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Full Length Research Paper

Prevalence of extended-spectrum *b*-Lactamases producing *Aeromonas hydrophila* isolated from stool samples collected in the Limpopo province, South Africa

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Aeromonas producing extended-spectrum ß- lactamases (ESBLs) have been reported in many countries, but there is no information on the prevalence of ESBL-producing clinical Aeromonas in South African. A total of 230 isolates of *Aeromonas hydrophila* were isolated from 660 stool samples and 709 water samples collected in different municipalities in Limpopo province, South Africa over a period of three month. Isolates were screened for the production of ESBLs by the double disk diffusion test and for AmpC production by assessing resistance to cefoxitin. *bla*$_{SHV}$, *bla*$_{TEM}$, *bla*$_{CTX-M}$ and *bla*$_{CMY-2}$ were isolated from all ESBL-positive and cefoxitin-resistant isolates. Only 21 isolates were found to be ESBL producers. All 21 isolates were screened for the production of *bla*$_{SHV}$, *bla*$_{TEM}$, *bla*$_{CTX-M}$ and *bla*$_{CMY-2}$ ESBLs. Only (1/21) was found to produce *bla*$_{TEM\ 63}$ and *bla*$_{CMY-2}$. All transconjugants were resistant to amoxicillin, piperacillin, the cephalosporins and aztreonam but remained susceptible to cefoxitin and imipenem. Crude extracts of ß-lactamase-producing transconjugants were able to reduce the diameters of inhibition zones around disks containing penicillin, but had no effect on such zones around cefoxitin, imipenem and amoxicillin-clavulanate disks. In conclusion, the occurrence of ESBLs in *A .hydrophila* emphasizes the importance of constant surveillance of clinical isolates to determine the prevalence of antibiotic resistance genes.

Key words: Extended-spectrum b-lactamases- Aeromonas hydrophilia , antibiotics , resistance.

INTRODUCTION

Many extended-spectrum ß -lactamases (ESBLs) are plasmid-mediated derivatives from TEM and SHV-type enzymes can cause resistance to expanded-spectrum cephalosporins. The use of broad-spectrum penicillins and cephalosporins has been associated with the emergence of extended-spectrum ß-lactamases (ESBLs) in Enterobacteriaceae (Hanson et al., 1998; Ndugulile et al., 2005). These have been described worldwide and they are the major cause of nosocomial infections associated with high mortality. ESBLs have been described in Africa (Kruger et al., 2004; Essack et al., 2001; Hanson et al., 1998; Ndugulile et al., 2005; Kariuki et al., 2001) and have considerable implications for the developing world, where there is limited access to more effective antibacterial agents. In recent years there has been an increase in the number of multidrug-resistant gram-negative organisms and a problem of reduced susceptibility to commonly used antibiotics amongst enteric diseases (Marchandin et al., 2003). Among the clinical populations of gram -negative microorganisms, the *bla*$_{TEM-1}$ gene is the most frequently detected plasmid-borne antimicrobial resistant gene. Among the Enterobacteriaceae, the TEM and SHV-derived ESBLs are often the predominant enzyme types among Enterobacteriae with an ESBL phenotype. There are more than 67 TEM derived and 12 SHV-derived ESBLs described to date and an increasing number of ESBL-

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Table 1. PCR products after amplification with different primers.

Isolate	*BlaSHV*gene	*BlaCMY2* gene	*BlaCTX-M*gene	*bla*TEM gene
Sesbl 01	-ve	+ ve	-ve	-ve
Sesbl 02	-ve	-ve	-ve	+ve
Sesbl 03	-ve	-ve	-ve	-ve
Sesbl 04	-ve	-ve	-ve	-ve
Sesbl 05	-ve	-ve	-ve	-ve
Sesbl 06	-ve	-ve	-ve	-ve
Sesbl 07	-ve	-ve	-ve	+ve
Sesbl 08	-ve	-ve	-ve	-ve
Sesbl 09	-ve	-ve	-ve	-ve
Sesbl 10	-ve	-ve	-ve	-ve
Sesbl 11	-ve	-ve	-ve	-ve
Sesbl 12	-ve	-ve	-ve	-ve
Sesbl 13	-ve	-ve	-ve	-ve
Sesbl 14	-ve	+ve	-ve	+ve
Sesbl 15	-ve	-ve	-ve	-ve
Sesbl 16	-ve	+ve	-ve	+ve
Sesbl 17	-ve	-ve	-ve	+ve
Sesbl 18	-ve	-ve	-ve	-ve
Sesbl 19	-ve	-ve	-ve	-ve
Sesbl 20	-ve	-ve	-ve	-ve
Sesbl 21	-ve	-ve	-ve	-ve

-ve: Negative results, +ve: positive results, Sesbl: Stool isolates producing extended-spectrum -Lactamases.

producing bacteria have been reported (Fosse et al., 2004). Although reports of ESBLs associated with *Aeromonas hydrophila* are relatively rare compared to those of the family Enterobacteriaceae, the number of reported cases is steadily increasing in recent years (Marchandin et al., 2003). Aeromonas spp have been found to express a wide variety of ESBL types, including TEM and SHV (Fosse et al., 2004, Marchandin et al., 2003). The infection of ESBLs and AmpC b-lactamases in *A. hydrophila* species is of paramount importance in developing countries where infections with these organisms are numerous. Resistance to ampicillin, aztreonam, cefixime, cefepime, cefoxitin, cefotaxime, cefpodoxime, ceftazidime, ceftriaxone, amoxicillin-clavulanic acid and chloramphenicol is rapidly increasing, necessitating the use of fluoroquinolones or extended-spectrum cephalosporins as treatment of choice for extraintestinal infections (Crump et al., 2003). Widespread fluoroquinolone use in children has been discouraged because of the potential adverse effects on cartilage development. Therefore, extended-spectrum cephalosporins such as cefotaxime or ceftriaxone are becoming the option of treatment of enteric bacterial infections in children. ESBLs production has considerable implications in medical health practitioners in this era of HIV/AIDS with Aeromonas species gradually becoming ESBLs producers. ESBLs have been found in many Enterobacterciae including enteric bacteria such as *salmonella* in South Africa (Belle et al., 2002; Hansen et al., 2001).

The aim of this study was to determine the prevalence of ESBLs in 21 *A. hydrophila* isolates from stool samples of HIV/AIDS individuals in Limpopo province of South Africa using competitive synergistic disc diffusion and PNA-based multiples polymerase chain reaction (PCR). Deoxyribonucleic acid sequencing was used to confirm the identity of $bla_{\text{TEM-63}}$ and $bla_{\text{CMY-2}}$ genes identified during the study.

MATERIALS AND METHODS

Detection of isolates for the production of ß-lactamases

A total of 1,369 samples (660 stool samples and 709 water samples) were collected during 2005 and 2008 and were screened for the presence of ESBL producing *Aeromonas* species. Stool specimens with and without diarrhea were cultured on blood agar (Oxoid Ltd, Basingstoke, UK) and MacConkey agar (Difco/BD Diagnostics Systems, Sparks, MI, USA) and water samples were plated on cysteine lactose electrolytes deficient (CLED) agar and MacConkey agar (Difco/BD Diagnostics Systems, Sparks, MI, USA) . Isolated strains were stored in tubes containing 1.5 ml brain heart infusion broth with 10% v/v glycerol at -70°C until the time of analysis. The isolates were identified and confirmed using the API 20E and API 20 NE identification systems (bioMerieux, Marcy-l'Etoile, France).

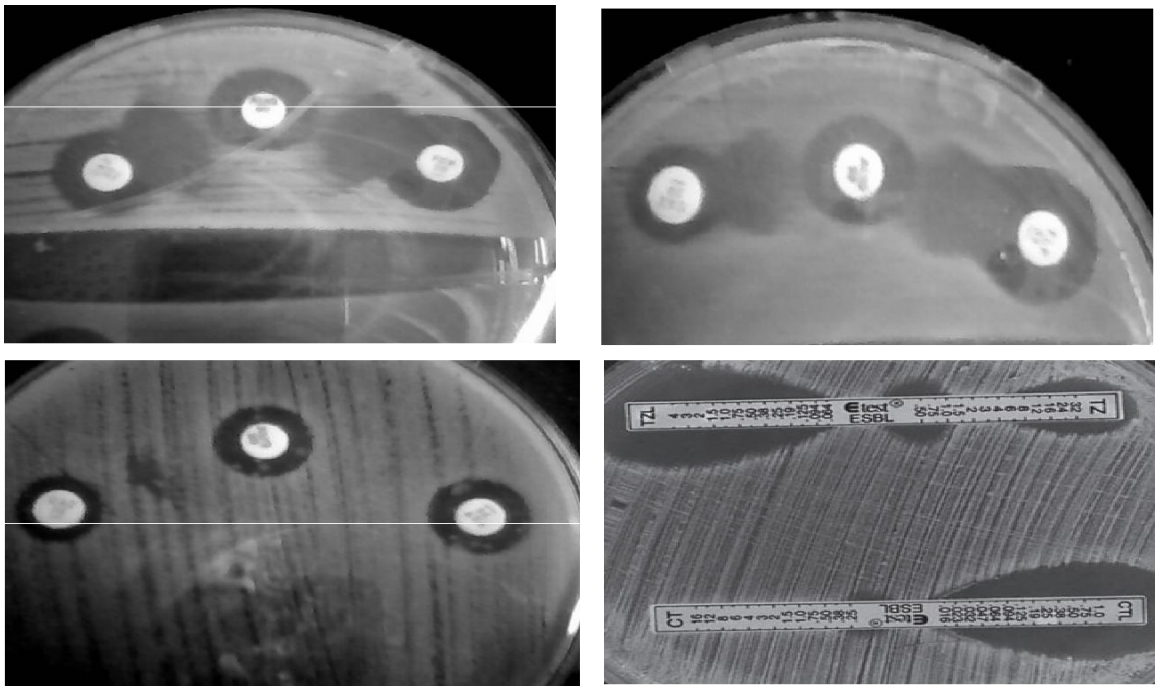

Figure 1. Detection of ESBL production using the double disc method.

The iodometric tube method, using penicilin G as the substrate (Livermore and Williams, 1996)

Crude extracts of the ß-lactamases were obtained by sonication and centrifugation of overnight cultures of the test isolates. Twenty (20) yl of starch indicator, (containing 1% soluble starch), 20 yl iodine reagent (containing 2% iodine in 53% potassium iodide) and 100 yl benzyl penicillin were pipetted into a glass test tube. Crude enzyme (100 yl) was added and the mixture vigorously shaken at room temperature. When beta-lactamase was present, the blue-black colour of the mixture disappeared and the solution became milky white within 5 min.

The nitrocefin method

Cells were mixed directly into the moist nitrocefin on the filter paper and left up to 1 h for any colour reaction to occur. A change from yellow to red indicated the production of ßeta-lactamase.

Antibiotic susceptibility testing

The susceptibility of isolates of *A. hydrophila* to antimicrobial agents was examined by an agar diffusion method using paper disks containing the following antibiotic concentrations: amikacin (30 µg), ampicillin (10 µg), gentamicin (10 µg), cefalotin (30 µg), cefotaxime (30µg), cefoxitin (30 µg),ceftazidime (30 µg),piperacillin/tazobactam (100/10 µg), amoxicilin/clavulanicacid (20/10 µg), profloxacin (5 µg), imipenem (10 µg), cefuroxime (30 µg), cefepime (30 µg), meropeenem (10 µg), cefpdoxime (10 µg),trimethoprim/sulfathoxazole (1.25/23.75 µg), nitrofurantoin (300 µg), norfloxacin(10 µg), ofloxacin (5 µg), piperacillin (100 µg), tobramycin (10 µg), colistin (10 µg) , aztreonam (30 µg), cefpirome (30 µg), isepamicin (30 µg), netilmicin (30 µg), pefloxacin (30 µg), ticarcillin (75 µg), ticarcillin/clavulanic acid (75/10 µg), cefaclor (30 µg), nalidixic acid (30 µg) and ertapenem (10 µg). Disks were purchased from Oxoid. Antimicrobial activities interpreted according to National Committee for Clinical Laboratory Standards (NCCLS). *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as quality control strains**.** E-test strips with gradient concentrations of cefotaxime, ceftazidime and cefepime at one end and cefotaxime, ceftazidime, cefepime with Clavulanic Acid (CA) at the other end were used in accordance with the guidelines of the manufacturer (catalogue no. 16V03228, 16V03258 and 16V03478; AB BIODISK, Solna, Sweden).

Detection of ESBL production

Production of ESBLs was studied by the double disc synergy and inhibition potentiated disc diffusion tests. Isolates were inoculated on Muellar- Hinton (MH) agar plates. Discs containing ceftazidime (30 yg), cefazolin (30 yg), cefoxitin (30 yg) and aztreonam were placed 25mm (centre to centre of the discs), from a disc containing ampicillin/clavulanic acid (20 yg). After overnight incubation at 37°C, the diameters of inhibition zones around the antibiotic discs were measured using a Venier caliper. A clear extension of the edges of the inhibition zone of any of the antibiotics towards the disc containing clavulanic acid was regarded as a phenotypic confirmation of the presence of ESBL (Figure 1). Isolates positive for ESBL production were subjected to PCR amplification using primers designed for the detection of *bla*$_{TEM}$, *bla*$_{CMY-2}$, *bla*$_{CTX-M}$ and *bla*$_{SHV}$ genes.

Crude enzyme extraction

Overnight cultures in brain heart infusion broth were centrifuged at 15 000g for 30 min at 4°C. The pellet was washed with 0.1 M phosphate buffer (pH7) and resuspended in the fresh buffer. Cells were disrupted by French pressure cell press (American Instrument

company, Maryland, USA) at 12 000lb/in2 and the cell debris removed by centrifugation (40 000g, 30 min, 4°C) Beckman, model L3-50 ultracentrifuge, USA. The supernatant was used as the crude extract containing the beta-lactamase.

Whole-cell DNA extraction

Extraction of whole-cell DNA was performed by a precipitation–based method, as descrided previously (Philippon and Arlet, 2006). Briefly one single colony-forming unit (CFU) of *A. hydrophila* was re-suspended in 5 ml brain heart infusion broth (Oxoid Ltd., Hampshire, UK) and incubated overnight at 37°C (WBM SPL 25 Labcon shaking water bath,Laboratory Marketing Services , Marisburg) while shaking at 50 revolutions per minute (RPM).One ml of the overnight culture was pelleted in a centrifuge at 3000 x g (Laboguge 400r, Heraeus Instruments, Germany) for 10 min at 4°C. The pellet was resuspended in 1 ml sterile distilled water and re-pelleted as described above. The resulting pellet was resuspended in 500 µl STE buffer containing 75 mM NaCl (Promega, Madison, WI), 20 mM Tris (Sigma Chemical Co. St. Louis, MO) and 25 mM EDTA (Promega, Madison, WI) at pH 7.5.

Cells were lysed by adding 25 yl of a 20% SDS (Promega, Madison, WI) solution (pH 12.45) and 1 yl of a 50mg/ml stock solution lysozyme (Sigma Chemical Co. St. Louis, MO) with incubation at 37°C(QBT2 heating block, Grant Instruments Ltd., Cambridge ,United Kingdom) for one hour. On completion of cell lysis, 220 yl of 5 M NaCl (Promega, Madison, WI) was added followed by the addition of 700 yl chloroform/isoamyalcohol (24:1) (Sigma Chemical Co. St.Louis, MO) to separate DNA and protein phases. Following centrifugation at 5000 x g (Z233 m-2 , Hermle Labortechnik ,Wehingen ,Germany) for 10 min at room temperature (25°C), the upper phase containing whole-cell DNA was removed and transferred to a new tube. The DNA was precipitated with 700 yl isopropanol (Merck, Darmstadt, Germany) at -20°C for 1 h or overnight. The precipitated DNA was pelleted by centrifugation at 5000 x g (Z233 m-2, Hermle Labortechnik, Wehingen, Germany) for 10 min at room temperature (25°C) followed by two washing steps with 800 µl of a 70% ethanol solution (Merck, Darmstadt, Germany). After air-drying, the DNA pellet was re- suspended in 1 ml TEM buffer (Promega, Madison, WI), containing 10 mM Tris/HCL and 1 mM EDTA at pH 7.4. 500 µl of the extracted DNA was stored at 4°C and -20°C until further analysis.

PCR amplification of *bla*TEM, *bla*CMY-2, *bla*CTX-M and *bla*SHV genes

Polymerase chain reaction analysis was performed with the *bla*TEM, *bla*CMY-2, *bla*CTX-M and *bla*SHV genes specific primer set using whole-cell DNA extracted from 21 ESBLs producing *A. hydrophila* isolates as template. Primer synthesis and purification was conducted by Inqaba biotechnology Industries Pty. Ltd., Pretoria South Africa. The reaction consisted of 12.5 µl GoTag Green Master Mix (Promega, Madison, WI), 0.8 µl of each primer (20 mM), 4 µl whole–cell DNA as template and molecular garden water (Promega, Madison, WI) prepared to a final reaction volume of 25 µl. The amplicons obtained were analyzed by gel electrophoresis.

Sequence –specific competitive PNA-based muliplex PCR

Two sets of primer pairs were used in a multiplex assay together with a *bla*TEM, *bla*CMY-2, *bla*CTX-M and *bla*SHV genes specific PNA-probe (Applied Biosystems, Rotkreuts, Switzerland). The selected primer pairs were specific for the detection of *bla*TEM, *bla*CMY-2, *bla*CTX-M and *bla*SHV genes and amplified 306 and 505 bp products respectively. The multiplex PCR mix (reaction volume 50 µl) consisted of 10 x Mg-free PCR buffer, 1.5 mM MgCl2, a 200µM concentration of each deoxynucleoside triphoshate, 1.25 U of Taq DNA polymerase (Promega, Madison, WI), a 0.32 mM concentration of each primer 0.32 yM PNA –probe and 2 yl DNA template. Multiplex PCR amplification was performed on a gene Amp 9600 thermocycler (Perkin Elmer Cetus, Emeyville, CA). The PCR cycle programme consisted of an initial denaturation step at 95°C for 2 min, followed by 35 amplification cycles each at comprising a denaturation step at 95°C for 30 s ,followed by an annealing step at 50°C for 1 min and an extension step at 72°C for 1 min. After the completion of 35 amplification cycles, a final extension step was performed at 72°C for 5 min.

Verification of DNA extractions and PCR products

All DNA extractions were verified by gel electrophoresis at 2 V/cm (Eilte -300 Power supply Wealtec Corp., Kennesaw ,GA) for 1 h in a 1% agarose gel (Pronadisa ,Madrid ,Spain) containing ethidium bromide (Promega, Madison, WI) (0.5µl/ml) at 4 V/cm(Eilte 300 Power supply Wealtec Corp., Kennesaw ,GA) for 45 min in 1 x TBE running buffer (pH8.3), with a 100 bp DNA ladder (Promega, Madison, WI) as molecular size marker. All agarose gels were visualized under UV illumination (TFM-26 Ultarviolet Transilluminator, UVP, Upland, CA) and the images captired using a digital gel documentation system (DigiDoc-It imaging system, UVP, Upland, CA).

DNA sequencing of *bla*TEM, *bla*CMY-2, *bla*CTX-M and *bla*SHV genes

The *bla*TEM, *bla*CMY-2, *bla*CTX-M and *bla*SHV specific PCR amplicons were sequenced with the forward primer using a SpectruMedix model SCE 2410 automated sequencer (SpectruMedix ,State College ,PA), incorporating the ABI Bih Dye terminator cycle Sequencing kit version 3.1(Aplied Biosystems ,Foster City ,CA) to determine the *bla*TEM, *bla*CMY, *bla*CTX-M and *bla*SHV genes identity. Sequencing was performed by Inqaba biotechnical industries Pty. Ltd., South Africa. Electropherograms of the sequences generated were inspected with Chromas software (version 1.45; Technelysium Pty. Ltd., Helensvale, Queensland, Australia). PCR products obtained with the *bla*TEM, *bla*CMY, *bla*CTX-M and *bla*SHV specific PCR from Enterobacteriaceae (Hanson et al., 2001) and non-typhodial *salmonella* (Kruger et al., 2004) were used as sequencing controls.

RESULTS AND DISCUSSION

The results of ESBLs production as shown in (Figure 1) demonstrate an increase in resistance of *A. hydrophila* to the most commonly used antibiotics in clinical settings. The sequence-selective competitive PNA-based multiplex PCR assay was used to determine the prevalence of *bla*TEM, *bla*CMY-2, *bla*CTX-M and *bla*SHV genes in clinical isolates of *A. hydrophila*. The amplification products generated during the PNA-based multiplex PCR was subjected to gel electrophoresis after which two distinct patterns were detected. The presumptive *bla*TEM, *bla*CMY, *bla*CTX-M and *bla*SHV possessing *A. hydrophila* clinical isolates are shown (Figures 2a - 2c and Table 1). The results indicated that one (4.76%) of the 21 *A. hydrophila* isolates tested positive for a possible *bla*CMY when analysed with competitive PNA-based multiplex PCR. one (4.76%) of the 21 *A. hydrophila* isolates tested positive for a possible *bla*TEM with this technique. This

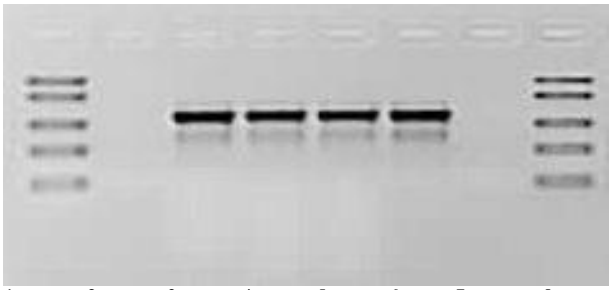

Figure 2a. Detection and identification of *A. hydrophila* Tem 63 genes by amplification of fragments in the PCR. Lanes 1 and 8,100 - 5000 bp ladder (invitrogen); Lanes 2 and 7 is water PCR negative control and Lanes 3 ,4 ,5 and 6 different isolates of *A. hydrophila* showing the TEM 63 genes.

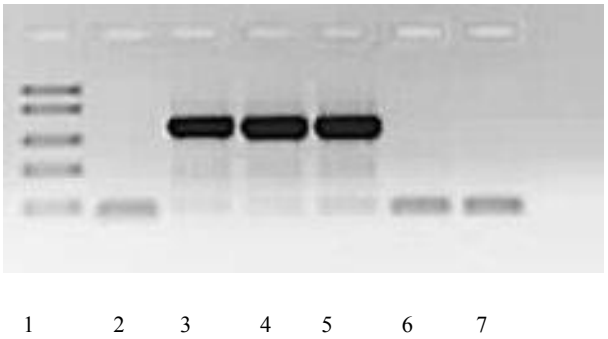

Figure 2b. Detection and identification of *A. hydrophila* CMY-2 genes by amplification of fragments in the PCR. Lanes 1, 100- 5000 bp ladder (invitrogen); Lanes 2 and 7 is water PCR negative and Lanes 3 , 4 and 5 different isolates of *A. hydrophila* showing the CMY-2 genes.

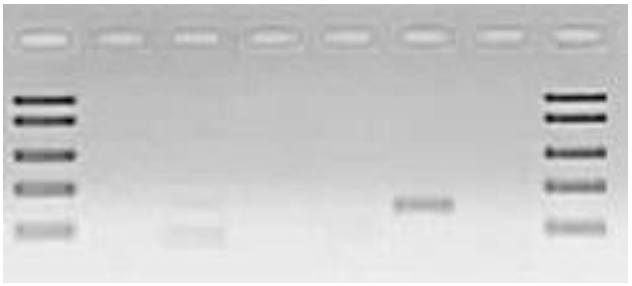

Figure 2c. Detection and identification of *A. hydrophila* CTX-M genes by amplification of fragments in the PCR. Lanes 1 and 8 ,100-5000 bp ladder (invitrogen); Lanes 2 and 7 is water PCR negative control and Lanes 3 ,4 and 5 different isolates of *A. hydrophila* showing the absence CTX-M genes.

result of ESBLs was also obtained in the study done by Kruger et al. (2004) on *Klesiella Pneumonia* and study done by Hanson et al. (2001) on non-typhodial *salmonella*. Sequencing of the amplicons obtained from the possible bla_{TEM} and bla_{CMY} positive isolates revealed these genes to be bla_{TEM-63} and bla_{CMY-2} with a 100% identify to the bla_{TEM-63} gene (GenBank accession number AF 332513) published by (Katherene et al., 2006) and bla_{CMY-2} gene (GenBank accession number AY 581207) published by (Svetlana et al., 2007).

Attempts to amplify the bla_{TEM} and bla_{CMY} gene in Premega Taq in Buffer A (Promega, Madison, WI) produced little product. Sufficient amplification was achieved using GoTaq Green Master Mix (Promega, Madison, WI). GoTaq Green Master Mix (Promega, Madison, WI) gave better results compared to Premega Taq in Buffer A. Optimised master mixes were thus a better choice of PCR reagents to be used in screening projects, resulting in greater sensitivity. The PNA-based multiplex PCR technique has been reported to be sensitive to template quality which might have resulted in the false-negative results. The advent of resistance to expanded–spectrum cephalosporins antibiotics in *Aeromonas* species and in other natural AMPC beta-lactamase producing members of the family Enterobacteriaceae is produced mainly by a constitutive over expression of chromosomal enzymes as previously suggested (Ehrhart et al., 1993). A lot of ESBLs including TEM 24 have also been described in Aeromonas species and have been responsible for several diarrheal diseases (Mohammad and Hassan, 2004; Alberto et al., 1996; Wang et al., 2002; Marchandin et al., 2003). ESBLs among *Aeromonas* species are of great concern since they are causative agents of diarrhea. The prevalence of ESBL–producing *Aeromonas* spp. was less in comparison to other ESBLs isolated from other members of Enterobacteriaceae. We have found that of all 21 ESBL producing isolates from different patients produced CMY2; this enzyme was also isolated by other researchers in South Africa from non-typhoidal *Salmonella* (Kruger et al., 2004), which demonstrated that *Aeromonas* might have acquired this enzyme from other species which belonging to the family Enterobacteriaceae. We also identified TEM 63 (Figure 2a) from our isolates which were also identified in many studies worldwide among the Enterobacteriaceae. The presence of CMY-2 (Figure 2b) demonstrates the need for identification of these ESBLs from *Aeromonas hydrophila*. Moreover, the absence of the CTM and SHV enzymes may be related to the mode of transfer of these enzymes from the environment to patients. However, the spread of ESBL-producing strains among different countries has been previously reported (Canton et al., 2002).

Conclusion

The increase in occurrence of ESBLs in *A. hydrophila* emphasizes the importance of constant surveillance of

clinical isolates to determine the prevalence of antibiotic resistant genes. The dissemination of beta-lactamases may play an important role in the spread of antibiotic resistance and may limit future choices of antibiotic regimens for the treatment of diarrheal infections due to ESBL-producing *A. hydrophila*. Continuous monitoring and evaluation of emerging antibiotic resistance genes in bacteria such as *A. hydrophila* is of great importance and would serve to enforce adequate control measures including review of policy guidelines for antibiotic usage in health facilities.

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