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Bacteriological Analysis of Siran River System for Fecal Contamination and Metallo-β-Lactamase *bla*NDM-1 Gene

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Abstract.- Musa ka Musalla glacier is situated in the Allai, Manshera District, Khyber Pakhtunkhwa, Pakistan **(**34.713678, 73.360806). There were 20 different water samples sites moving downstream from the glacier, which is the source of river Siran, during winter, summer and monsoon seasons taken for the analysis of water quality destruction in sense of total viable count, total coliform count, fecal coliform count, fecal *E.coli* count, which are indicator organisms of fecal contamination, as well as the prevalence and distribution of MBL *bla*NDM-1 producing Gram negative bacteria. Total viable counts were found in the range of 3×10^2 to 40×10^2 CFU/mL in winter, 5×10^2 to 45×10^2 CFU/mL in summer and 2×10^2 to 40×10^2 CFU/mL in monsoon. The range of MPN 100 mL^{-1} for total coliform was found 11–2400, 150–2400 and 80–2400, for fecal coliform, it was 3–700, 2–900, 2–800 and for fecal *E.coli*, it was 1–300, 2–450 and 1–400 in winter, summer and monsoon, respectively. While, out of total 20 samples from different sites 07 were positive to MBL producing Gram negative bacteria and among 07 Only 03 isolates including Dadar (U-4), Shinkiari (M-10) and Khaki (M-13) were *bla*NDM-1 positive. The overall objective of this work is to examine the incidence of these indicator organisms, coliforms, fecal coliforms, fecal *E.coli* and as well as *bla*NDM-1 gene occurrence in isolates in runoff of the Musa ka Musalla glacier**,** Khyber Pakhtunkhwa, Pakistan.

Keywords: Fecal Indicators, Metallo-beta-lactamases, *bla*NDM-1, Musa ka Musalla glacier.

INTRODUCTION

Water contaminated with Human feces is generally considered to be great risk to human health because it may include human enteric pathogen (Esomonu *et al*., 2012); Vinay *et al*., 2005; Kistemann *et al*., 2002; Harwood *et al*., 2001; Vaidya *et al*., 2001). Concurrently, contamination of water by enteric pathogen has increased worldwide (Islam *et al*., 2001). According to the WHO, the mortality of water associated diseases exceeds 5 million people per year. From these, more than 50% are microbial intestinal infections (João, 2010; Esomonu *et al.*, 2012).

The microorganisms are widely scattered in nature and their plenty and multiplicity may be used as an indicator for the suitability of water. The most important aspect of water is its free will from contamination with fecal matter (Tambekar and Neware, 2012; *Khan et al.,* 2011). Coliform bacteria are used as microbiological indicators for water quality.

A wide range of pathogenic microorganisms can be transmitted to humans via water contaminated with fecal material. These include enteropathogenic agent such as salmonella species, shigella Species, enteroviruses and multicultural parasites as well as opportunistic pathogens like *Pseudomonas aeruginosa*, Klebsiella, *Vibrio parahaemolyticus* and *Aeromonas hydrophila* (Dziuban *et al.,* 2006; Hodegkiss, 1988).

Wide range of bacteria resistant to antibiotics have been found in the surface water, ground water, rivers, drinking water and lakes (Agnese *et al.*, 2012). Most resistant bacteria produce β-lactamases in response to different antibiotic producing organisms. The β-lactam antibiotics are inactivated by Beta-lactamases that hydrolyze the amide bond that exists in the beta-lactam ring, disrupting the ring structure and make the antibiotics nonfunctional against bacteria.

NDM-1 is a broad-spectrum β-lactamase which has the ability to inactivate most of the β-

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lactam antibiotics (Walsh *et al.*, 2005). Infections with NDM-1 positive pathogens were first reported from UK, India and Pakistan (Kumarasamy *et al*., 2010). It is similar to other MBLs but their similarities are more related to enzymes VIM-2 and VIM-1 with (32% amino acid identity), rarely it is found on chromosome but mostly encoded by plasmid of various sizes and incompatible groups (Walsh *et al*., 2005). The plasmids encoding carbapenemases among bacterial pathogens show resistance to β-lactam and carbapenems antibiotics, which in turn reduced the treatment options (Livermore, 2009). This is mostly important for NDM-1 β-lactamase producing *Enterobacteriaceae* and this gene has the ability to move from one plasmid to other very easily and increased their global dissemination (Samuelsen *et al*., 2010). Transfer is most efficient around 30.8°C, corresponding to the average temperature during several months of the year in many Asian subcontinent (Walsh *et al*., 2011).

The antibiotic resistant bacteria in any type of water pose a public health threat through recycling by agriculture (swine, cattle, pigs and poultry), aquaculture (fish and birds) and anthropogenic activities of the human itself. Now the presence of NDM-1 in drinking water pose a direct threat to human health because the gene is encoded by incompatible plasmids with various kind and could be easily transferred to non pathogenic organisms render them resistant and will promptly increase the mortality issue in children, adults and immuno-compromised patients, Therefore, identification of a significant number of NDM-1 producers in the environment is of prior concern (Nordmann *et al.*, 2011).

To the best of our knowledge, no report is available on bacterial and molecular analysis of River Siran water in KPK, Pakistan for quality assessment. The river Siran originates from Musa ka Musalla, Allai, Mansehra District, Khyber Pakhtunkhwa, Pakistan (34.713678, 73.360806) and runoff to Tarbela Dam, Topi Tehsil, Khyber Pakhtunkhwa, Pakistan. The river Siran provides water and drainage for million people living along its runoff. The overall objectives of this work were to investigate the incidence of total viable count, total coliforms, faecal coliforms, *E. coli* as indicator organisms and the presence of MBL producing gram negative bacteria from different sites of river Siran KPK, Pakistan, as well as detection of the *bla*NDM1 gene in the isolated strains using PCR amplification.

MATERIALS AND METHODS

Bacteriological water analysis

Samples collection

Study area is divided into three parts in which upper stretch constituted five sampling sites, which are Allai, Sacchan, Jabori and Andrasi (U1-U5), middle stretch are Dadar, Sum, Sum Combine, Tanda, Shinkiari, Baffa, Trangrian, Khaki and Icher (M6-M14) and lower stretch are Nawashehar, Perhina, Lassanawab, Jura and Soha (L15-L19).

Water samples were collected from 20 different sampling sites of Siran river system, in sterile glass bottles, transported on ice to the laboratory and processed within 6–8 h of collection. Samples were collected during the winter, summer and monsoon seasons Figure 1b.

Screening for total viable count

For total viable count, the sterile spreader was used to spread the 10 µL water sample on Nutrient agar and then incubation at 37±1°C for 24 h. After the incubation the colonies were counted.

Screening for total coliform, feacal coliform and E. coli

The water quality was determined by the standard three-tube most probable number (MPN) method. Coliforms were detected by inoculation of samples into tubes of MacConkey broth and incubation at 37±1°C for 48 h. To detect fecal coliform, the positive tubes were subcultured into brilliant green bile broth (BGBB) and incubate at 44.5±1°C. Gas production in BGBB at 44.5±1°C was used for the detection of fecal coliform after 48 h incubation.

The positive tubes were subcultured into EC broth and were incubated at 44.5±1°C for 48 h. Gas production in EC at 44.5±1°C was used for the detection of *E. coli* after 48 h incubation. For confirmation positive tubes were streaked to Eosine Methylene Blue (EMB) plates. *E. coli* colonies were purple with green metallic sheen.

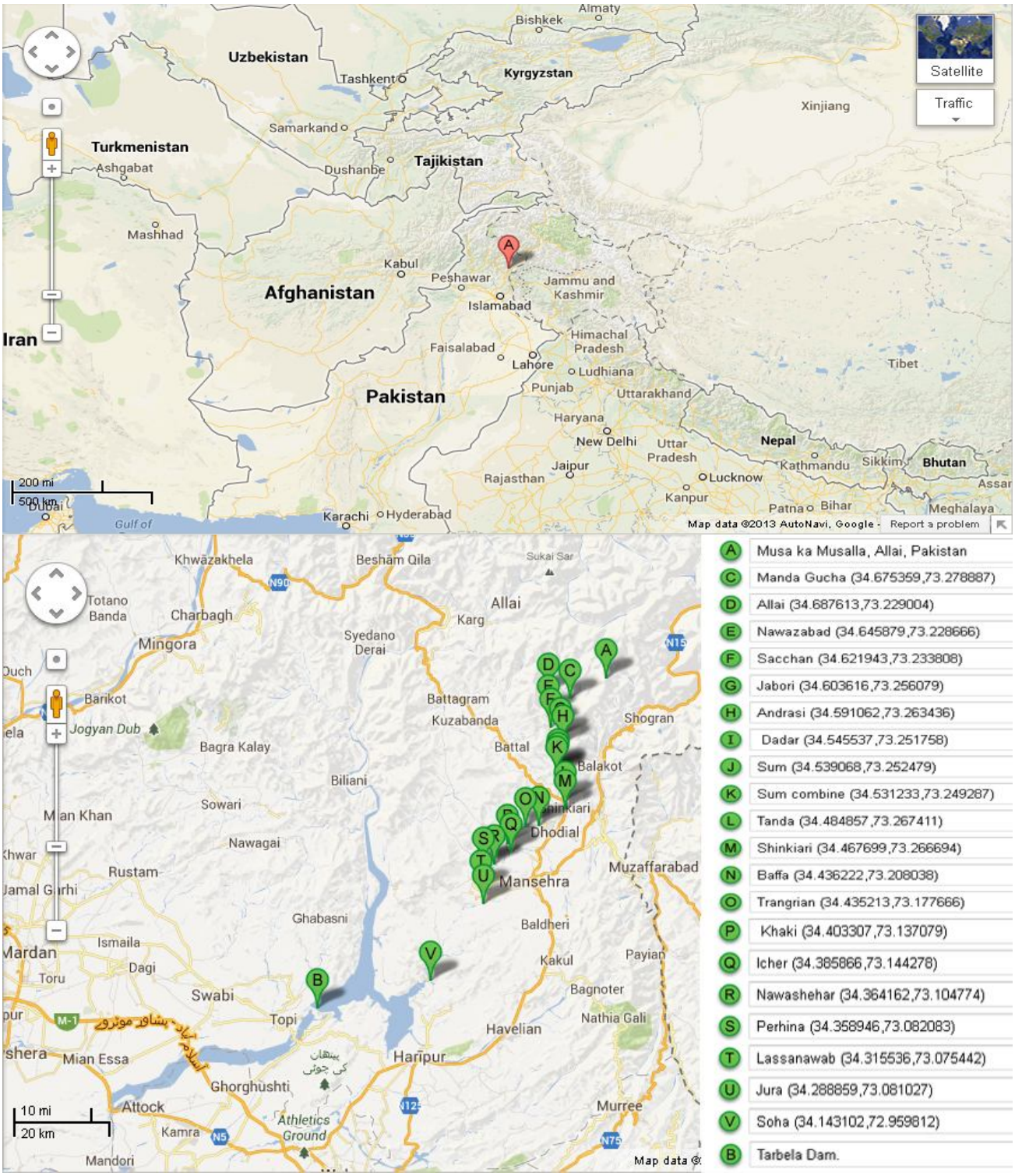

Fig.1. (a) Location map of Musa ka Musalla glacier; (b) Location map of the study area.

Molecular analysis for the presence of metallo-β-lactamase blaNDM-1 gene

Isolation of resistant strains

Each water sample was also inoculated on MacConkey's media supplemented with meropenem antibiotic (0.5g/1000mL) to isolate resistant gram negative bacteria producing metallo betalactamase. Isolated strains were further subcultured on meropenem containing MacConkey's media plates to get pure and resistant strains.

Combined disk for MBL detection

The isolated resistant strains were further detected for metallobetalactamase (MBL) production by combined disc method (Yong *et al.*, 2009). Isolates grown overnight on Nutrient Agar and were resuspended in normal saline to get inoculums with density equivalent to 0.5 M McFarland. A lawn was prepared on Muller Hinton Agar plate using cotton swab dipped in inoculums by streaking three times rotating the plate at 60° angle each time. The combined disc was prepared by pouring 4µL of 5M EDTA (3.72gm/20 mL) on 10µg meropenem disc and incubates for 5 minutes at 37±1°C and then kept at -20°C freezer until used. Combined disc and meropenem disc alone were placed adjacent to each other. The plate was incubated at 35±1°C. After 18-24 h zone diameter was measured. An increase in the zone of inhibition of combination compared with Meropenem alone was taken as positive control for MBL production. All the pure MBL producing strains were preserved on nutrient agar slants and were stored at 4˚C.

DNA extraction

Boiling method was used for the isolation of DNA (Shibata *et al.*, 1988; Coates *et al.*, 1991). Resistant strains were first inoculated on nutrient agar media and were incubated at 37±1°C for 24 h. 3-4 colonies were suspended into 40µL autoclaved distilled water using sterilized inoculation loop and was then mixed on vortex for 20 sec and was incubated at 95˚C for 5 min in PCR machine and then freeze for 20 min. Each sample was again mixed on vortex for 20 sec and then boiled for 15 minutes and was finally stored at -4°C.

PCR reaction

The strains were further screened for the presence of known mobile MBL genes (*bla*NDM1) by PCR with the primers reported in GenBank (National Center for Biotechnology Information, National Institutes of Health, Bethesda, MD, USA) database entry AB571289.1 (http://www.ncbi.nlm.nih.gov/nuccore/300422615). The forward primer NDM-1F 5′-ACC GCC TGG ACC GAT GAC CA-3′ and reverse primer NDM-1R 5′-GCC AAA GTT GGG CGC GGT TG-3′ were used. Amplification was performed using PCR program consisting of 30 cycles under following conditions: Initial denaturation at 95°C for 5 min; 95°C for 1 min, primer annealing at 52°C for 1 min followed by final extension at 72°C for 5 min. The amplified DNA is stored at -4°C.

Gel electrophoresis

The extracted DNA and PCR products were loaded on 1% agarose gel (Mickel *et al.*, 1977) with amount of 0.8g agarose in 0.5X solution of TBE (Bio life, Italina S.r.l, Italy). The agarose was heated in microwave oven for 1 min and upon cooling upto 45°C , 0.3 µg/mL ethidium bromide was added (Roche, Germany) for staining. Electrophoresis was carried out for 1 hour at 80 V. The bands were visualized in UV Transilluminator (UVItec, EEC) and digital photograph was taken.

RESULTS AND DISCUSSION

Bacteriological water analysis

Total viable counts were found in the range of 3×10^2 to 40×10^2 CFU/mL in winter, 5×10^2 to 45×10^2 CFU/mL in summer and 2×10^2 to 40×10^2 CFU/mL in monsoon (Fig. 2).

It is apparent from the data that in Musa ka Musalla glacier upper Stretch area, ''Jabori'' shows the number of total coliforms, *i.e.* 350, 400 and 300 MPN 100 mL^{-1} in winter, summer and monsoon, respectively. Furthermore, in middle stretch, Baffa and Khaki always show high count of total coliform of 950, 1300 and 1200 MPN 100 mL^{-1} and 900, 1600 and 1600 MPN 100 mL^{-1} winter, summer and monsoon, respectively. Always high count of total coliforms was found at all the study sites of lower stretch (Fig. 3).

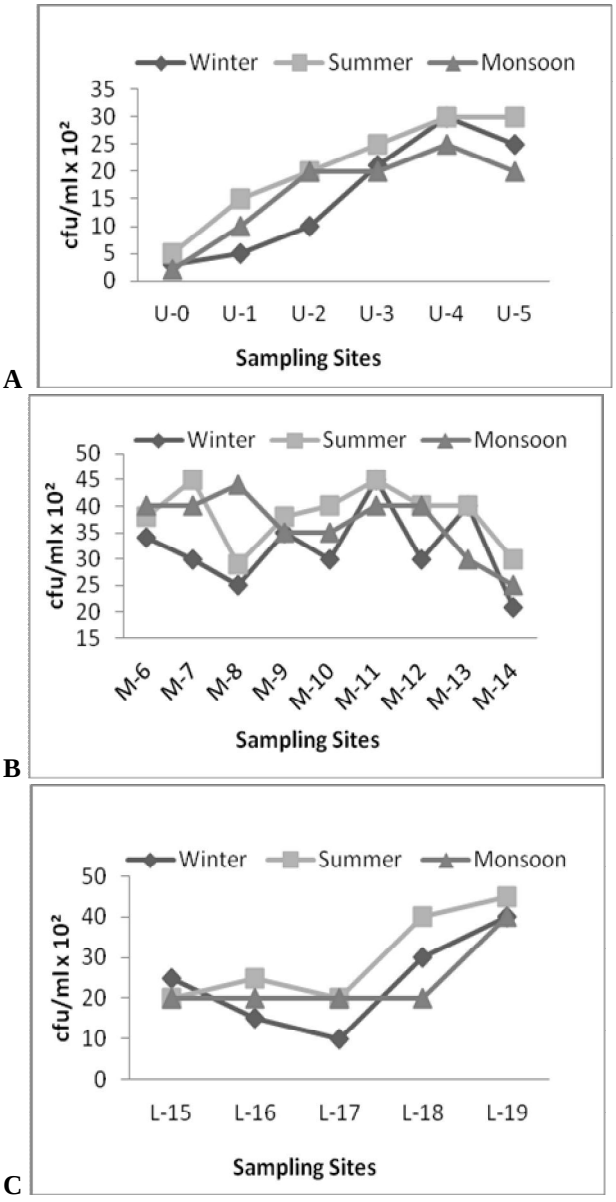

Fig. 2. Total viable count of the various sites of the runoff of Musa ka Musalla glacier: (a) upper stretch; (b) middle stretch; (c) lower stretch.

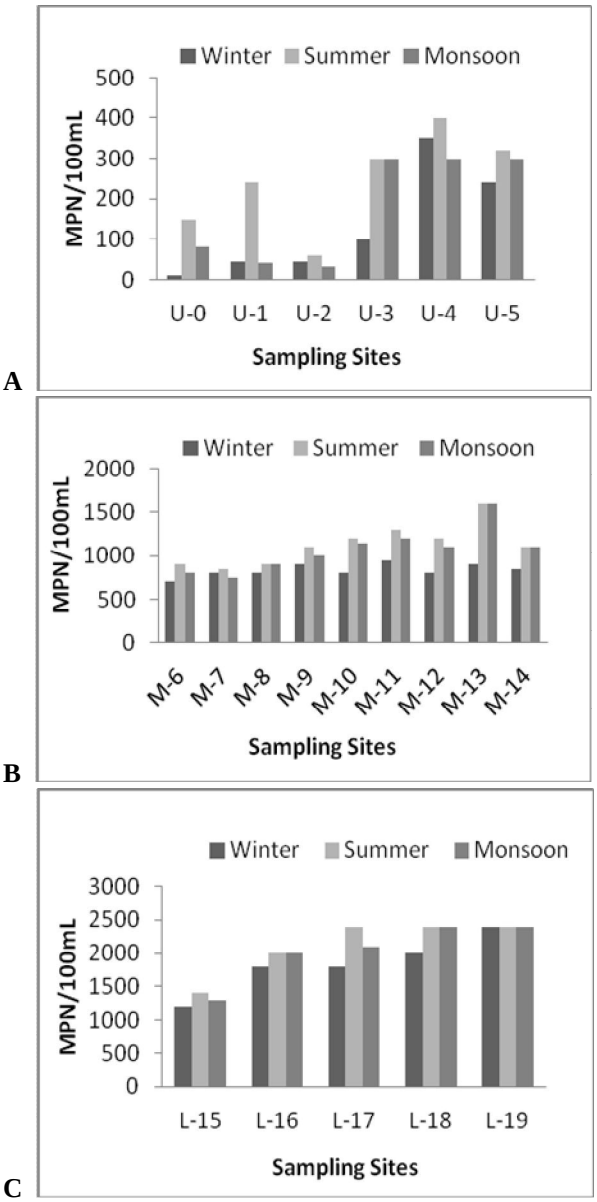

Fig. 3. Total coliform count of the various sites of the runoff of Gangotri glacier: (a) upper stretch; (b) middle stretch; (c) lower stretch.

In upper stretch, maximum thermotolerant coliforms count of 30 MPN 100 mL^{-1} was observed in Jabori in summer. In middle stretch at Khaki, high thermotolerant coliforms count *i.e.,* 500 MPN 100 mL^{-1} in summer, whereas, it was reduced to 350 MPN 100 mL^{-1} during winter was noticed and in lower stretch higher thermotolerant coliforms count was found (Fig. 4).

It is clear from our result that high count of fecal *E. coli*, *i.e.*, 10, 15 and 10 MPN 100 mL^{-1} in Jabori area and 200, 300, 250 MPN 100 mL^{-1} in middle starch at Khaki area in winter, summer and monsoon, respectively. While at lower stretch, Nawashehar, Perhina and Lassanawab always depicted high count of fecal *E. coli, i.e.* 300 MPN 100 mL^{-1} (Fig. 5).

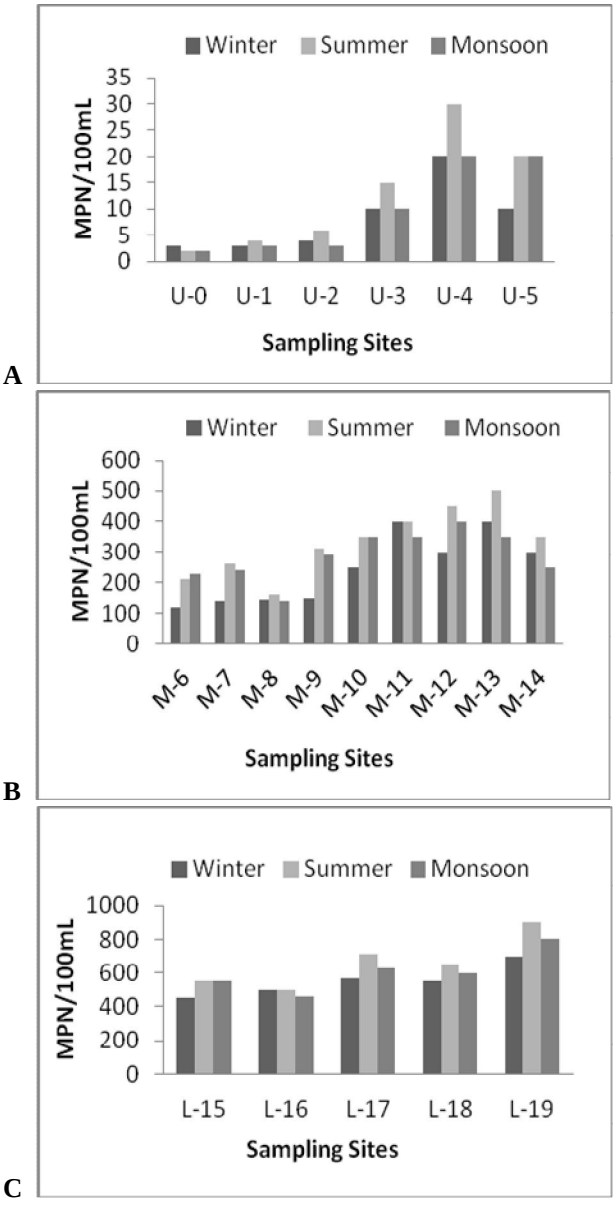

Fig. 4. Fecal coliform count of the various sites of the runoff of Musa ka Musalla glacier: (a) upper stretch; (b) middle stretch; (c) lower stretch.

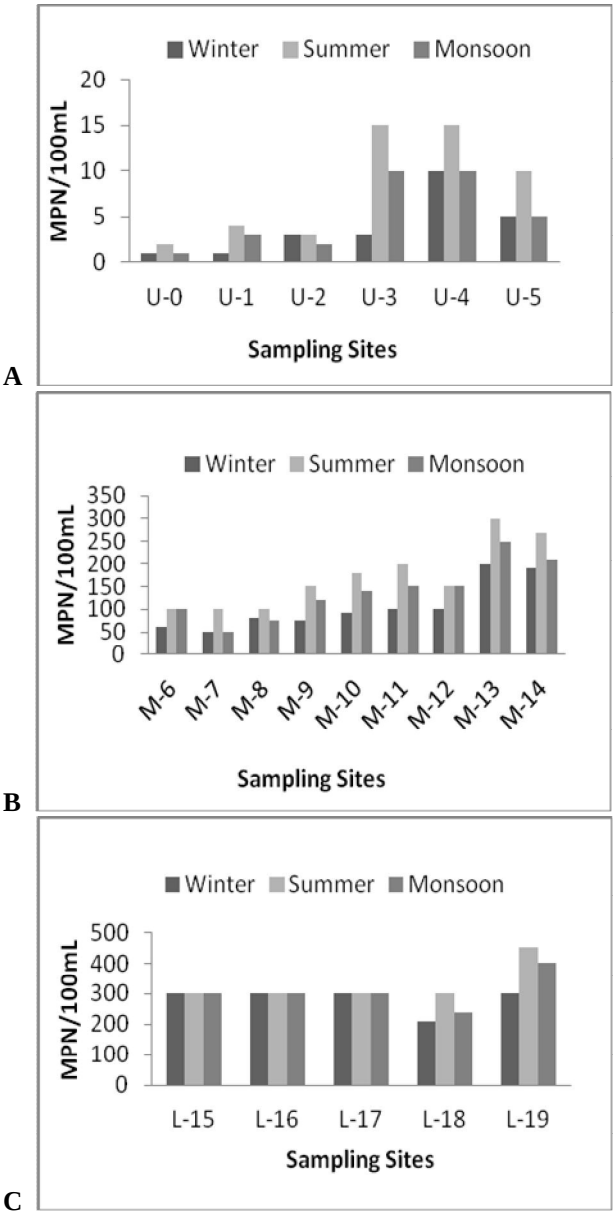

Fig. 5. Fecal *E. coli* count of the various sites of the runoff of Musa ka Musalla glacier: (a) upper stretch; (b) middle stretch; (c) lower stretch.

The bacteriological analysis exposed that all the samples collected from 20 different sites of Musa ka Musalla glacier runoff were contaminated with coliforms, fecal coliform and *E. coli*. It was pragmatic that maximum total coliform and thermotolerent coliforms were found during summer followed by monsoon and winter at most of the study sites. This may be because large number of tourist visiting the area in summers or may be due to temperature, as, temperature is directly co-related with the fecal coliform count, which is in accordance with the prior studies (Vinay *et al*. 2005).

As the results indicate population in upper

stretch area not high but *E. coli* count towering, this may be due to the animal's feces runoff from roads and yards to streams and river through storm sewers, this fact is supported by Doyle and Erickson (2006), according to their view, failing home septic systems can allow coliforms in the effluent to flow into the water table, aquifers, drainage ditches and nearby waters. Sewage connections that are connected to storm water drainage pipes can also allow human sewage into surface waters and during high rainfall periods flow to a nearby stream or river.

The above results showed that the bacterial contamination increases as we move from upper stretch to lower stretch. The anthropogenic and socio-cultural activities may also play their significant role at different middle and lower stretch sites. Rapid development of town ships of the Middle and lower stretch may also have added strains in the runoff and to an extent resulted in the dilapidation of its water quality probably the sites which have more fecal coliform, may have contamination by human fecal matter which relates to the finding of Walsh *et al*. (2011) and Kumarasamya *et al*. (2010).

Molecular detection of blaNDM1 gene

Out of 20 samples analyzed for detection of *bla*NDM1 gene 11 positive for harboring resistant strains, 7 for producing metallo-beta-lactamase and three for producing NDM1 were isolated. The three *bla*NDM1 carrying Gram negative bacteria were from Dadar (U-4), Shinkiari (M-10) and Khaki (M-13) sites of River water (Table I).

The isolated resistant strains were screened for metallo-beta-lactamase (MBL) production. Formaton of an inhibition zone shows the presence of MBL producers which show complete resistance to simple meropenem. All MBL producing strains were identified by the same double disc method as shown in Figure 6.

All the samples from Dadar (U-4), Shinkiari (M-10) and Khaki (M-13) demonstrated 784 bp segments of *bla*NDM-1 gene (Fig. 7).

Antibiotics have also been detected in the aquatic environment through hospital effluent, agriculture runoff and domestics discharge which result in creation of major place for incoming of resistant bacteria (Kummerer, 2009). Resistance to antibiotic is common in most of bacterial strains that contain the chromosomally encoded multidrug efflux pumps, MDR ampC β-lactamase genes and other resistance genes present in different source of water (Agnese *et al.*, 2012).

Table I.- The total samples sites with their names, codes and isolated resistant , metallo-beta-lactamase strains, their location and molecular amplification for the presence of *bla*NDM1 metallo-beta-lactamase gene.

S. No	Codes (Site name)	Resistant Strain	MBL Positive	NDM1 Positive
0	U-0 (Manda Gucha)	No	No	No
1	U-1 (Allai)	No	No	No
2	U-2 (Nawazabad)	No	No	No
3	U-3 (Sacchan)	Yes	No	No
4	U-4 (Jabori)	Yes	yes	No
5	U-5 (Andrasi and Granthalli)	No	No	No
6	M-6 (Dadar)	Yes	Yes	Yes
7	M-7 (Sum)	No	No	No
8	M-8 (Sum combine)	Yes	No	No
9	M-9 (Tanda)	No	No	No
10	M-10 (Shinkiari)	Yes	Yes	Yes
11	M-11 (Baffa)	Yes	Yes	No
12	M-12 (Trangrian)	No	No	No
13	M-13 (Khaki)	Yes	Yes	Yes
14	M-14 (Icher)	Yes	No	No
15	L-15 (Nawashehar)	Yes	Yes	No
16	L-16 (Perhina)	Yes	Yes	No
17	L-17 (Lassanawab)	No	No	No
18	L-18 (Jura)	Yes	No	No
19	L-19 (Soha)	No	No	No

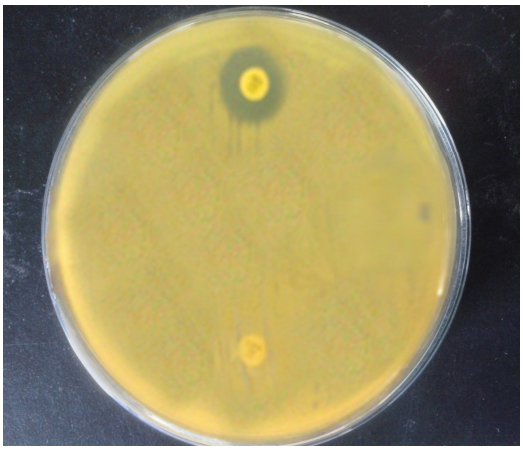

Fig. 6. MBL detection assay. Image indicates combined disk method with EDTA with zone which confirms MBL production.

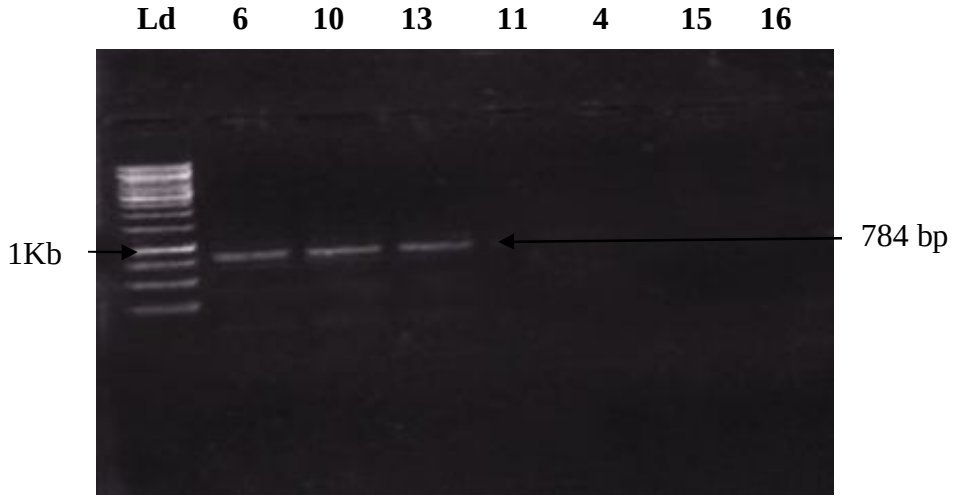

Fig. 7. Resolving pattern of PCR amplified NDM1 gene on 1% Agarose gel. The gel image showing (from left to right): Ladder (Ld), Dadar (6), Shinkiari (10), Khaki (13, Baffa (11), Jabori (4), Nawashehar (15) and Perhina (16).

Since after earlier cases of NDM-1 (New Delhi metallo-beta-lactamase) identification from India and Pakistan (Yong *et al*., 2009; Castanheira *et al.,* 2011), so many researches have been done on NDM-1 all over the world (Perry *et al.,* 2011; Pfeifer *et al*., 2011; Mazzariol *et al*., 2012). In our study, we reported 07 MBL producing Gram negative bacteria out of which Only 03 isolates were *bla*NDM-1 positive by PCR, including Dadar (U-4), Shinkiari (M-10) and Khaki (M-13) which relates to the finding of Walsh *et al*. (2011).

The NDM-1 strains are mostly reported in Enterobacteriaceae, the presence of this gene made the strains to resist almost all types of carbapenem and β-lactam antibiotics which are often used against Gram-negative bacterial infections. In our investigation, all of the *bla*NDM-1 positive strains showed complete resistance against imipenem, Cefotaxime, cefotaxidine, Aztreonam, but they were sensitive against Polymyxin B and Colistin, similar results were obtain by Kumarasamy *et al*. (2010).

CONCLUSIONS

Our study on microbial environmentalism in the runoff of the Musa ka Musalla glacier have clearly exposed that there is momentous presence of bacterial indicators of fecal contamination and *bla*NDM-1 carrying Gram negative bacteria which indicates that the situation of glacier runoff is not very serious but alarming. The presence of fecal indicator organisms provides warning of water-borne problems which is a direct threat to human and animal health. For this reason, monitoring of microbial contamination in the runoff of Musa ka Musalla glacier should be a very important section of the safeguard approach in glacier area. The base line data generated in this study may give us biomonitoring standard and comparisons for other glaciers which give rise to other rivers of Pakistan and may be useful for all researchers, decision makers and resource managers working with environmental planning and management of such areas.

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