ABSTRACT

Most precious resource of any nation is a healthy population. However, it is impossible to achieve good health without safe drinking water supply and basic sanitation. Safe water is not even readily available in urban areas of Nepal and water quality remains very poor. Nepal Water Supply Corporation, which operates, maintains and develops water supply system in urban centers in the country, has a full-fledged water quality-testing laboratory only in capital city Kathmandu, but do not have water quality monitoring facilities elsewhere in the country. There is no regular national water quality monitoring and surveillance program in place in the country.

Main aim of the present study was to find out occurrence and diversity of *Salmonella* serovars in urban drinking water supply systems of three major cities in Nepal. A total of 200 samples from Kathmandu, 50 from Pokhara and 50 from Biratnagar were collected and analysed. Amongst the water supply systems included in this study, only Kathmandu has a complete water treatment system including coagulation, sedimentation, slow sand filtration, and disinfection. Other two cities Pokhara and Biratnagar do not have complete water purification system. Chlorination is done at the catchments and reservoirs infrequently. In all the city systems, distribution pipelines are quite old and lie closer to wastewater drainage pipelines. Existing microbiological quality was assessed on the basis of total coliform count, thermotolerant coliform count, presence of *E. coli* and *Salmonella* (Chapter2).

The *Salmonella* isolates from urban drinking water supply systems of Nepal were characterized with respect to serotype, phage type, antibiotic sensitivity and virulence genes (Chapter3). In present study the *Salmonella* bacteriophages from river water of Nepal were isolated and identified. Efficacy of isolated phages in reduction of *Salmonella* in waste-water spiked with *Salmonella* was evaluated. Results indicated that the *Salmonella* bacteriophages could be an alternative option in the control of *Salmonella* in the environment. Prior to present study, no other reports are available on isolation, characterization of Bacteriophages in Nepal (Chapter 4). In the present study bioactive *Bacillus* spp. from soil samples

of Nepal against multi drug resistant *Salmonella* and other waterborne pathogens were isolated and screened. One of the isolate *Bacillus subtilis* KBB was studied in detail viz; anti *Salmonella* compound production, extraction and characterization (Chapter5).

This study gives background information on microbiological quality of drinking water from urban water supply systems of Nepal. In the present study, free residual chlorine concentration was found in trace amount in drinking water supply from Kathmandu, whereas in the water samples from other two cities free residual chlorine was undetectable. Majority of the samples as analysed by Membrane filtration method contained large number of total coliform bacteria and thermotolerant coliform bacteria, which indicate faecal contamination, improper water treatment, poor distribution and serious lapses in drinking water supply systems in Nepal. The highest mean count of total coliform and thermotolerant coliform bacteria was observed in second quarter of the year (also known as summer and pre monsoon period in Nepal) in the samples from Pokhara (March April and May), whereas for Kathmandu and Biratnagar it was observed in the third quarter (June, July, August), also known as rainy season in Nepal. In the present study many multi drug resistant *E. coli* (resistant to more than 3 antibiotics) from urban water supply systems of Nepal were isolated which indicated that they might be responsible for increasing case fatality rates (national Case Fatality Rate for diarrhoea & gastroenteritis is 0.6%) of diarrhoea and gastroenteritis in Nepal. No other reports have been available on the isolation of multi drug resistant *E. coli* from drinking water supply systems of Nepal. In this study 10 antimicrobials were tested against *E. coli* isolates and highest resistance was observed with ampicillin. However, all the isolates were sensitive to ciprofloxacin, chloramphenicol and ceftazidime. The results of the present study indicate that if the existing conditions of water supply systems in Nepal are not improved there are always the chances of sudden outbreak of water borne infections. Present study will be useful in water borne diseases control and prevention strategy formulation in Nepal and in the global context.

The present study was conducted to study occurrence and diversity of *Salmonella* serovars in urban water supply systems of Nepal (Chapter 3). Occurrence of

Salmonella was detected in 42 out of 300 water samples by enrichment culture technique in selenite F broth followed by plating on SS agar. Total 54 isolates identified to genus level by standard tests, were subsequently confirmed by serotyping, phage typing and PCR detection of virulence genes (*inv A* and *spv C*). Predominant serotype was *Salmonella enterica* serovar Typhimurium followed by *Salmonella enterica* serovar Typhi, *Salmonella enterica* serovar Paratyphi A and *Salmonella enterica* serovar Enteritidis. Most of the isolates of *Salmonella enterica* serovar Typhi were E1 phage type followed by UVS4, A and UVS1. All isolates of *Salmonella enterica* serovar Paratyphi A and *Salmonella enterica* serovar Enteritidis were an untypable (UT) phage type. Majority of isolates were multi-drug resistant as revealed by Kirby-Bauer disc diffusion technique. Ceftriaxone resistant isolates of *Salmonella enterica* serovar Enteritidis indicated presence of one of the ESBL genes, blaSHV, whereas, the genes blaTEM and blaCTX were absent. Presence of *Salmonella* in most of the water samples provided an undeniable evidence of the poor microbiological quality of the public water supply in urban Nepal (Chapter 3). The isolation and characterization of Bacteriophages from different environments have been reported rarely in developing countries including Nepal due to the lack of essential technical skills and technical facilities such as electron microscopy. Present study is the first study on the isolation and characterization of Bacteriophages in Nepal. Water samples collected from river Bagmati in Kathmandu, Nepal were clarified and inoculated in the phage broth along with host strains of *Salmonella* and incubated overnight at 37°C and two lytic *Salmonella*-bacteriophages (Nephage 1 and Nephage 2) were isolated. Morphology of both the isolates as studied by electron microscopy revealed that they belong to Myoviridae and Siphoviridae family respectively. One of the phage isolates (Nephage 1) belonged to Myoviridae with a very big head resembled with phage 121Q, but seems to have shorter tail than previously reported (Ackermann *et al.* 1992). Other isolates (Nephage 2) with an isometric head belonged to family Siphoviridae. By the use of Nephage 1, the numbers of *Salmonella* in experimentally inoculated wastewater samples were reduced by 99.95%, while Nephage 2 reduced the numbers of *Salmonella* by 95.47% after 72 h of incubation. It indicates that the isolated bacteriophages could be used in

reduction of multidrug resistant *Salmonella* in the polluted environment viz; from wastewater, poultry and slaughterhouse offal, hospital wastewater discharge etc (Chapter 4).

The microorganisms are developing resistance to antibiotics currently in use and there is a need for searching new potential antibiotics to curb the infections. *Bacillus* spp. have long been recognized for their potential to produce many types of commercially important, low molecular weight peptides and most of them possess antibacterial and antifungal properties. In the present study bioactive *Bacillus* spp. were isolated against *Salmonella* and other gram-negative waterborne pathogens. Soil samples from Kathmandu Nepal were collected and five bioactive *Bacillus* viz; *Bacillus licheniformis* KBR; *Bacillus subtilis* KBB; *Bacillus pumilus* KBY; *Bacillus cereus* KBC; and *Bacillus subtilis* KBA were isolated and screened as potential antagonists of multi drug resistant *Salmonella.* However, previous reports have shown the bioactivity of such isolates mostly against gram-positive bacteria in contrast to present study.

In the present study, one of the strains *Bacillus subtilis* KBB was found superior antagonist of many gram negative bacteria such as *Proteus mirabilis*, *E. coli*, *Enterobacter agglomerans*, *Citrobacter frundii*, and *Morganella morganii* and also inhibited the growth of MDR *Salmonella* serovars.

Isolation of *Bacillus subtilis* KBB makes the study significant as the findings have established this strain as the potential broad-spectrum antagonists of waterborne pathogens.

The culture condition optimization for antimicrobial compound production by *Bacillus subtilis* KBB and extraction, partial purification and characterization of low pH antimicrobial compound of thermostable nature makes this study useful for further research. In the present study, the antimicrobial compound production from *Bacillus subtilis* KBB was optimized using different liquid media such as nutrient broth, Mueller Hinton broth, LB broth, and LB broth with additional carbon source (1% glucose, sucrose, and maltose) along with source of manganese. LB broth medium containing 1% maltose and 0.1% $MnCl_2$ and 0.1% KNO_3 was found most suitable for the production of anti-salmonella compound. The time course of production of antimicrobial compound indicated that the

maximum antimicrobial activity was produced on the 3rd day of fermentation and decreased thereafter.

In the present study, antimicrobial activity of the culture supernatant of *Bacillus subtilis* KBB was tested after the treatment with enzymes and organic solvents. The antimicrobial activity of culture supernatant was also studied at different temperature. The antimicrobial activity of culture supernatant was partially susceptible to proteinase K, pronase and chymotrypsin suggesting that the active molecule is proteinaceous. The common organic solvents such as methanol, ethanol, and chloroform did not affect antimicrobial activity of culture supernatant. Antimicrobial activity of culture supernatant was observed at high temperature and over a wide range of acidic and basic pH and indicated that active molecule is highly thermostable in nature. The antimicrobial activity of culture supernatant remain stable even after storage at cold temperature for a month and at room temperature for 4 month, which suggested that it did not undergo any kind of degradation.

One-step solvent extraction procedure (Bligh and Dyer method, 1959) with slight modification) optimized in present study was found to be suitable method to extract active antimicrobial compound from culture supernatant of *Bacillus subtilis* KBB. Protein nature of the partially purified compound was confirmed by Biuret test and Ninhydrin test, TLC, UV and IR. Thin Layer Chromatography confirmed the purity of the compound. MIC values of the extracted antimicrobial compound were 125µg/ml for *Staphylococcus aureus* and 250µg/ml for *Salmonella enterica* serovar Typhi W7. In the present study UV, IR, GCMS analysis indicates peptide nature of the compound. The ^{1}H NMR (Nuclear magnetic resonance, 500 MHz) $^{13}CNMR$ spectra of the antimicrobial compound indicated the presence of 13 H and 19 C in the sample. However, complete structure elucidation of the compound, mechanism of action and toxicity of the compound, amino acid profiling, and sequencing remains the topic for further study. The low pH antimicrobial compound of thermostable nature extracted in the present study indicates that the compound could withstand gastric acidity of animal stomach; hence it could be a potential antagonist to use against *Helicobacter pylori* also. Further studies in this regard are recommended. Due to

GRAS status, the *Bacillus subtilis* KBB may be used in animal clinical trials. The results also indicated that the low pH and thermostable antimicrobial compound produced by *Bacillus subtilis* KBB could be used as broad-spectrum disinfectant, since it showed antimicrobial activity against both the gram-negative and gram positive bacteria. The findings of this study indicated strong possibilities that *Bacillus subtilis* KBB strain could be used to eliminate *Salmonella* from wastewater and other sources of water pollution. However, further studies are recommended in regards to survival of this *Bacillus* in water environment.