Identification of *Klebsiella* species isolated from Modimola dam (Mafikeng) North West Province – South Africa

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Life on earth depends on water. However, there is scarcity of portable water supplies in South Africa especially in rural areas. This results to the consumption of untreated water that may be contaminated with microbial species such as *Klebsiella*. The aim of the study was to investigate *Klebsiella* contamination levels in water from the Modimola dam situated adjacent the Modimola village, in the North West Province, Mafikeng – South Africa. Nine water samples (three each) were collected from the upstream, middle-stream and down-stream of the dam. The samples were analyzed by plating on mFC and Eosin methylene blue agar plates. The blue isolates on mFC were counted and results recorded. The identities of metallic sheen colonies on EMBA were determined using Gram staining, preliminary (TSI and oxidase test) and confirmatory (API 20E, gapA specific PCR) tests. A total of 504 presumptive isolates were screened and 28 (35.9%) *Klebsiella* species were positively identified. Despite the fact that faecal coliform bacteria were not detected in tap water, the level of faecal contamination was higher in samples obtained from the upstream than the middle stream and; than the downstream. All water samples collected from the different sites of the Setumo dam contained *Klebsiella* species and this indicates the risk that water from this dam can pose threat to the inhabitants of the Modimola community who use the water for fishing and some daily activities.

**Key words:** *Klebsiella*, Setumo dam, TSI, API 20E, gapA specific polymerase chain reaction (PCR).

**INTRODUCTION**

Bacteria that belong to the genus *Klebsiella* are facultative, anaerobic, non motile, Gram-negative rods that possess a prominent polysaccharide capsule (Umeh and Berkowitz, 2009). *Klebsiella* species exist as normal flora in the gastrointestinal tract of animals and humans (Podschun et al., 2001). Despite this, *Klebsiella* species can cause severe infections that include meningitis, bronchitis, bacteremia, pneumonia, urinary tract infections in humans and animals (Lai et al., 2000; Boye and Hansen, 2003; Jonas et al., 2004; Trautmann et al., 2004; Casolari et al., 2005; Maroncle et al., 2006; Lau et al., 2007). In humans these infections are common in patients who are admitted in hospitals and those who are immunocompromised.

Domestic animals such as cattle and horses are principal hosts for *Klebsiella* species (Podschun et al., 2001). Improper farm management techniques and/or improper hygiene may facilitate contamination of water sources with *Klebsiella* species (Podschun et al., 2001). Thus most infections caused by *Klebsiella* species result from consumption of contaminated food such as rotten fish and/or water (Podschun et al., 2001; Kanki et al., 2002; Haryani et al., 2007). *Klebsiella* species have been found to cause infections in babies through the consumption of powdered infant formula that was contaminated with pathogenic strains (Liu et al., 2008). Improper deposition of human faeces can lead to contamination of the soil with *Klebsiella* species hence *Klebsiella pneumonia* has been isolated from vegetables such as dried bush okra (*Corchorus olitorius*) and African spider herb (*Cleome gynandra*) (Mpuchane and Gashe, 1996).

Virulence in *Klebsiella* species results from the presence of capsular polysaccharides (CPS),
lipopolysaccharides, adhesions (Lai et al., 2000; Cartés et al., 2002). Moreover, the acquisition of iron exchange systems may result to severe clinical signs on infected individuals (Lai et al., 2000; Cartés et al., 2002). Klebsiella express two types of cell surface antigens viz; the O and K antigens that contribute to pathogenicity in these species (Umeh and Bertowitz, 2009).

Treatment of infections caused by Klebsiella species is usually achieved by administering antibiotics. However, treatment options become limited when infections are caused by Klebsiella species that are resistant to multiple antibiotics and this presents a severe challenge in immunocompromised individuals (Gupta et al., 2003; Seid and Asrat, 2005; Schelenz et al., 2007; Umeh and Bertowitz, 2009). Based on the aforementioned, there is a need to frequently assess the levels of contamination with Klebsiella species in water. This study was carried out to measure the level of contamination of Klebsiella species in water samples collected from Setumo dam adjacent Modimola Village. This dam is located near the sludge treatment plant and water from this plant is deposited into the dam. Animals in the nearby village drink water from the dam and also contribute in contaminating the water with pathogenic microorganisms. Individuals who live nearby this dam lack proper water facilities and they use water from the dam for household activities. Moreover, some individuals fish in the dam and these are consumed. This explains the need to determine the level of bacterial contamination in the water as this may have serious health implications on humans.

MATERIALS AND METHODS

Sampling site

Samples that were tested were collected from Modimola dam, in the Modimola Village. Modimola Village is about 30 km from the North-west University, Mafikeng campus. It is situated adjacent to Unit 14, a residential area in Mmabatho, North West Province, South Africa. Modimola dam is located near the Mmabatho sewage treatment plant that effluent is discharged into. Due to the fact that the vegetation next to the dam is most often green, livestock graze near the dam and as such also drink water from this dam. The water is also used for other human activities such as swimming and laundry. This increases the level of pollution.

Sample collection

Nine (triplicates) water samples (three from each of the three different sites of the Modimola dam) were collected into sterile one liter Duran Schout bottles. These sites were assigned as; up-stream which is the inlet where water that has undergone secondary treatment and chlorination from the treatment plant is discharged. The next site was the middle-stream which is about 2 km from the inlet and finally the down-stream which is about 1.5 km from the middle-stream. Samples were collected while avoiding contamination and collection were done only in spring. The water samples were labeled properly, placed on ice to inhibit the growth of microorganisms and transported immediately to the Microbiology research laboratory for analysis. Tap water was used as a control during analysis.

Analysis of water samples using conventional microbiological techniques

Determination of bacteria load in water samples

Aliquots of 50 ml of each water sample was filtered through a 0.45 µm Grid filter-unit (Type HA) using a Gelman Little Giant Pressure/vacuum pump machine (model 13156-Gelman Sciences, Michigan-USA). The filters were placed on m-FC agar plates using sterile forceps and plates were incubated aerobically at 44°C for 24 h. After incubation, blue colonies were observed on m-FC agar indicating faecal coliforms. A colony counter was used to count these isolates and the results were recorded, as cfu/50 ml of water.

Selective isolation of Klebsiella species

Ten-fold serial dilutions were prepared from each water sample using 2% peptone water and aliquots of 100 µl were spread plated unto (EMBA). The plates were incubated at 37°C for 24 h. Presumptive colonies were sub-cultured on EMBA and the plates were incubated at 37°C for 24 h. Pure isolates were Gram stained.

Gram staining

Presumptive isolates were Gram stained using standard methods (Cruikshank et al., 1975). Gram negative rods were retained for identification using biochemical preliminary tests.

Preliminary biochemical tests

Oxidase test: The reagents used in performing the Oxidase test were (Pro-Lab Diagnostics-United Kingdom) products. In carrying out this procedure, a sterile wire loop was used to transfer a colony unto a filter paper (Whatman International Ltd, Maidstone, England) and a drop of oxidase reagent was added to it. A sterile wire loop was used to mix the reagent and the bacteria cells. Results were read within 30 s and the formation of a purple colour was recorded as a positive test while no colour reported as oxidase negative. Klebsiella spp. are oxidase negative, thus all isolates that were oxidase negative were retained for confirmatory biochemical tests. Triple sugar iron agar test (TSI): Triple sugar iron (TSI) agar obtained from Biolab, Merck, S.A. was used to determine the ability of presumptive Klebsiella isolates to utilize the three sugars; glucose, sucrose and lactose. In conducting this test, 63 g of TSI agar was dissolved into 1 L of sterile distilled water and 5 ml was dispensed into McCartney bottles. The bottles were autoclaved. The media were allowed to solidify while the bottles were placed in slanting positions so as to create a slant and butt. The media was stab inoculated at the butt and streaked on the slant. Bottles were loosely closed and incubated at 37°C for 24 h. The results were read and recorded based on colour change from red to yellow, gas production and H2S production as determined by Forbes and Weisfeld (1998).

Confirmatory biochemical test

Analytical profile index 20E (API 20E)

API 20E is a standard test kit that is designed for the identification of bacteria that belong to the family Enterobacteriaceae. The test was carried out according to the instructions of the manufacturer (BioMérieux, France). The results were read after incubation based on color changes with or without the addition of reagents.
Table 1. Total number of bacterial count for water samples obtained at different points in the dam.

| Bacterial count (fecal coliforms)/50 ml | Upstream (cfu/50 ml) | Middle stream (cfu/50 ml) | Downstream (cfu/50 ml) | Tap water (control) (cfu/50 ml) |
|----------------------------------------|----------------------|---------------------------|------------------------|---------------------------------|
|                                        | (US1) 720            | (MS1) 83                  | (DS1) 51               | (TW1) 0                         |
|                                        | (US2) 921            | (MS2) 103                 | (DS2) 43               | (TW1) 0                         |
|                                        | (US3) 1230           | (MS3) 68                  | (DS3) 18               | (TW1) 0                         |

US=Upstream, MS=Middle stream, DS=Downstream, TW=Tap water.

Confirmatory identification of Klebsiella isolate(s) using polymerase chain reaction (PCR)

Nutrient broth was prepared and pure isolates from nutrient agar were inoculated into it. These were incubated overnight culture in a shaking incubator at 37°C at a rotation speed of 120 rpm to enhance bacterial growth. PCR was then used to further confirm the identities of all the presumptive Klebsiella isolates.

Extraction of genomic DNA

DNA genomes were extracted from the bacterial isolates using a DNA extraction kit (Catalog# 732-6030-Biolab, South Africa) as directed by the manufacturer and the supernaths were stored in a freezer at -20°C for PCR analysis. DNA was of good quality with no fragmentation.

DNA amplification

A total of 78 Klebsiella pneumoniae isolates were screened by specific PCR to confirm their identities through amplification of the gapA gene fragment. The primers are designed to amplify specific sequences on the chromosome that code for the gapA gene that is responsible for synthesizing the protein glyceraldehyde 3-phosphate dehydrogenase. PCR amplifications were performed using a mini cycler (CG1-96 Corbett Research, Australia). The reactions were prepared in 25 µl volumes which were made up of 10 µg/µl of the template DNA, 50 pmol of each oligonucleotide primer set (F: GGTTCATTCAGGACGTTGGAATGACTTACCTCA CG; R: TTGAGGCGGATTAACACTTGTCCAGAACGCGGT TGGT), 1X master mix, and RNase free distilled water. All PCR reagents were obtained from Fermentas, USA but supplied by the Ignaba Biotec Ltd, Sunnyside South Africa. The cycling conditions included an initial DNA denaturation step at 94°C for 3 min; 34 cycles of denaturation step at 94°C for 20 s; primer annealing at 60°C for 30 s and primer elongation at 72°C for 30 s. A final elongation was carried out at 72°C for 5 min and the PCR products were stored at 4°C. The proportion of Klebsiella species obtained at the different positions in the dam was determined and percentages were calculated by dividing the number of isolates confirmed by PCR in a particular site by the total number of isolates screened.

RESULTS

Determination of bacterial load in water samples

The faecal coliform counts are shown in Table 1. The result indicates that water samples obtained from the upstream had a high colony count than those from the middle stream and the downstream. However, no bacteria were isolated from tap water.

Gram staining and biochemical tests

Results in Table 2 indicate that all the isolates were Gram-negative rods that were oxidase positive except for two from the middle stream. The isolates also ferment sugars glucose, lactose and fructose in the TSI medium producing a pattern that is characteristic for Klebsiella species (Forbes and Weissfeld, 1998).

API 20E analysis

The number of K. pneumoniae isolated was higher in the upstream (37) than the middle stream (25) and the downstream (16). The presence of Klebsiella species indicate faecal contamination and the potential of water...
Table 2. Proportion of presumptive *Klebsiella* species identified using the Gram Staining, Triple Sugar Iron (TSI) Test, Oxidase Test and the API 20ETest.

| Location         | Sugar fermentation (+ve) | Gas (+ve) | H₂S (-ve) | Oxidase test | API 20E test |
|------------------|--------------------------|-----------|-----------|--------------|--------------|
| Up-stream        | 168                      | 102       | 160       | 168          | 168          |
| Middle stream    | 168                      | 73        | 153       | 166          | 166          |
| Down stream      | 168                      | 52        | 80        | 168          | 168          |
| Total            | 504                      | 227       | 393       | 502          | 504          |
| Control strain   | -ve rod                  | +ve       | +ve       | -ve          | +ve          |

+ve = Positive for the test; -ve = Negative for the test; GS= Gram staining.

**Figure 1.** GapA gene fragments amplified from *Klebsiella* species obtained from the Modimola dam. Lane 1: 100 bp DNA maker, Lanes 2-7: gapA gene fragments from *Klebsiella* isolates obtained from the up-stream; middle stream and downstream; respectively: Lane 8: *K. pneumoniae* strain ATCC 15611 (Positive control).

Confirmation of *Klebsiella* species using the PCR technique

Amplification of the gapA gene from suspected *Klebsiella* isolates using specific PCR analysis

Specific PCR analysis of the gapA fragments did not produce the expected amplicon sizes (450 bp) in both the control and test strains. The fragments obtained were 700 bp as shown in Figure 1. Based on the PCR results a total of 28 isolates that belong to the genus *Klebsiella* were identified. The proportions of the isolates were higher in the up-stream (15; 53.6%) than the middle stream (8; 28.6%) and the downstream (5; 17.8%).

**DISCUSSION**

It has been stipulated that the bacterial load in potable drinking water must be zero in 100 ml of water sampled (WHO, 2003). The detection of bacteria of faecal origin in the present study indicated that the water was not safe from the dam to transmit these pathogens to humans who use the water.
and could serve as a potential source for the transmission of these pathogens to individuals who use the water. Fish obtained from the water could harbor parasites of some trematode species that are known to cause gastrointestinal diseases in humans when ingested (Abdussalam et al., 1995). These parasites are resistant to heating, freezing and salting (Abdussalam et al., 1995). Human infection with food borne trematodes may result from the ingestion of raw or inadequately processed food such as contaminated fish. Prevention of infections in humans in the Modimola Village may be achieved through environmental control of the water in the dam where fishes are caught and control of the first intermediate host (snails). Moreover, it is suggested that individuals of the Modimola Village be on the importance to properly cook fish from the dam before eating.

A further objective of the study was to confirm the identities of Klebsiella species using primary and PCR analysis. Several studies have been conducted to evaluate the efficiency of methods in identifying Klebsiella species (Lai et al., 2000; Haryani et al., 2007; Lau et al., 2007). PCR analysis has been reported to be more accurate in the identification of Klebsiella species when compared to other preliminary identification tests (Diancourt et al., 2005). A similar observation was obtained in the present study in which case some isolates that were preliminarily identified as Klebsiella species were not picked up in an optimized gapA specific PCR analysis. In the present study a 700 bp partial nucleotide sequence of Klebsiella genome was amplified as opposed to the expected 450 bp. A similar observation has been reported with the same primer sequence (Kong et al., 2002). The gapA gene sequences code for glyceraldehyde-3-phosphate dehydrogenase enzyme in Klebsiella species (Brisse et al., 2009). This enzyme plays an important role in glycolysis and gluconeogenesis by reversibly catalysing the oxidation and phosphorylation of G3P to the energy-rich intermediate 1,3BPG. NAD⁺ is a co-substrate for this reaction. GAPDH displays diverse non-glycolytic functions as well; hence its role depends on its location within the cell. Translocation of GAPDH to the nucleus acts as a signalling mechanism for programmed cell death, or apoptosis. The accumulation of GAPDH within the nucleus is involved in the induction of apoptosis. This explains the need to control the synthesis of this enzyme.

Conclusion

All water samples collected from the different sites of the Modimola dam contained Klebsiella isolates when analyzed. Klebsiella being a normal inhabitant of the intestinal tract of human and animals has acquired plasmids that carry virulence genes and increases their pathogenicity. Water from the dam showed high levels of faecal bacteria contaminant that indicate a potential threat to human life as stipulated by WHO. It is therefore suggested that humans in the Modimola Village should limit contact with water from the dam.

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