Contrasting occurrence of *Chromobacterium violaceum* in tropical drinking water springs of Uganda

Denis Byamukama, Andreas H. Farnleitner, Frank Kansiime, Mohamad Manafi, Martina Burtscher and Robert L. Mach

ABSTRACT

Occurrence of *Chromobacterium violaceum* in six protected drinking water springs in Uganda was investigated. *C. violaceum* showed a contrasting occurrence, which was independent of human impact as assessed by faecal pollution indicators. It was isolated from two springs (S1 and S2) that were located close to each other (3 km) but not in the rest. In S1 *C. violaceum* was continuously detected, in concentrations ranging from 6 to 270 cfu 100 ml^{-1}, while in S2 it was detected on only one sampling occasion. *C. violaceum* was never detected in the investigated upper soil layers (down to 15 cm) in the immediate surroundings (50 m radius) of the springs, despite continued isolation of faecal indicators. The results of the study indicate that *C. violaceum* may not be ubiquitous in spring water, but could occur in significant numbers in particular potable groundwaters as an autochthonous member.

Key words | *Chromobacterium violaceum*, faecal indicators, protected springs, tropical waters, water quality

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INTRODUCTION

On a worldwide scale, *Chromobacterium violaceum* is considered a rarely occurring bacterium, but is thought to be commonly found in tropical and subtropical waters and soils (Hazen 1988; Richard 1993; Midani & Rathore 1998; Lee *et al.* 1999). It is of potential public health concern given its high virulence with a reported mortality rate as high as 65–80% (Macher *et al.* 1982; Ponte & Jenkins 1992; Midani & Rathore 1998; Bilton & Johnson 2000), depending on the kind of symptoms presented by the infection, and how fast it is diagnosed and antibiotic therapy is started (Moss & Ryall 1981; Kaufman *et al.* 1986; Ti *et al.* 1993). Reported *C. violaceum* infection symptoms mainly include sepsis and abscesses in multiple organs such as the liver, skin, lungs, lymph nodes and the brain (Macher *et al.* 1982; Richard 1993; Midani & Rathore 1998; Chou *et al.* 2000, Perera *et al.* 2003, Chen *et al.* 2003).

Most *C. violaceum* strains produce a pigment violacein, which gives colonies on agar plates a distinctive dark purple to black colour (Chong & Lam 1997), though non-pigmented strains have also been isolated (Sivendra *et al.* 1975; Richard 1993). *C. violaceum* may cause infections mainly through the oral route and open skin (Kaufman *et al.* 1986; Ponte & Jenkins 1992; Chong & Lam 1997; Lee *et al.* 1999). In addition to the reported human infections, fatal animal cases have also been reported (Liu *et al.* 1989; Dyer *et al.* 2000). Death from *C. violaceum* has been observed to occur between 7 days to 15 months from the time of infection (Macher *et al.* 1982; Perera *et al.* 2003). With the recent completion of the *C. violaceum* genome sequence (Brazilian National Genome Project Consortium 2003), more information that could be vital in investigating and understanding this pathogen is now available.

Despite the potential public health implications, and the wide information available in the medical literature, little is

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known about the environmental occurrence, behaviour and factors affecting *C. violaceum* distribution. *C. violaceum*, a Gram-negative motile saprophytic bacterium, has been mainly isolated from soil and water in tropical and subtropical regions (Koburger & May 1982; Richard 1993). Some researchers have reported that *C. violaceum* is found between latitudes 35°N and 35°S (Macher *et al.* 1982; Moore *et al.* 2001; Dromigny *et al.* 2002). In these regions, it is thought to be a member of the natural flora in waters and soils. However, detailed studies on its distribution, taking into account differences in physical, chemical and biological environments of habitats from where infections have been reported are sparse and hence need for more investigations.

In an investigation on the applicability of the indicator bacteria *Escherichia coli* and total coliforms for water quality monitoring in Uganda, colonies that resembled those of *E. coli*, but not exactly fitting the description of the manufacturer were observed on some chromocult coliform agar plates. On API 20E characterization, these dark purple-black colonies were identified as *C. violaceum*, whose description was that of a highly virulent pathogen commonly found in tropical and subtropical environments. At times, these colonies outnumbered those fitting the description of typical *E. coli*. In the context of this observation and description, we carried out this study in which six protected drinking water springs were investigated for the spatial and temporal occurrence of *C. violaceum* in the water, and the upper (15 cm) layer of the soil in the immediate surroundings (50 m radius) of the spring outlet.

The springs were selected on the basis of differing anthropogenic influence as well as environmental habitat characteristics. Protected springs in Uganda are used as sources of untreated potable water supply, mainly for rural and peri-urban human settlements that are not served by the treated water network. The protection of springs generally includes a concrete retention wall, a spring box for trapping the water and pipe(s) outlets. The spring box is then filled with gravel and covered with soil; grass is also often planted on the backfill as part of catchment protection. Though the springs are considered protected for potable water, at times some of them are poorly maintained and liable to contamination, and knowledge about their microbial water quality is thus very important.

There are many rampant and more widely occurring waterborne pathogens, especially in the tropics, that could be considered to be of much greater concern, but a number of factors, including the high virulence, make further investigation into *C. violaceum* presence in untreated potable water a necessity. Unlike most of the countries where a number of infections have been reported, most of them resulting in fatalities, such as the United States, Australia and Singapore (Macher *et al.* 1982; Chong & Lam 1997; Chen *et al.* 2003), Uganda is a poor developing country with a poor medical care system, where such infections might not be properly diagnosed and the required treatment administered. The fewer than 50 cases reported worldwide (Chong & Lam 1997) have mostly been from such countries with an advanced medical infrastructure, suggesting that the cases that might have occurred in the developing countries, which coincidentally form a bigger part of the tropics and subtropics, could have gone either undiagnosed or unreported. It is also important to note that the number of immunocompromised individuals in developing countries such as Uganda is much higher because of ailments ranging from opportunistic diseases, inadequate housing and malnutrition (Hazen 1988) especially in children. *C. violaceum* has been reported to have more severe effects on people in a state of malnourishment, immune deficiency and who are immunocompromised (Macher *et al.* 1982; Bilton & Johnson 2000; Moore *et al.* 2001). Additionally, the poor access to sanitation and safe water service in some low-income areas in both rural and urban Uganda has been linked to waterborne disease outbreaks and is a cause of concern (Legros *et al.* 2000; Tumwine *et al.* 2002).

In the prevailing circumstances, effective monitoring of the water sources and the necessary interventions could help reduce possible infections from this virulent pathogen. The results of the study suggest that *C. violaceum* may not be ubiquitous in spring water, but could occur in significant numbers in particular potable groundwaters independent of human impact but, most probably, influenced by the habitat type. For enumerating the presumptive target organism, we used a selective chromogenic medium which allowed us to enhance simultaneous enumeration of *C. violaceum* as well as the presumptive identification of total coliforms (TC) and *Escherichia coli* (*E. coli*) while minimizing the growth of background bacteria.

METHODS

Study sites and sampling

Six protected drinking water springs from different parts of Uganda (Figure 1) were investigated over a 12-month period. Two of the springs (S1and S3) had minimum anthropogenic influence. Spring S1 is located about 100 m from the nearest human settlement and has a sheltered and highly vegetated catchment. S3 is located about 50 m from human settlements but is more open; part of the catchment is bare ground. The rest of the springs (S2, S4, S5, S6) are located within urban areas close (within 20 m) to human settlements; S4 is in a shanty town where it is directly affected by wastewater from the nearby houses. One water sample was taken from each of the springs every two months between October 2001 and October 2002. Sampling was done according to *Standard Methods* (APHA 1995) using sterile glass bottles. Samples were transported to the laboratory on ice and analysed within 8 h of taking the first sample.

General water quality characteristics

Electrical conductivity (EC), temperature, pH and dissolved oxygen (DO) were measured *in situ* with WTW meters (Wissenschaftlich Technische Werkstätten GmbH, WTW, Germany), calibrated at 25°C. Total suspended solids (TSS) were determined in the laboratory using the drying method (APHA 1995).

Sample processing and simultaneous enumeration of target organisms

A range of volumes (1 ml, 10 ml, 50 ml and 100 ml) of the water samples was filtered using 0.45 μm pore size and 47 mm diameter cellulose nitrate membrane filters (Sartorius, Vienna, Austria), and thereafter placed on Chromocult Coliform Agar (CCA, Merck Darmstadt, Germany) plates. To minimize possible interference from background bacterial flora, the culture medium was amended with Cefsulodin antibiotic (Sigma, Vienna, Austria) at a final concentration of 5 mg l^{-1}. The plates were incubated at 37 °C for 24 h under dry conditions.

Enumeration of *E. coli* and TC on CCA was undertaken according to the manufacturer's guidelines and previous studies using this medium (Byamukama *et al.* 2000). Pink colonies resulting from the cleavage of the chromogenic substrate, Salmon Galactoside by β-D-galactosidase were scored as presumptive coliforms other than *E. coli*. The deep blue to violet colonies resulting from the combined breakdown of Salmon Galactoside and X-glucuronide by β-D-galactosidase and β-D-glucuronidase, respectively,

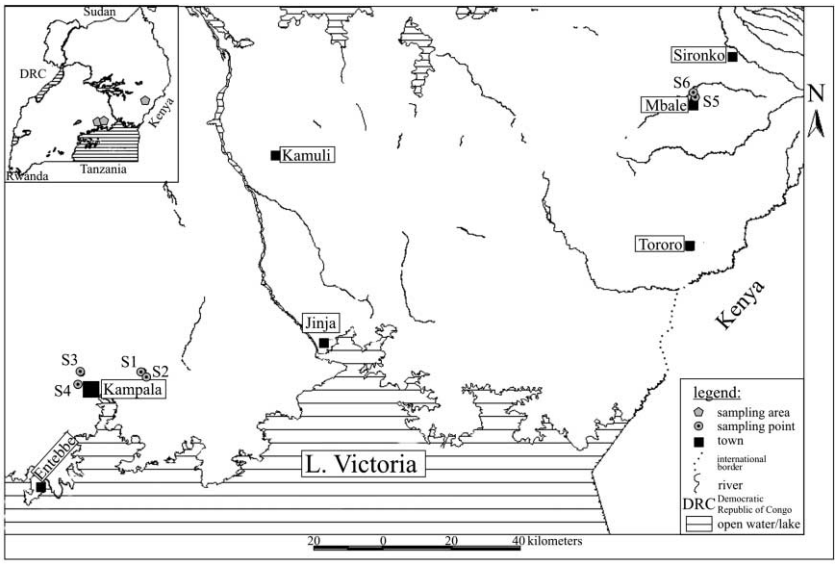

Figure 1 | Map of the study area showing the locations of the sampled protected springs (S1–S6).

were scored as presumptive *E. coli*. Presumptive TC was calculated as a sum of *E. coli* and non *E. coli* coliforms. For presumptive enumeration of *C. violaceum* from the analysed drinking water samples, all colonies showing a dark violet to black pigmentation on CCA plates after the 24 h incubation at 37°C were quantified. In the cases where coliforms appeared in higher levels compared to either *C. violaceum* or *E. coli*, colonies of the respective targeted groups were counted from plates with the appropriate dilutions and finally expressed as colony forming units (cfu) per 100 ml. Possible growth inhibition by excessive numbers of TC on *C. violaceum* and *E. coli* was tested. This was performed by using environmental isolates of *C. violaceum*, TC and *E. coli* in differing ratios of cell abundances of each of the three growing both as single and mixed cultures.

Soil sampling and processing

A soil auger was used to obtain an integrated soil sample core of the top 15 cm soil layer from 10 randomly selected spots within a 50 m radius of the sampled spring. To avoid cross contamination of the soil samples from different sites, for each site, a separate sterile spoon was used to scrape a spoonful (10 cm^3) aliquot from the outer part of the soil core not in contact with the auger. The aliquots from each of the 10 spots were then homogeneously mixed to form a composite soil sample for that respective sampling site. Samples were put in sterile plastic bags (Packaging Industries Limited, PIL, Kampala, Uganda) and transported to the laboratory for analysis under conditions similar to those for the water samples.

To enumerate the respective indicator bacteria in the soil, 10 g of soil were added to 100 ml of distilled sterile water, hand shaken and sonicated for 1 minute in a Bransonic PC-650 (Branson Ultrasonics Corp, Danbury, Connecticut) bath sonicator and allowed to settle for 1 h. The supernatant was collected in sterile 100 ml flasks, then either diluted or whole volumes (10^{-4} ml to 10 ml) were filtered and treated as for the water samples and the respective indicator bacteria. For both water and soil, triplicate incubations were made per volume filtered and for each media; the final colony count for the respective volume was then obtained by taking the average of the three plates.

For further identification and detailed characterization, 30 randomly selected colonies of presumptive *C. violaceum* were purified and analysed throughout the study period. These included 24 from spring S1, where four well spaced colonies were randomly selected on each of the six sampling dates, and six colonies from spring S2 selected on the last sampling day, the only time *C. violaceum* was ever detected in this spring during the whole study period. Pure cultures of the dark violet to black colonies were prepared in a two phase purification step by streaking the representative colonies on CCA, incubating at 37°C for 24 h and transferring them to nutrient agar (NA, Merck) plates, and incubating them at similar conditions to CCA. As a positive control, *C. violaceum* type culture strain DSM 30191 from the German Collection of Microorganisms and Cell Cultures (DSMZ) was used during the investigation.

Characterisation of selected presumptive *C. violaceum* isolates

Biochemical characterization was carried out by inoculating API 20E test strips (Biomerieux Sa, Marcy-I'Etoile, France) with a suspension of pure culture isolates in a sterile 0.85% sodium chloride saline solution. Results were read after a 24 h dry incubation at 37 °C. The APILAB–PLUS version 3.1.1 identification software (Biomerieux) was used to identify the strains based on the obtained reaction profiles. Gram stain tests on pure cultures were carried out according to *Standard Methods* (APHA 1995). Grown cultures were transferred to vials containing 2 ml of 0.85% sodium chloride saline solution and stored in a freezer at −80°C pending DNA isolation. Before DNA isolation, the samples were kept at room temperature for about 30 minutes to allow them to thaw completely. Genomic DNA was isolated using the QIAGEN DNeasy Tissue Kit (Cat. No. 69506) according to the manufacturer's instructions (QIAGEN Laboratories, California). The isolated genomic DNA was stored at −20°C pending further processing and analysis. This DNA was ready to use for PCR applications and no further purification steps were done.

Genetic ribosomal 16S DNA characterization was done using the information from the V3 16S rDNA hypervariable region by eubacterial specific primers PRBA 338f and PRUN

518r (modified with GC rich region for denaturing gradient gel electrophoresis (DGGE) screening) for PCR (Farnleitner *et al.* 2001). PCR amplicons were screened afterwards by means of DGGE for DNA band pattern differences. The DGGE band patterns were analysed by the gelcompar II software (Applied Math, Belgium). Representative bands were subjected to DNA sequencing (Farnleitner *et al.* 2001). DNA sequences were then submitted to BLAST search (http://www.ncbi.nlm.nih.gov) in order to allocate their closest DNA sequence affiliation, thereby allowing rapid identification of the isolates. Culturing of strains, API 20E biochemical characterization and DNA isolation was done at Makerere University Institute of Environment and Natural Resources in Kampala, Uganda, while the molecular analyses were carried out at the Institute of Chemical Engineering, Vienna University of Technology, Vienna, Austria.

Statistical analysis

Non-parametric tests and rank correlation data analysis was performed using SPSS for Windows Version 11.0 (SPSS Inc., Illinois). Kruskal Wallis and Mann-Whitney U rank tests for grouped data were used to detect differences among and between springs while Spearman's rank was used to test for correlation between the parameters. A general 95% confidence level was set.

RESULTS

General water quality

The water quality characteristics of the springs tended to reflect the nature of the selected habitats in terms of differing anthropogenic influence as well as habitat type. Springs located within urban areas close to human settlements (S2, S4, S5, S6) showed significantly increased values ($p < 0.05$) in EC and *E. coli* ranging from 122 to 428 $\mu S\,cm^{-1}$ and 0 to 2.0×10^3 cfu $100\,ml^{-1}$ compared to the less influenced springs (S1 and S3), ranging from 41.3 to 88.5 $\mu S\,cm^{-1}$ and 0 to 1.0×10^2 cfu $100\,ml^{-1}$, respectively (Tables 1 and 2). TC correlated significantly with *E. coli* (Spearman's, $r = 0.75$, $p < 0.05$). In terms of TSS, temperature, pH and DO, no profound differences could be observed throughout the studied springs, except a significantly lower temperature ($p < 0.05$) and a trend of higher TSS for S1 (Table 1). The lower temperature was most likely an effect of the tree canopy and not due to climatic differences.

Occurrence of *C. violaceum* and the indicator bacteria

C. violaceum showed contrasting occurrence in the studied springs. It was isolated in S1 and S2 but not in S3, S4, S5 and S6 (Table 2). In contrast to S2, where *C. violaceum* was detected in only one sample taken at the end of the study period, in spring S1, *C. violaceum* was continuously isolated

Table 1 | Chemophysical parameters measured for the six springs; median (M) and range (R) values of the six sample data sets

	TSS ($mg\,l^{-1}$)		Temp. (°C)		EC ($\mu S\,cm^{-1}$)		pH		DO ($mg\,l^{-1}$)	
Spring	M	R	M	R	M	R	M	R	M	R
S1	5.6	0.6–20	22.7	22.2–23.1	45.5	41.3–48.8	5.4	5.2–5.7	2.3	2.0–2.9
S2	3.3	0.1–7.4	24.5	24.0–28.1	413	356–428	5.1	5.0–5.5	3.6	3.1–4.7
S3	2.0	0.3–3.5	23.9	22.8–26.7	71.3	57.9–88.5	5.7	5.3–6.9	2.4	2.0–2.8
S4	2.2	0.8–5.0	24.7	24.3–25.7	268	245–286	5.7	5.5–6.0	2.7	2.4–3.6
S5	2.5	1.0–17	26.5	26.5–27.3	271	122–273.3	6.1	5.8–6.5	2.4	1.4–2.6
S6	2.3	0.8–17	26.0	24.3–27.2	271	122–286	5.9	5.5–6.5	2.5	1.4–3.6

S1 to S6 = selected spring sites, TSS = total suspended solids ($mg\,l^{-1}$), EC = electrical conductivity ($\mu s\,cm^{-1}$), DO = dissolved oxygen ($mg\,l^{-1}$)

Table 2 | Indicator bacteria in the water of the six springs; median, minimum and maximum values of the six sample data sets

	Bacterial counts (cfu 100 ml^{-1})					
	TC		*E. coli*		*C. violaceum*	
Spring	M	R	M	R	M	R
S1	1.1×10^2	6.0×10^1–4.2×10^2	1.0×10^0	0.0×10^0–2.0×10^0	4.0×10^1	6.0×10^0–2.7×10^2
S2	1.0×10^3	2.6×10^2–5.5×10^3	6.8×10^1	5.0×10^0–1.8×10^2	0.0×10^0	0.0×10^0–8.0×10^0
S3	5.4×10^1	1.4×10^1–8.0×10^2	1.5×10^0	0.0×10^0–1.0×10^2	ND	ND
S4	1.0×10^4	4.5×10^2–2.2×10^5	5.0×10^2	2.0×10^0–2.0×10^3	ND	ND
S5	3.9×10^3	1.8×10^3–3.2×10^6	8.2×10^1	0.0×10^0–3.0×10^2	ND	ND
S6	1.1×10^3	6.1×10^1–3.4×10^4	2.2×10^2	2.7×10^1–3.8×10^2	ND	ND

TC = total coliform; ND = not detectable

during the study period, ranging from 6 to 270 cfu 100 ml^{-1} (Figure 2). TC abundance was detectable in the same range as *C. violaceum*, but *E. coli* showed lower numbers compared to *C. violaceum* and TC ($p < 0.05$). No significant correlation could be observed between *C. violaceum* and TC ($r = 0.26$, $p = 0.62$), and *E. coli* ($r = 0.24$, $p = 0.65$), though there was correlation between the levels of TC and *E. coli* in the water ($r = 0.75$, $p < 0.001$). For the whole study period, *C. violaceum* was not detected in soil at any of the sampled springs despite continuous isolation, and sometimes high levels of TC and *E. coli* (Table 3). No significant differences could be detected between TC ($p = 0.67$) and *E. coli* ($p = 0.27$) levels in soil at the six different spring sites. However, significant correlation was observed between TC and *E. coli* in the soil ($p < 0.05$).

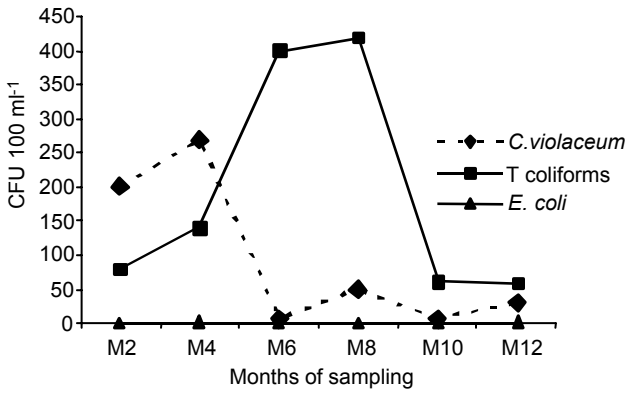

Figure 2 | Presumptive *C. violaceum*, *E. coli* and the total coliforms in spring S1 over the 12 months; values of the six data sets are plotted, representing the six sampling occasions

Characteristics of *C. violaceum*

Using Chromocult Coliform Agar, *C. violaceum* formed violet to black colonies after a 24 h dry incubation period at 37°C. This was a result of pigment production, not by cleaving the chromogenic substrate (e.g. Salmon Gal and X-glucuronide), as proven by the use of NA, where transferred selected strains also formed violet colonies. The reference strain displayed the same characteristics as the environmental strains. All 30 randomly isolated and tested *C. violaceum* strains stained as Gram-negative bacillus rods of varying length and were confirmed as *C. violaceum* by the API 20E technique. To overcome the problem of purple pigmentation which interfered with the oxidase test, anaerobic incubations (Sivendra *et al.* 1975; Kaufman *et al.* 1986) were performed to obtain non-pigmented strains. Strong oxidase reactions were observed in 26 (86.7%) of the colonies. These included 21 (87.5%) of the 24 colonies isolated from spring S1 and five (83.3%) of the six colonies from S2. Four colonies, three from S1 and one from S2 were weakly positive. However, it is important

Table 3 | Indicator bacteria in the soil around the six springs; median, minimum and maximum values of the six sample data sets

	Bacterial counts (cfu g^{-1})				
	TC		*E. coli*		
Spring	M	R	M	R	*C. violaceum*
S1	2.0×10^5	$2.4 \times 10^4 - 6.1 \times 10^7$	2.4×10^2	$0.0 \times 10^0 - 1.1 \times 10^3$	ND
S2	1.6×10^5	$2.4 \times 10^4 - 8.0 \times 10^7$	1.9×10^3	$0.0 \times 10^0 - 1.9 \times 10^6$	ND
S3	1.1×10^5	$1.7 \times 10^4 - 1.0 \times 10^6$	1.6×10^3	$6.1 \times 10^1 - 5.8 \times 10^4$	ND
S4	1.3×10^5	$4.7 \times 10^4 - 3.0 \times 10^7$	3.0×10^3	$5.0 \times 10^2 - 4.2 \times 10^5$	ND
S5	6.5×10^4	$1.2 \times 10^4 - 2.1 \times 10^5$	7.6×10^2	$4.0 \times 10^2 - 2.0 \times 10^3$	ND
S6	4.1×10^5	$3.0 \times 10^4 - 6.4 \times 10^6$	1.1×10^3	$0.0 \times 10^0 - 3.6 \times 10^5$	ND

to note that the oxidase test alone did not change the outcome of the identification for *C. violaceum*. Positive and negative oxidase reactions resulted in identification levels of 78.2 and 90.0%, respectively, for *C. violaceum*.

Despite the differences in oxidase reactions, DGGE profiling on the 30 *C. violaceum* strains resulted in a single, clearly focused band of identical migration behaviour. Figure 3 shows 11 representative bands from the DGGE, indicating the strains, the water source and oxidase reaction characteristics. Seven strains in lanes 1–7 are from spring S1; two of them, lanes 1 and 2, are those with weak oxidase reactions. Four strains in lanes 8–11 were from spring S2; one strain, in lane 11, showed weak oxidase reaction. Four representative strains, with the following water source and oxidase reaction characteristics were selected for the determination of the sequences. One strain each from S1 weak oxidase (lane 1), S1 strong oxidase (lane 3), S2 strong oxidase (lane 9) and S2 weak oxidase (lane 11) were sequenced and were found to have exactly identical partial sequences (Gene bank accession number AY273182). BLAST search resulted in closest alignment to *C. violaceum*.

DISCUSSION

The findings presented herein on the occurrence of *C. violaceum* confirm that *C. violaceum* can also exist in tropical spring water, though apparently not as frequently and widely as some previous reports have observed for other environmental habitats (Sivendra *et al.* 1975; Macher *et al.* 1982; Chou *et al.* 2000). The dominant presence in only one spring and isolation once in the most closely (3 km) located source (Figure 1), for the whole year does not suggest the ubiquitous occurrence of *C. violaceum* in tropical spring waters. Nonetheless, as this study indicates, in particular habitats *C. violaceum* could occur in

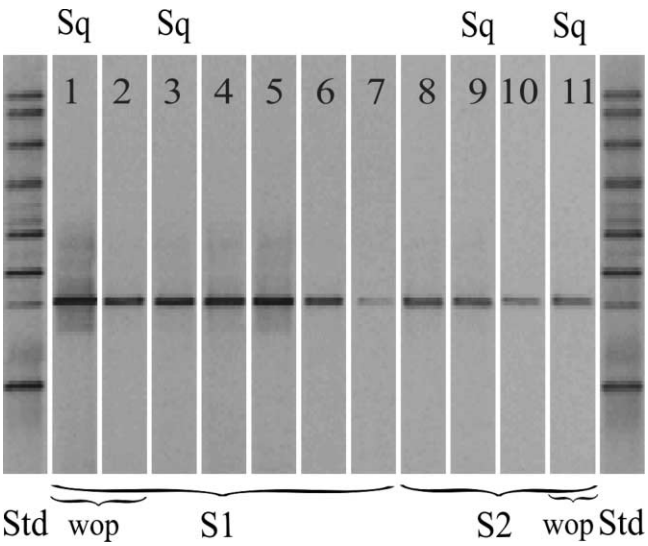

Figure 3 | DGGE profiles after analysis with the Gelcompar software of the 11 selected *C. violaceum* strains representing isolates from the two springs and the various oxidase reactions. Sq = lanes of strains that were sequenced; wop = weak oxidase positive strains; Std = standard DGGE marker.

significant and persistent concentrations, which should be of public health concern. The observed low numbers notwithstanding, the mere isolation of such a virulent pathogen in water used for drinking cannot be ignored. Unlike the diarrhoeal waterborne infections such as cholera that have been a cause of concern in Uganda (Legros *et al.* 2000; Tumwine *et al.* 2002), an infection with symptoms such as sepsis and abscesses in multiple organs (Macher *et al.* 1982; Richard 1993; Midani & Rathore 1998; Chou *et al.* 2000; Perera *et al.* 2003) might not be easily diagnosed or even linked to water.

Most reports on the occurrence of *C. violaceum* based their conclusions on the prevalence of clinical reported cases that occurred in these tropical regions (Chong & Lam 1997; Midani & Rathore 1998; Lee *et al.* 1999; Moore *et al.* 2001; Perera *et al.* 2003; Chen *et al.* 2003); we base our conclusion about the occurrence on environmental investigations. These infections have mainly been associated with either recreational or stagnant muddy water (Macher *et al.* 1982; Martin & Brimacombe 1992; Chong & Lam 1997; Huffam *et al.* 1998; Midani & Rathore 1998; Lee *et al.* 1999); we report about the presence of *C. violaceum* in potable water. To our knowledge, this is the first report about the presence of *C. violaceum* in water designated for drinking.

The lack of a significant correlation of *C. violaceum* with either total coliforms ($r = 0.26$, $p = 0.311$) or *E. coli* ($r = 0.24$, $p = 0.324$) in S1 suggests that its presence is not related to these two pollution indicators of water quality. In contrast, compared to the other springs, especially S4, which had elevated numbers of TC and *E. coli*, S1 was more or less pristine with a recorded maximum *E. coli* concentration of 2 cfu $100\,ml^{-1}$ over the 1-year sampling period (Table 2, Figure 2). The presence of *C. violaceum* could not therefore be attributed to faecal/anthropogenic pollution. In some previous studies, *Chromobacterium* spp. have been isolated as part of the heterotrophic plate count communities (Sarmiento *et al.* 1986). So far, no clear explanation can be given for the occurrence of *C. violaceum* in this particular spring throughout the 1-year study period (Figure 2), and its sporadic appearance in S2 at the end of the monitoring period. Its presence in S1, however, might be related to the higher TSS and wider root network (rhizosphere) of the forested catchment compared to the other investigated springs (Table 1).

In the possible event that *C. violaceum* isolated from spring water was autochthonous to the inner soil layers, soil particles and suspended solids could have carried the cells into the spring water (Moss & Ryall 1981). But, it is important to note that the soil investigations were conducted in the upper 15 cm of the topsoil; presence in the much deeper layers in contact with the spring water was not investigated. Because *C. violaceum* is a facultative anaerobe (Sivendra *et al.* 1975; Kaufman *et al.* 1986), it could inhabit deeper soil layers, including those in contact with the water, and occasionally be released into the water column. Another important factor is that the catchment of the spring spreads beyond the 50 m radius in which soil sampling was done, so the distribution outside this 50 m area and any subsequent effect on the water in the spring is not known. Hussain & Vancura (1970) have reported isolating *C. violaceum* in rhizosphere soils while Dudchenko *et al.* (1973) recorded higher numbers of chromobacteria in the rhizosphere of some crop plants than in that of perennial plants.

C. violaceum requires special media for isolation (Chong & Lam 1997), and some form of enrichment to enhance chromobacteria growth in the presence of large numbers of other bacteria might be needed (Moss & Ryall 1981). However, CCA as an isolation medium can be assumed to be favourable for *C. violaceum* growth because of its formulation. It contains tryptophan which is an important growth requirement for *C. violaceum*, and part of the violacein biosynthetic pathway (Davis *et al.* 1975; Rivero *et al.* 1989; August *et al.* 2000; Moore *et al.* 2001), as well as Cefsulodin, a selective β-lactam antibiotic, a group to which *C. violaceum* is resistant (Kaufman *et al.* 1986; Martin & Brimacombe 1992; Moore *et al.* 2001). With the media selectivity in favour of *C. violaceum*, and the incubation temperature of 37°C used, which is in the favourable range for *C. violaceum* (Midani & Rathore 1998), it is our view that optimum growth conditions were provided during the study.

When tests for possible growth inhibition by excessive numbers of TC on *C. violaceum* and *E. coli* were done, at least up to a ratio of $1{:}10^4$ either *C. violaceum* or *E. coli* to total coliforms, no inhibition effect could be detected. Because this was less than the anticipated maximum ratio of $1{:}10^3$ (derived from Table 2, assuming a minimum of 1

C. violaceum cfu per 100 ml and the median TC values), inhibition of *C. violaceum* during the investigation was unlikely. The negative results of *C. violaceum* in the other investigated springs, and in the soil at all the spring sites thus show that *C. violaceum* was not inhibited by either the culture media or growth conditions but was instead not present in these habitats, at least not in a viable and culturable state. But the finding of exactly the same strain sequences in two different springs seems to suggest that the *C. violaceum* strains found in the studied environment are phylogenetically closely related. However, this observation is based on information from the V3 16S rDNA hypervariable region which is especially useful for differentiation of taxonomic units up to species level but not for subspecies level typing (Farnleitner *et al.* 2004). With the recently completed *C. violaceum* genome (Brazilian National Genome Project Consortium 2003), other segments could be investigated for typing procedures in the future.

CONCLUSION

The outcome of this investigation points to the fact that *C. violaceum* is likely to occur in significant concentrations in particular groundwaters, which is of potential health relevance. The level of vulnerability may be high as the two main ports of entry for these microorganisms, the oral route and the skin, are exposed in this situation. These findings also show that, for public health protection, reliance on the faecal indicators alone is not enough; pathogens can exist in untreated water sources that seem to be acceptable by indicator quality analysis. However, the six springs and a 1-year monitoring programme cannot be considered exhaustive and representative of the status of all tropical springs. Further microbiological and epidemiological studies are warranted to clarify its environmental behaviour, distribution and the potential public health implications.

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