear red in color.
- Observed under the microscope in 10x,50x and using immersion oil in 100x.

3.2.7.2 Biochemical Tests

- **MR-VP TEST:** MR -VP broth is allowed to come in room temperature, light inoculum from the isolated colony and resuspended in 5 ml MR-VP broth tube, and are incubated for 48 hours in incubator at 37^0c.

- **Citrate Test:** This test is also called Simmons's citrate test. In this test organism give positive test which are capable of fermenting citrate in the presence of citratase enzyme.
- **Triple Sugar Iron (TSI) TEST:** This test checks the ability of organism to ferment the sugar and their ability to produce the H_2S gas.

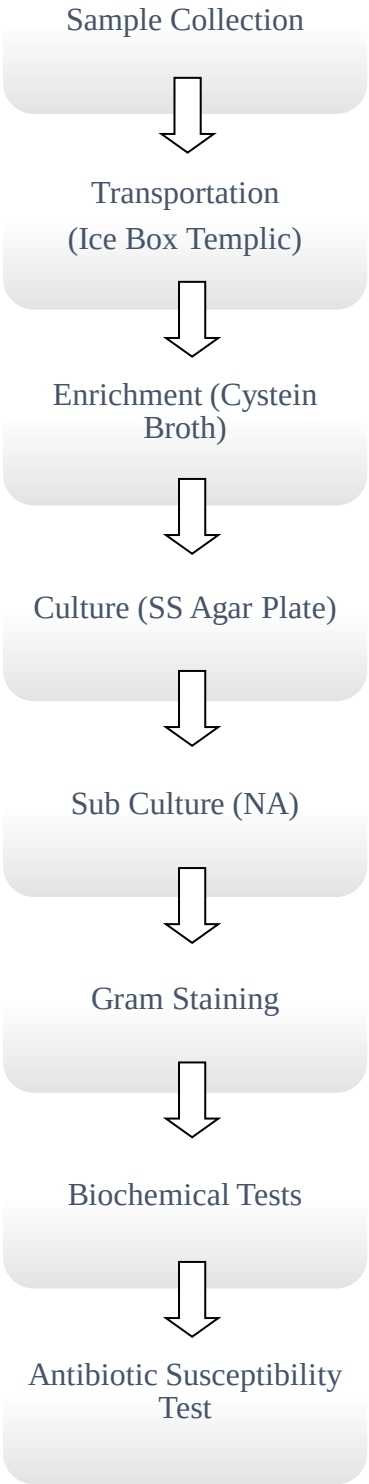
3.2.8 Antibiotic Susceptibility Test

Antibiotic susceptibility test was done by Kirby Bauer disc diffusion method. For this procedure, Mueller-Hinton agar was prepared on which the bacterial sample was inoculated. Paper antibiotics discs were then placed on the inoculated agar surface. Plates were then incubated at 37^0C for 24 hours and observations were made.

In this test 7 different antibiotics were used, and their zone of inhibition was measured following CLSI (Clinical and laboratory Standard Institute).

This test helps health care practitioner to determine which drugs are likely to be most effective in treating the person's infection.

3.3 Research Work Method in Flow Chart



CHAPTER-IV

RESULTS

Out of total water sample collected from River water, Sewage and Tap water. From all above sources 100 samples were collected. Out of 100 samples in 76 samples *salmonella* were present whereas 24samples there was absence of *salmonella.*

4.1 Present and absent percentage of *salmonella*:

Table No. 1: Showing frequency and percentage of Salmonella

Salmonella	Frequency	Percent
Absence	24	24.0
Presence	76	76.0
Total	100	100.0

The above table shows that 100 sample were collected. Out of 100 sample 76 of the sample shows positive that means *salmonella* were present in 76 samples and 24 samples shows negative that their samples show absence of *salmonella*. Calculating the frequency and frequency percent the total frequency of absent of *salmonella* are 24 and 24% and frequency percent and frequency.

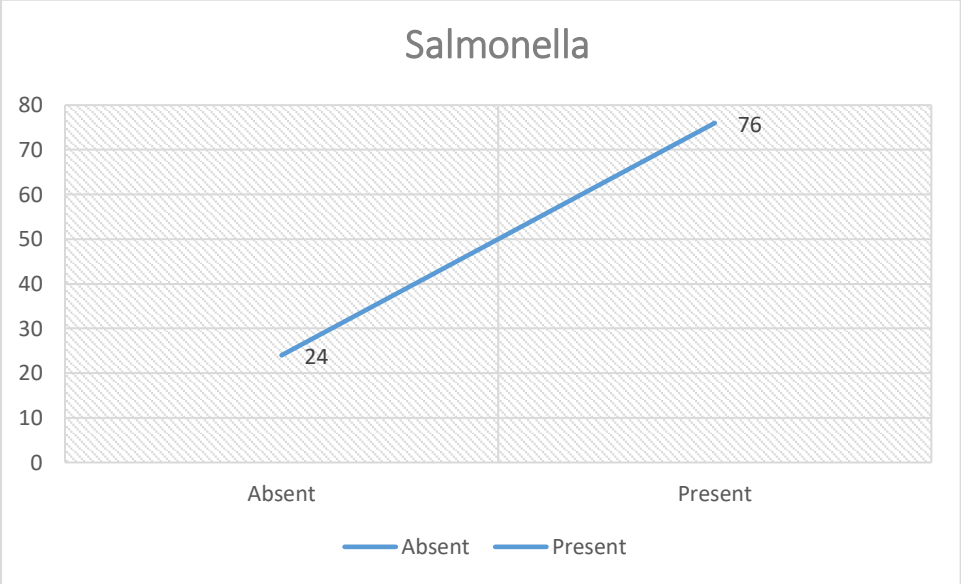


Fig 1: showing absence and presence of Salmonella

Out of total 100 sample collected from February 2017 march13 to February 2020. In 76 samples *salmonella* were present and in 24 samples *salmonella* were absent.al the retrospective data obtained from the water sample were used for analysis. The baseline characteristics of our study population is shown in Table 1.

The 76 samples in which *salmonella* were present, all their lab work performance was done. All their lab work activities will be explained in following pages.

4.2 Presence and absence of *salmonella* based on place:

Table No. 2: Showing absent and present according to place

	Dharan	Itahari	Biratnagar
present	48	14	14
Absent	11	5	8
Total	59	22	19

The above table shows present, and absence of *salmonella* based on sources. Out of total sample the highest sample (59) was collected from Dharan in which 48 samples shows presence of *salmonella* and 11 samples shows absence of *salmonella*. Similarly, the second highest sample was collected from Biratnagar (22) out of 22,14 samples shows presence of *salmonella* and 5 of them shows absence of *salmonella*. And finally, least sample was collected from (19) Biratnagar, in which 14 samples were present *salmonella* and 8 samples shows absence of *salmonella*.

4.3 Prevalence of *salmonella* on basis of place:

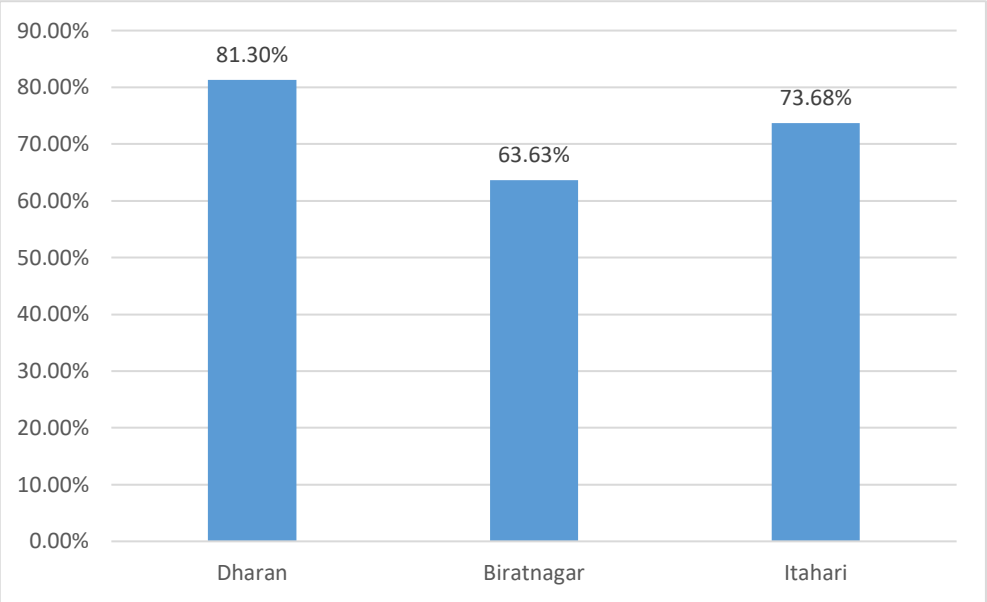


Fig No. 2: Prevalence of salmonella based on places

From above figure and table, we can see that out of total 100 water sample in 76 of them *salmonella* was present. Based on place, we can see that 59 samples were collected from Dharan, whereas out of 59 48 were positive and 11 samples no *salmonella* were found. Similarly, 22 samples were collected from Biratnagar, where as in Biratnagar *salmonella* were present in 14 samples and in 8 samples no *salmonella* were found. And finally, 19 samples were collected from Itahari in which 14 samples were positive and 5 samples were negative. Since the total samples collected were 100, calculating their frequency percentage shows that 22% were positive from Dharan,19% positive from Biratnagar and 22 % were positive from Itahari.

The bar graph represents the prevalence of *salmonella* based on place. Calculating the prevalence rate of each place the Dharan represents 81.3% (48/58 ×100) which is obtained like total present sample of Dharan divided by total collected sample of Dharan multiplied by 100.Similarly following same process the prevalence rate of Itahari and Biratnagar are 63.63% and 73.68% respectively.

4.4 Presence absence of *salmonella* based on sources:

Table No. 3: Showing absent and present *salmonella* based on Sources

	River	Sewage	Tap water
Absent	7	4	16
Present	20	18	35
Total	27	22	51

The above table shows that out of total sample (100) collected from provenience no 1. The total 100 sample were collected from three different sources they are River, Sewage and tap water. From river total 27 samples were collected in which 20 samples shows the presence of *salmonella* and 7 of them shows absence of *salmonella*. Similarly, from sewage also 22 samples were collected in which 18 samples were positive and 4 of them were negative. Finally, the maximum samples were collected from tap water that is 51. Out of 51, 35 samples show positive in which *salmonella* were present whereas 16 samples show absence of *salmonella*.

4.5 Prevalence of *salmonella* based on Sources:

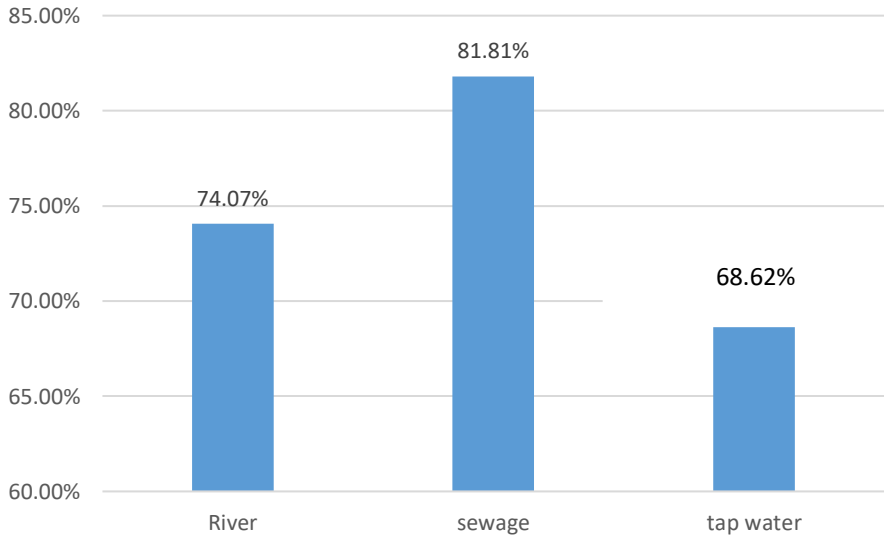


Fig No. 3: Prevalence on basis of sources

As we collected the 100 samples, the different samples were collected from River, Sewage and Tap water. The above table no 3 represents that out of total 100 samples 27 of them were collected River in which 20 of them were positive and 7 of them were negative, similarly 22 samples were sewage in sewage also 18 were positive and 4 of them were negative. Finally, the

last sources are Tap water in tap water also 35 samples show the presence of *salmonella* whereas 16 samples show absence of *salmonella*.

Similarly, the graph represents the prevalence of *salmonella* is high in sewage i.e., 81.81%, the prevalence of *salmonella* in river is 74,07% and finally the prevalence *of salmonella* in tap water is 68.62%.

4.6 Laboratories activities done for the identification of *salmonella*:

Table No. 4: Showing cultural characteristic, gram staining and biochemical tests.

Culture	Gram Staining	Biochemicals			
Black head colony were present in salmonella shigella agar	Gram-negative rod-shaped bacteria were observed	MR	VP	Citrate	TSI
		+	-	+	+

The above table represents that after the collection of samples the samples were run under the different laboratory work so that we can confirm that weather the collected sample consist of *salmonella*. At first after enrichment the culture was done in salmonella shigella agar, whole 100 samples were cultured .out of 100 olly76 samples shows black head colonies. Then gram staining and biochemical tests of only 76 samples were done for the conformation of *salmonella* in the sample.

Gram staining was done, which shows the gram-negative rods under the 100x of microscope and confirms that Salmonella is there in the sample. After gram staining different biochemical tests are carried out like MR, VP, TSI and Citrate among these tests all tests show positive is *salmonella* is present in *salmonella,* but VP is negative if *salmonella* is present.

Out of total 100 samples all the laboratory works shows that in 24 samples there is absence of *salmonella* whereas the 76 of the samples shows confirms that there is presence of *salmonella*.

4.7 Different antibiotic test of *salmonella*:

Table No. 5: Shows mean, maximum and minimum values of antibiotics

Antibiotics	Mean (mm)	Minimum-Maximum (m)
AZM	15.89	7-22
A/C	14.99	8-33
CIX	13.82	8-28
NA	14.89	0-20
AMP	0.00	0
C	13.37	0-21
CIP	14.09	8-22

After the isolation and identification of *salmonella* from the sample they were tested against different antibiotics like Azithromycin, Amikacin, Cefotaxime, Nalidixic, Ampicillin, Chloramphenicol, Ciprofloxacin.

The above table shows the mean value and maximum minimum range value of antibiotics that we observed during the antibiotic susceptibility test process. From this we can see that highly sensitive antibiotics is azithromycin whose mean value is 15.89 then nalidixic acid and amikacin. Least sensitive antibiotics are ciprofloxacin, cefotaxime and chloramphenicol .and resistance antibiotics is ampicillin. Whose mean value is 0.

4.8 Antibiotic sensitivity pattern of the isolate from different sample:

Table No. 6: Shows mean Resistance, Intermediate, Sensitivity of antibiotics.

SN.	Antibiotics	Resistance	Intermediate	Sensitive	P-value
1	AZM	29(38.15) %	-	47(61.84%)	0.040
2	NA	39(51.31)	24(31.57%)	13(17.10)	0.027
3	AK	34(44.7%)	16(21.05%)	26(34.21%)	0.032
4	AMP	76(100%)	0	0	0.057
5	C	37(48.68%)	28(36.84%)	11(14.47%)	0.052
6	CTX	69(90.78%)	44(5.26%)	3(3.9%)	0.036
7	CIP	48(63.15%)	21(27.63%)	7(9.21%)	0.032

Above table represents total number of resistances, intermediate and sensitivity of total 76 samples along with their percentage.

Following CLSI guidelines the zone of inhibition was measured ;7 antibiotics drugs were used. Each drug shows their own resistance, intermediate and sensitive value following CLSI guidelines.

From above table we can see that out of 76 samples which were tested azithromycin shows that 29 (38.15%) were resistance whereas 47(61.84%) samples were sensitive. Among 7 antibiotics used azithromycin is the drugs which shows maximum number 47(61.84%) of sensitivity. Whereas ampicillin is the drugs which shows highest number of resistances i.e., it shows 100% resistance.

At the last column there is a p -value which represents weather the used drugs are significant or not. P -value is defined as a statistical measurement used to validate a hypothesis against observed data. Comparing the P – value with 0.05 we can say that which drugs is statically significant or not. From above we can say that azithromycin, nalidixic acid, amikacin, cefotaxime and ciprofloxacin have the p-value which is less than 0.05 so we can say that these drugs are significant. Similarly, the ampicillin and chloramphenicol have p-value which is greater than 0.05 which shows that they are not significant.

CHAPTER-V

DISCUSSION

The study was conducted during March 2020 to 2021. Since the actual duration of time frame for our research study is 6 months, but due to covid outbreak the duration of time was extended. The targeted sample of my thesis work is water and the main organism to be isolated is *Salmonella.* Total 100 samples were collected from different places of Dharan, Itahari and Biratnagar. The main samples were river, tap and sewage. In 100 sample 76 of the sample shows the presence of *salmonella* whereas 24 of the sample shows absence of *salmonella*.

From this study we can see that the water sample of these area have poor sanitation. Similarly, from the study (John et al 2005) shows that out of total 132 drinking water samples collected from different places of Kathmandu, Bhaktapur and Lalitpur the study results clearly indicates that most of natural sources of water are highly contaminated. Relating our study research to this study we also can relate that the water sources of Dharan, Itahari and Biratnagar are highly contaminated as like that of Kathmandu area.

When we see the prevalence rate of *salmonella* based on place. Out of total 100 samples Dharan (48) prevalence rate is 81.30% likewise Itahari (14) whose prevalence rate is 73.68% and finally the Biratnagar (14) has least prevalence rate 68.62%. From this study we can see that the maximum data were collected from Dharan and the prevalence rate of Dharan is also high, and the equal data were collected from Itahari and Biratnagar, but the prevalence rate of *salmonella* shows high of Itahari than Biratnagar. It means the sources of water are highly contaminated following poor sanitation in Dharan and Itahari than that of Biratnagar. (Narayan et al 2010) also the prevalence of *salmonella* in Kathmandu 59.8%, Bhaktapur 7.6% and in Lalitpur 32.6%. This study also shows that the prevalence of *salmonella* is high there in water of Kathmandu than that of Bhaktapur and Lalitpur. In Between Bhaktapur and Lalitpur also the prevalence of Lalitpur is high. So regular monitoring of water should be done it not only monitors the water but also prevents diseases and hazards and checks the water resources from going further pollution.

Similarly, when we see the prevalence rate of *salmonella* based on sources. Three major sources where sample were collected are river, tap water and sewage. Among these three sources of water the highest prevalence of *salmonella* is seen sewage and less prevalence was seen in tap water. From the study (Ghimire et al 2018) compares the prevalence of *salmonella* in ground water and municipal drinking water in which they find the prevalence of *salmonella* in ground water was 80.3% and in ground water 19.7% which clears that the ground water is highly contaminated. This prevalence study of *salmonella* in water sources indicates that

pollution of water is increasing alarmingly and that it has created serious threat to human health and environment.

From our study we also can see that though the maximum number of samples were collected from tap water, but the prevalence rate of *salmonella* is least. Whereas the least samples were collected from sewage, but the prevalence rate of sewage is high this indicates that number of total samples collected from tap water (51) only 35 shows presence of *salmonella*, but the total sample collected from sewage is 22 in which out of 22 ,18 samples almost all of the sample shows the presence of *salmonella* which indicates that the all the sewage samples are contaminated which shows high prevalence of *salmonella*.

The total present sample (76) were tested against different antibiotics, after testing with antibiotics their zone of inhibition was measured and figured out that weather the tested antibiotics was sensitive, resistant and intermediate. Antimicrobial susceptibility testing of the isolated strains of *Salmonella* was carried out using the disk diffusion method (modified Kirby–Bauer method) on Mueller–Hinton agar (Hi-Media, India) following standard procedures recommended by the Clinical and Laboratory Standards Institute (CLSI). We analyzed the susceptibility of common therapeutic antimicrobial agents including ampicillin (10µg), nalidixic acid (30µg), ciprofloxacin (5µg), chloramphenicol (30µg), amikacin (30µg), Cefotaxime (30µg), and azithromycin (15μg), (Hi-Media Laboratories, India).

The results of the antibiotic susceptibility were determined based on interpretative zone diameters suggested by CLSI. On Kirby–Bauer disk diffusion antimicrobial susceptibility testing, *Salmonella* isolates were highly susceptible to azithromycin and amikacin whereas Amikacin and ciprofloxacin are less sensitive. But ampicillin was resistant to *salmonella* which did not show any zone of inhibition. This AST helps health care practitioner to determine which drugs are likely to be most effective in treating the person's infection.

Since here ampicillin shows the resistant, this antibiotic is not effective at inhibiting the growth of the organism or may not be an appropriate choice for treatment. Resistance may be innate or acquired. Innate is a part of microbes due to physical and genetic characteristics. If treatment is stopped before all the pathogens are killed. The survivors may develop resistance to that drug.

(Karkey A et al 2016) study says that Third generation cephalosporins are the safest choice for empirical use but ampicillin, cotrimoxazole, azithromycin, and chloramphenicol can be effective alternatives. This study also shows that cefotaxime shows the highest susceptibility to *salmonella*, whereas in contrast Nalidixic acid shows resistance to the *salmonella*. This study says that Nalidixic acid resistance was very much common among the isolates of *Salmonella enterica*. Overall, 92.2% of the isolates were NA resistant.

Similarly, now comparing the P value we can calculate the significance of antibiotic. P -value is defined as a statistical measurement used to validate a hypothesis against observed data. Comparing the P – value with 0.05 we can say that which drugs is statically significant or not. From above we can say that azithromycin, nalidixic acid, amikacin, cefotaxime and ciprofloxacin have the p-value which is less than 0.05 so we can say that these drugs are significant. Similarly, the ampicillin and chloramphenicol have p-value which is greater than 0.05 which shows that they are not significant. (Dekker et a l2012) The smaller the p- value the stronger the evidence that should reject the null hypothesis and more significant is our results. (Adhikari et al 2016) study research says that Chloramphenicol was once considered the drug of choice for enteric fever acquired resistance within few years of its introduction, but later, MDR *Salmonella* resistant to chloramphenicol, ampicillin, and trimethoprim sulfamethoxazole emerged in the late 1980s and early 1990s Reemergence of susceptibility to chloramphenicol and other first-line drugs in previously resistant areas has been reported in studies This study of Mahindra also shows the similar results of like ours study which shows that ampicillin is highly resistant to *salmonella* isolates.

CHAPTER VI

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion:

For the isolation and identification of *Salmonella* different sources of water from different places of province no 1 were selected. From the statistical study different results were obtained. Since we all know that *salmonella* is a gram-negative rod which is responsible for causing different types of water borne diseases.

From above results portion we came to know that out of total 100 sample, most of the samples were collected from Dharan, since highest sample were collected from Dharan, Dharan shows high prevalence rate of *salmonella* (48) 81.30%, Itahari shows the second highest prevalence rate of *salmonella* i.e. (14) 73.68%, and Biratnagar shows the less prevalence rate i.e. (14) 68.53%. From this data we can conclude that *Salmonella* is uniformly present in different places of water sources. But mostly Dharan water shows highly infected.

This obtained data represents that water sources of Itahari and Biratnagar are less polluted than that of Dharan. Which shows that concerted efforts should be made by control program to make the sources of water free from organism and waste products. Similarly, this finding of present study may provide useful information for such integrated strategies to overcome the public health burden of water borne diseases in Dharan, Biratnagar and Itahari.

6.2 Recommendation:

- Awareness on water borne diseases, improving hygiene should be conducted based on the result of this study
- Several supportive program for cleanliness of sources of water should be conducted.
- the study result should draw the attention of government to conduct different environmental programs in different places of province no 1

The above listed are the recommendations that we can draw from our thesis result.

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PHOTOGRAPHS

Fig No. 1: Showing how & where we collected sample and the collected sample was kept in ice bag

Fig No. 2: showing black head colonies of *salmonella* in *salmonella* shigella agar

Fig No. 3: Showing different biochemical tests of *salmonella*

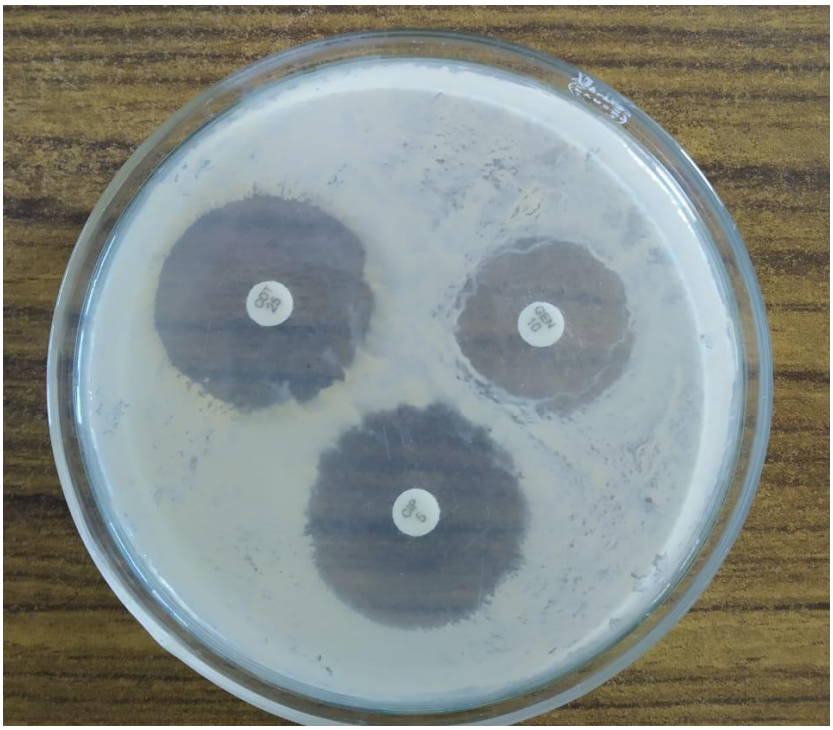

Fig No. 4: Showing AST of *salmonella* which shows the zone of inhibition shown by antibiotic against Salmonella.

List of Appendix

APPENDIX -I

1. Composition and Preparation of Different Culture Media

The culture media used were from Hi-Media Laboratories Pvt. Limited, Bombay, India. (Final pH of media was tested by pH meter; Sterility testing was performed by incubating prepared plates at 37^0C for 24 hours in an incubator and observed for any growth.)

1.1 Selenite-F broth

Ingredients	gm/liter
Part A	---
Casein enzymic hydrolysate	5.00
Lactose	4.00
Sodium phosphate	10.00
Part B	---
Sodium hydrogen selenite	4.00

Final pH (at 25^0C) 7.0±0.2

0.4 grams of Part B was suspended in 100 ml distilled water and 1.9 grams of Part A was added and mixed well. It was then warmed to dissolve and transferred to sterile test tubes.

1.2 Nutrient Broth (NB)

Ingredients	gm/liter
Peptone	5.0
Sodium Chloride	5.0
Beef Extract	1.5
Yeast Extract	1.5

Final pH (at 25^0C) 7.4±0.2

1.3 grams of the medium was dissolved in 100 ml distilled water, dispensed about 3-5 ml into clean test tubes and autoclaved at 121^0C for 15 minutes.

1.3 Salmonella Shigella Agar (SHA)

Ingredients	gm/liter
Beef Extract	5.00
Enzymatic Digest of Casein	2.50
Enzymatic Digest of Animal Tissue	2.50
Lactose	10

28 grams of the medium was suspended in 1000 mi distilled water and the medium was warmed to dissolve no autoclaving is required and then the media was plated in sterile Petri plate for inoculation of organism.

Lactose is the carbohydrate present in *salmonella* shigella agar. Thiosulfate and ferric citrate permit detection of hydrogen sulfide by the production of colonies with black centers. Neutral red turns red in the presence of an acidic PH, this showing fermentation has occurred.

1.4 Mueller Hinton Agar (MHA)

Ingredients	gm/liter
Beef, Infusion form	300.0
Casein Acid Hydrolysate	17.5
Starch	1.5
Agar	17.0

Final pH (at 25^0C) 7.4±0.2

38 grams of the medium was suspended in 1000 ml distilled water and the medium was warmed to dissolve. Then the medium was sterilized by autoclaving at 121^0C (15lbs pressure) for 15 minutes, cooled and plated in sterile petri plates.

1.5 Biochemical Test Media

1.5.1 MR-VP Medium

Ingredients	gm/liter
Buffered Peptone	7.0
Dextrose	5.0
Dipotassium Phosphate	5.0

Final pH (at 25^0C) 6.9±0.2

1.7 grams of powder was dissolved in 100 ml distilled water. 3 ml of medium was distributed in each test tube and autoclaved at 121^0C for 15 minutes.

1.5.2 Simmons Citrate Agar

Ingredients	gm/liter
Magnesium Sulfate	0.2
Mono-ammonium Phosphate	1.0
Dipotassium Phosphate	1.0
Sodium Citrate	2.0
Sodium Chloride	5.0
Agar	15.0
Bromothymol Blue	0.08

Final pH (at 25^0C) 6.8±0.2

2.42 grams of the medium was dissolved in 100 ml distilled water, warmed to dissolve completely. 3ml medium was distributed in test tubes and sterilized by autoclaving at 121^0C for 15 minutes. After autoclaving tubes containing medium were tilted to form slant.

1.5.3 Triple Sugar Iron (TSI) Agar

Ingredients	gm/liter
Peptone	10.0
Tryptone	10.0
Yeast Extract	3.0
Beef Extract	3.0
Lactose	10.0
Sucrose	10.0
Dextrose	1.0
Ferrous Sulphate	0.2
Sodium Chloride	5.0
Sodium Thiosulphate	0.3
Phenol Red	0.024
Agar	12.0

Final pH (at 25^0C) 7.4±0.2

6.5 grams of the medium was dissolved in 100 ml of distilled water, warmed to dissolve and about 5 ml medium was distributed into test tubes. It was then sterilized by autoclaving at 15lbs (121^0C) pressure for 15 minutes. The medium was allowed to set in sloped form with a butt about 1 inch of thickness.

1.6 Staining and Test Reagents

1.6.1 For Gram's Stain

a) Crystal Violet solution

Crystal Violet	20.0 g
Ammonium Oxalate	9.0 g
Ethanol or Methanol	95 ml
Distilled Water(d/w)	1 liter

Preparation: In a clean piece of paper, 2 gm of crystal violet was weighed and transferred to a clean brown bottle. Then, 10 ml of ethanol was added and mixed until the dye was completely dissolved. To the mixture, 1 gm of ammonium oxalate dissolved in 20 ml of D/W was added. Finally, the volume was made 100 ml by adding D/W.

b) Lugols's Iodine

Potassium Iodide	20.0 g
Iodine	10.0 g
Distilled Water	1000 ml

Preparation: To 25 ml of D/W, 2 gm of potassium iodide was dissolved. Then 1 gm of iodine was mixed to it until it was dissolved completely. Finally, the volume was made 100 ml by adding D/W.

c) Acetone-Alcohol Decolorizer

Acetone	500 ml
Ethanol (Absolute)	475 ml
Distilled Water	25 ml

Preparation: To 5ml D/W, 50ml of absolute alcohol was added, mixed and transferred into a clean bottle. Then immediately, 50 ml acetone was added to the bottle and mixed well.

d) Safranin (Counter Stain)

Safranin	10.0 g
Distilled Water	1000 ml

Preparation: In a clean piece of paper, 1 gm of safranin was weighed and transferred to a clean bottle. Then 100 ml D/W was added to the bottle and mixed well until safranin dissolved completely.

1.6.2 For Methyl Red Test

Methyl Red Solution

Methyl red	0.05 g
Ethyl alcohol (absolute)	28 ml
Distilled Water	22 ml

Preparation: To 28 ml ethanol, 0.05 gm of methyl red was dissolved and transferred to a clean brown bottle. Then 22 ml D/W was added to that bottle and mixed well.

1.6.3 For Voges-Proskauer Test (Barritt's Reagent)

Solution A

α-Naphthol	5.0 g
Ethyl alcohol (absolute)	100 ml

Preparation: To 25 ml D/W, 5 g of α-Naphthol was dissolved and transferred into a clean brown bottle. Then the final volume was made 100 ml by adding D/W.

Solution B

Potassium hydroxide	40.0 g
Distilled Water	1000 ml

Preparation: To 25 ml D/W, 40 gm of KOH was dissolved and transferred into a clean brown bottle. Then the final volume was made 100 ml by adding D/W.

APPENDIX II

List of Equipment's, materials and supplies

A. EQUIPMENT

- Autoclave
- Incubator
- Hot air oven
- Microscope **(**Olympus)
- Refrigerator 4-8^{0}C
- Weighing machine
- Water Bath
- Gas burners
- Glassware's
- Inoculating wire and loops

B. MICROBIOLOGICAL MEDIA

- Salmonella Shigella Agar
- Selenite Broth
- Nutrient Broth
- Mueller Hinton Agar
- MRVP Broth
- Triple Sugar Iron Agar
- Citrate

C. CHEMICALS AND REAGENTS

- Catalase reagent (3% H_2O_2)
- Oxidase reagent (1% Tetramethyl *p*-phenylene diamine dihydrochloride)
- Conc. H_2SO_4
- Gram's reagent

D. ANTIBIOTIC DISKS

The antibiotic disks used for the susceptibility tests that were from Hi-media Laboratories Pvt Ltd. India are as follows:

- Amikacin (**30**µg)
- Ampicillin (**10**µg)
- Cefotaxime (**30**µg)
- Ceftazidime (**30**µg)
- Ciprofloxacin **(5**µg)
- Chloramphenicol (**30**µg)
- Nalidixic acid (**30**µg).

E. ZONE SIZE INTERPRETATION CHART OF ANTIBIOTIC SUSCEPTIBILITY TESTING

Antibiotics	Symbol	Disc content	Diameter of zone of inhibition in mm		
			Intermediate	Resistance	Sensitive
Azithromycin	AZM	15	≤ 13	14-17	≥ 18
Ampicillin	AMP	10	≤ 13	14-16	17
Amikacin	AK	30	≤ 14	15-16	≥ 17
Cefolaxime	CTK	30	≤ 22	2, 3-25	≥ 26
Necidixicacid	NA	30	≤ 13	14-18	≥ 19
Cholorompherical	C	30	≤ 12	13-17	≥ 18
Ciproflaxacin	CIP	30	≤ 20	21-30	31

APPENDIX-III

Gram-staining Procedure

First devised by Hans Christian Gram during the late 19th century, the Gram-stain can be used effectively to divide all bacterial species into two large groups: those that take up the basic dye, crystal violet (Gram-positive) and those that allow the crystal dye to was out easily with the decolorizer alcohol or acetone (Gram-negative). The following steps are involved in Gram-stain:

1. A thin film of the material to be examined was prepared and air dried.
2. The material on the slide was heat fixed and allowed to cool before staining.
3. The slide was flooded with crystal violet stain and allowed to remain without drying for 30 seconds.
4. The slide was rinsed with tap water, shaking off excess.
5. The slide was flooded with iodine solution and allowed to remain on the surface without drying for 60 seconds.
6. The slide was rinsed with tap water, shaking off excess.
7. The slide was flooded with alcohol acetone decolorizer for 10 seconds and rinsed immediately with tap water until no further color flows from the slide with the decolorizer.
8. The slide was flooded with counter stain (safranin) for 30 seconds and washed off with tap water.
9. The slide was blotted between two clean sheets of bibulous paper and examined microscopically under oil immersion at 100X (Gephart *et al.,1981)*

APPENDIX IV

Biochemical Tests for identification of bacteria

1.1 Methyl Red test

This test is performed to test the ability of an organism to produce sufficient acid from the fermentation of glucose to give a red color with the indicator methyl red (denotes changes in degree of acidity by color reactions over a pH range of 4.4-6.0).

A pure colony of the test organism was inoculated into 2 ml of MRVP medium and was incubated at 37°C for 24 hours. After incubation, about 5 drops of methyl red reagent was added and mixed well. The positive test was indicated by the development of bright red color, indicating acidity.

1.2 Voges Proskauer (VP) test

This test is employed to detect the production of acetyl methyl carbinol (a neutral end product) or its reduction product 2, 3-butanidiol during fermentation of carbohydrates.

A pure colony of the test organism was inoculated into 2 ml of MRVP medium and was incubated at 37°C for 24 hours. After incubation, about 5 drops of Barritt's reagent was added and shaken well for maximum aeration and kept for 15 minutes, positive test is indicated by the development of pink red colour.

1.3 Citrate Utilization test

This test is performed to detect whether an organism utilizes citrate as a sole source of carbon for metabolism with resulting alkalinity. Organisms capable of utilizing citrate as its sole carbon source also utilizes the ammonium salts present in the medium as its sole nitrogen source, the ammonium salts are broken down to ammonia with resulting alkalinity.

A loopful of test organism was streaked on the slant area of Simmon's Citrate Agar medium and incubated at 37°C for 24 hours. A positive test was indicated by the growth of organism and change of media by green to blue, due to alkaline reaction. The pH indicator bromothymol blue has a pH range of 6.0-7.6, i.e. above pH 7.6; a blue color develops due to alkalinity of the medium.

1.4 Triple Sugar Iron (TSI) Agar

The TSI agar is used to determine the ability of an organism to utilize specific carbohydrate incorporated in the medium (glucose, sucrose and lactose in concentrations

of 0.1%, 1.0% and 1.0% respectively), with or without the production of gas (indicated by cracks in the media as well as an air gap at the bottom of the tube) along with determination of possible hydrogen sulfide production (detected by production of black color in the medium).

The test organism was streaked and stabbed on the surface of TSI and incubated at 37°C for 24 hours. Acid production limited only to the butt region of the tube is indicative of glucose utilization, while acid production in slant and butt indicates sucrose or lactose fermentation. Phenol red is the pH indicator which gives yellow reaction at acidic pH, and red reaction to indicate an alkaline surrounding.

APPENDIX V

Zone size interpretation chart of antibiotics susceptibility testing

Antibiotics	Symbol	Disc content	Diameter of zone of inhibition in mm		
			Intermediate	Resistance	Sensitive
Azithromycin	AZM	15	$\leq$ 13	14-17	$\geq$ 18
Ampicillin	AMP	10	$\leq$ 13	14-16	17
Amikacin	AK	30	$\leq$ 14	15-16	$\geq$ 17
Cefolaxime	CTK	30	$\leq$ 22	2, 3-25	$\geq$ 26
Necidixicacid	NA	30	$\leq$ 13	14-18	$\geq$ 19
Cholorompherical	C	30	$\leq$ 12	13-17	$\geq$ 18
Ciproflaxacin	CIP	30	$\leq$ 20	21-30	31