Sudan Journal of Science and Technology
Journal homepage:
http://jst.sustech.edu/

Molecular Study of Bacterial Pathogens Isolated From Haemodialysis Patients

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ARTICLE INFO

ARTICLE HISTORY
Received: 5th April 2015
Accepted: 18 October 2015
Available online: June 2016

KEYWORDS:
Bacteria,
Haemodialysis,
Minimum Inhibitory Concentrations (MICs),
16S rRNA gene,
Integron integrase (intI) genes

ABSTRACT

Sixty-two bacterial pathogens were isolated from 201 blood culture specimens collected from chronic haemodialysis patients. All patients were suffering from signs and symptoms of bacteraemia which is a frequent complication associated with tunnelled, cuffed, permanent catheters (PCs). The study aimed to use molecular technique to diagnose bacterial pathogens isolated from haemodialysis patients. Identification and Minimum Inhibitory Concentrations (MICs) of commonly used antibiotics were determined by VITEK 2 Compact System and confirmed by amplification of species–specific primers targeted to the 16S rRNA gene for *Staphylococcus epidermidis, E. faecalis, E. faecium, Staphylococcus aureus* and *P. aeruginosa*. Class 1 and 2 integron integrase (intI) genes were amplified to determine whether integrons were present in all isolates genome. The amplification of genes was carried out by Polymerase Chain Reaction (PCR). The result showed that PCR assays employing 16S rRNA primer pair produced DNA products of the predicted size of 34 of 35 *Staphylococcus epidermidis*, 5 of 6 *E. faecium* and all *E. faecalis, P. aeruginosa* and *Staphylococcus aureus.* The result showed that class 1 intI gene was found in 54 (87.1%) of the isolates and the integron harbouring isolates were significantly more resistant to Erythromycin (54.2%), Clindamycin (31.3%), Ciprofloxacin (16.7%) and Rifampicin (6.3%). The class 2 intI gene was not found in these studied strains.

INTRODUCTION

Haemodialysis continues to be an important treatment option for persons with end-stage renal disease (Taylor *et al*., 2004). Haemodialysis catheter-related bloodstream infections (HD CRBSIs) are a serious complication of PC use and a common cause of catheter failure (Saad, 1999).

Identification of bacterial species from culture material by 16S rRNA gene sequencing is now widely used in many clinical microbiology laboratories (Hartmeyer and Justesen, 2010). The use of 16S rRNA gene sequences to study bacterial phylogeny and taxonomy has been by far the most common housekeeping genetic marker (Janda and Abbott, 2007). As a result, one of the major limitations is that when two bacterial species share a similar 16S rRNA gene sequence, this technology alone would not be useful for distinguishing them confidently (Teng *et al* .,2011).

Integrons were defined by Fluit and Schmitz, (2004) as elements which contain the genetic determinants of the components of a site-specific recombination system that recognizes and captures mobile gene cassettes. These can be subdivided into two main groups, the super integrons and the antibiotic resistance integrons (ARIs) (Mobarak-Qamsari *et al*., 2013). Based on the intI sequences, at least six classes of integrons have been described to date . Class 1 integrons are the most studied and are largely implicated in the dissemination of antibiotic resistance among clinical isolates (Severino and Magalha~es, 2004). All class 1 integrons possess the following key components: an integrase gene (*intI1*), a recombination site (*attI1*), and a promoter (Pc) for the transcription of cassette-associated genes (Holmes *et al*,. 2003; Zhao *et al*., 2011).

The goal of this study was to investigate the feasibility of developing a relatively rapid, inexpensive, PCR-based method for confirming bacterial isolates to be identified to the species level by biochemical testing and the presence of integrons, gene to determine their roles in disseminating antimicrobial resistance genes.

MATERIALS AND METHODS

A total of 201 blood culture samples were collected from end stage renal disease (ESRD) patients with the diagnosis of HD CRBSIs, The study was a cross-sectional conducted out in Khartoum State in the period from March 2013 to March 2014. Blood collection and patients' data were done in 17 Dialysis Units of Khartoum Teaching Hospital (KTH), Gaffer Iben ouf Specialized Hospital for Children (GIASH), Omdurman Teaching Hospital, Mohamed Elamin Hospital for Children, AL academy Teaching Hospital, Al waledain dialysis centres, Giad hospital, El gamea Hospital for renal disease, Ahmed Gasim Hospital, El Safia Dialysis Centre, Military Medical Hospital, Ribat University Hospital, Iben Seina Hospital, Alamal Alwatani Hospital, Ompada Hospital, Sharg Elneel Hospital and Elban Gadeed Hospital. Usually, one set was obtained by using clean technique as mentioned by Landry *et al*., (2010).

The isolate were 62% from 201 patients. There were isolated by VITEK 2 Compact system in previous work done by the first author that included *Staphylococcus epidermidis* 35 out of the 62 (56.5%) cases. Other prominent bacteria included were: *Enterococcus faecalis, Enterococcus faecium* each 6 (9.7%), *Staphylococcus aureus* 4(6.5%), *Pseudomonas aeruginosa*

ISSN (Print): 1605 427x e-ISSN (Online): 1858-6716

3(4.8%), *Staphylococcus vitulinus, Staphylococcus hominis, Staphylococcus simulans, Streptococcus uberis, Enterobacter cloacae, Serratia marcescens,* and *Escherichia coli,* each once (1.6%). And there antimicrobial susceptibility testing (AST) were also done pure cultures of isolates were prepared and genomic DNA was extracted using GF-1 bacterial DNA extraction kit, (Vivantis Technologies, Malaysia). A species–specific PCR assay with primers targeted to the 16S rRNA gene was done for *Staphylococcus epidermidis, E.faecalis, E. faecium, Staphylococcus aureus* and *P. aeruginosa* to confirm the VITEK 2 compact system species identification (Table1).

Using the primers
Forward: 5-TACATGCAAGTCGAGCGAAC-3
Reverse: 5-ATCATTTGTCCCACCTTCG-3
targeted to the *Staphylococcus epidermidis* 16S rRNA,
Forward: 5'-GTT TAT GCC GCATGG CAT AAG AG-3',
Reverse: 5'-CCG TCA GGG GACGTT CAG-3'
targeted to the *E. faecalis* 16S rRNA,
Forward: 5'ATCGCAAGATTGTTCGAAC3,
Reverse: 5'CTTAGAAAGGAGGTGATCCAG-3
targeted to the *E.faecium* 16SrRNA,
Forward: 5CGTCACACCACGAGAGTTTGTAA-3
Reverse: 5'ACCTTTCGACGGCTAGCTCC-3
targeted to the *Staphylococcus aureus* 16SrRNA and
Forward: 5'-GACGGGTGAGTAATGCCTA-3
Reverse: 5'-CACTGGTGTTCCTTCCTATA-3
targeted to the *P. aeruginosa* 16S rRNA. which designed on previously published studies (Vandecasteele *et al*., 2001, Dumani *et al.*, 2012,Galloway-Pen˜a *et al*., 2012, Zhang *et al*., 2000 and Hassan *et al*., 2012).

The amplification program of *Staphylococcus epidermidis, Staphylococcus aureus and* P. *aeruginosa* were run as follows: initial denaturation One pre cycle of 95°C for 2 minutes, 30cycles of denaturation at 92°C for 1 minute, annealing for 1 minute at 55°C for *Staphylococcus epidermidis* and *P. aeruginosa* and at 60°C for *Staphylococcus aureus* and extension at 72°C for 1 minute and One final extension cycle at 72°C for 10 minutes.

The temperature profile for *E. faecalis and E. faecium* included an initial denaturation step of 95°C for 15 min and followed by 35 cycles of a denaturation step at 94°C for 30 s and primer annealing step for 1 min at 60°C for *E. faecalis* and at 53°C for *E. faecium* an extension step at 72°C for 1 min, and a final step at 72°C for 2 min. The presence of class 1 and 2 integrons was tested by PCR using primers specific for the integron integrase genes intI1 and intI2 (Table1) (Chang *et al.,* 2011; Mobarak-Qamsari *et al.* 2013). The PCR conditions were as follows:- Initial denaturation at 95 °C for 1 min, 30 cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 65 °C and extension for 1 min at 72 °C, and a final extension for 7 min at 72 °C for intI1 and initial denaturation at 95 °C for 5 min followed by 35 cycles of 1 min at 94 °C, 1 min at 65 °C and 1 min at 72 °C with a final extension at 72 °C for 10 min for intI2. For negative control ultrapure water was used.

PCR products were analyzed by 1.5% agarose gel (Agarose, Vivantis Technologies, Sdn. Bhd, Malaysia), electrophoresis performed at 120 V in Tris-Borate EDTA buffer. The gels were stained with 1.5 µl of ethidium bromide and visualized under ultraviolet light, and photographed with the (BioRad Gel doc 2000, USA) Imaging System. 50-bp and 100-bp DNA ladder digest (Vivantis Technologies, Malaysia) were used as a molecular weight marker.

Table 1: Primers used for identification and bacterial susceptibility for bacterial isolates

Gene	Primer	Amplicon size (bp)	Reference
16S rRNA of *Staphylococcus epidermidis*	Forward: 5-TACATGCAAGTCGAGCGAAC-3 Reverse: 5-AATCATTTGTCCCACCTTCG-3	1443	Vandecasteele *et al.* (2001)
16S rRNA of *E. faecium*	Forward: 5- ATCGCAAGATTGTTCGAAC-3 Reverse: 5- CTTAGAAAGGAGGTGATCCAG-3	1569	Pena *et al.* (2012)
16S rRNA of *E. faecalis*	Forward: 5'-GTT TAT GCC GCATGG CAT AAG AG-3' Reverse: : 5'-CCG TCA GGG GACGTT CAG-3'	310	Dumani *et al.*(2012)
16S rRNA of *P. aeruginosa*	Forward: 5-GACGGGTGAGTAATGCCTA-3 Reverse: 5-CACTGGTGTTCCTTCCTATA-3	956	Hassan *et al*. (2012)
16S rRNA of *Staphylococcus aureus*	Forward: 5-CGTCACACCACGAGAGTTTGTAA-3 Reverse: 5-ACCTTTCGACGGCTAGCTCC-3	70	Zhang *et al.* (2000)
Class 1 intI	Forward: 5-CCTCCCGCACGATGATC-3 Reverse: 5- TCCACGCATCGTCAGGC-3	280	Zhao *et al. (*2011)
Class 2 intI	Forward: 5-TTATTGCTGGGATTAGGC-3 Reverse: 5- ACGGCTACCCTCTGTTATC-3	233	Qamsari *et al.* (2013)

RESULTS

PCR amplifications for classes 1 and 2 integrase Genes showed that 54 (87.1%) of the 62 isolates harboured class 1 (Figure 1) and the class 2 intI gene was not found in this study. Class 1 integrase Gene were present in all Gram negative isolate and in 48 of 56 Gram positive isolates (Table 2).

Table 2: The presence of integrons in 62 bacterial isolates

Type of organisms	N0	Integron positive isolates	Integron negative isolates
Gram-positive organisms	56	48	8
Gram-negative organisms	6	6	0
Total number	62	54	8

The integron harbouring isolates were significantly more resistant to erythromycin (54.2%), Clindamycin (31.3%), Ciprofloxacin (16.7%) and Rifampicin (6.3%) (Table 3). On the other hand, resistance to Levofloxacin, Moxifloxacin, and benzyl penicillin, Oxacillin, Gentamicin, Tetracycline and Trimethoprim /Sulphamethoxazole were significantly higher in integron negative isolates (Table 3). The association between integron carriage and antibiotic resistance was significant for oxacillin ($p = 0.002$) (Table 4).

Two primer pairs were used for each species in this study. These primers targeted the variable regions in the 16S rRNA gene. PCR assays employing this primer pair produced DNA products of the predicted size of 34 of 35 *Staphylococcus epidermidis* (1443 bp) (Figure 2), 5 of 6 *E. faecium (*1569bp) (Figure 3), and all *E. faecalis* (310 bp) (Figure 4), *P. aeruginosa* (956bp) (Figure 4) and *Staphylococcus aureus* (70 bp) (Figure 5), 16S rRNA gene sequence offered a useful method for the identification of bacteria.

Table 3: Association between antimicrobial susceptibility and integron carriage in 56 Gram positive clinical isolates

Antibiotic	Integron positive isolates (n= 48)		Integron negative isolates (n=8)		Level of significance*
	% R	% S	% R	% S	
P	91.7	8.3	100	0	NS
OX	81.3	18.7	100	0	**
GM	10.4	89.6	12.5	87.5	NS
CIP	16.7	83.3	12.5	87.5	NS
LEV	6.3	93.7	12.5	87.5	NS
MXF	2.1	97.9	12.5	87.5	NS
E	54.2	45.8	37.5	62.5	NS
CM	31.3	68.7	12.5	87.5	NS
LNZ	0	100	0	100	NS
VA	0	100	0	100	NS
TE	35.4	64.6	37.5	62.5	NS
TGC	0	100	0	100	NS
FT	0	100	0	100	NS
RA	6.3	93.7	0	100	NS
SXT	35.4	64.6	50	50	**NS**

The significance level was determined using the two-tailed Mann-Whitney U test. **, 1%, NS, non-significant; R, resistant; S, sensitive.

P=Benzyl penicillin, OX= Oxacillin, GM= Gentamicin, CIP= Ciprofloxacin, LEV= Levofloxacin, MXF= Moxifloxacin, E= Erythromycin, CM= Clindamycin, LNZ= Linozolid, VA= Vancomycin, TE= Tetracycline, TGC= Tigecycline, FT= Nitrofuranation, RA= Rifampicin, SXT= Trimethoprim/Sulphamethoxazole

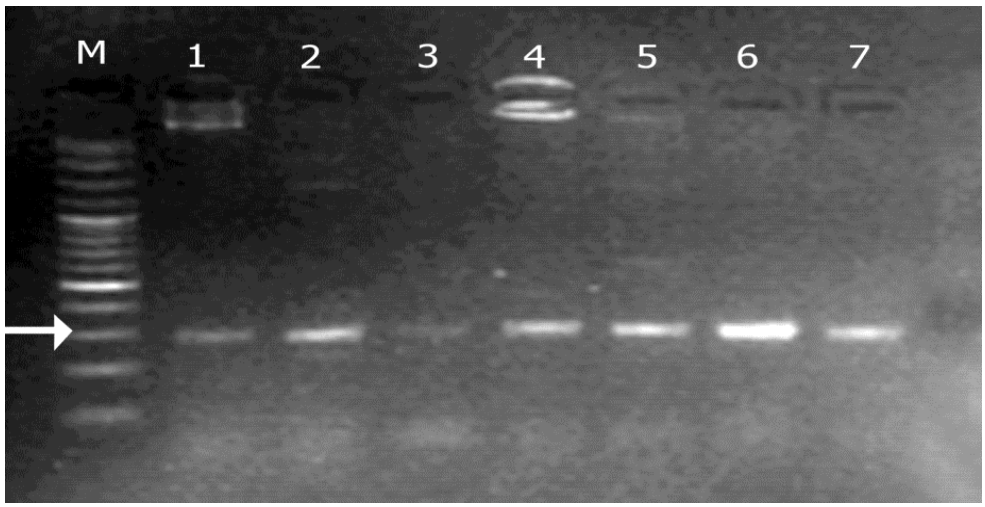

Figure 1: (1-7) 280 bp PCR amplification product of class 1 integrone integrase genes in 54 of 62 clinical isolates. M, Ladder, DNA molecular weight marker (100 bp)

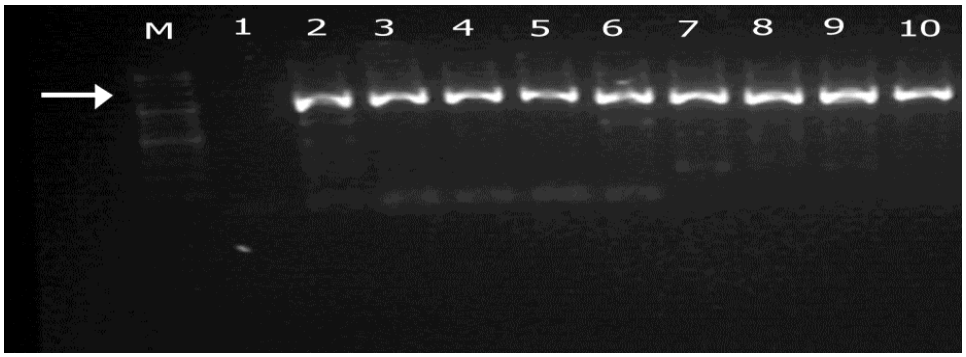

Figure 2: (2-10) 1443 pb PCR amplification product of 16S rDNA which was specific for *Staphylococcus epidermidis* were identified in 34 of 35 of *Staphylococcus epidermidis* isolates in 1.5% agarose gel electrophoresis. M (100 bp) DNA ladder.1, negative control (no DNA template).

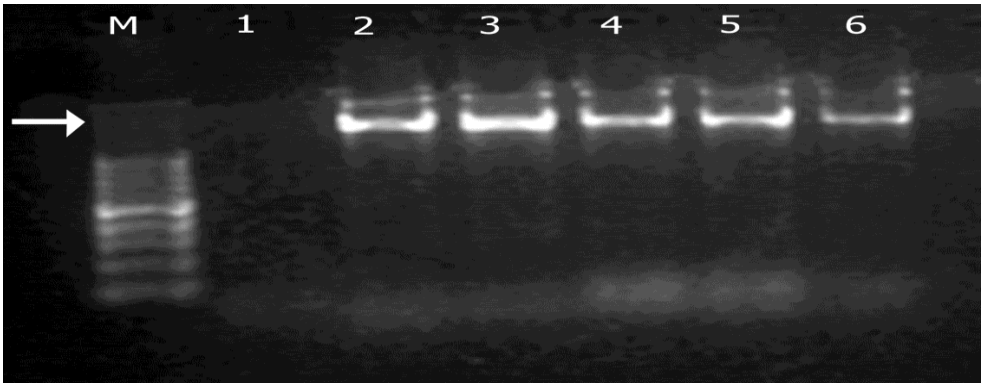

Figure 3: (2-6) 1569 pb PCR amplification product of 16S rDNA which was specific for *E. faecium* were identified in 5 of 6 of *E. faecium* isolates in 1.5% agarose gel electrophoresis. M (100 bp) DNA ladder.1, isolate negative for 16S rDNA specific for *E. faecium*

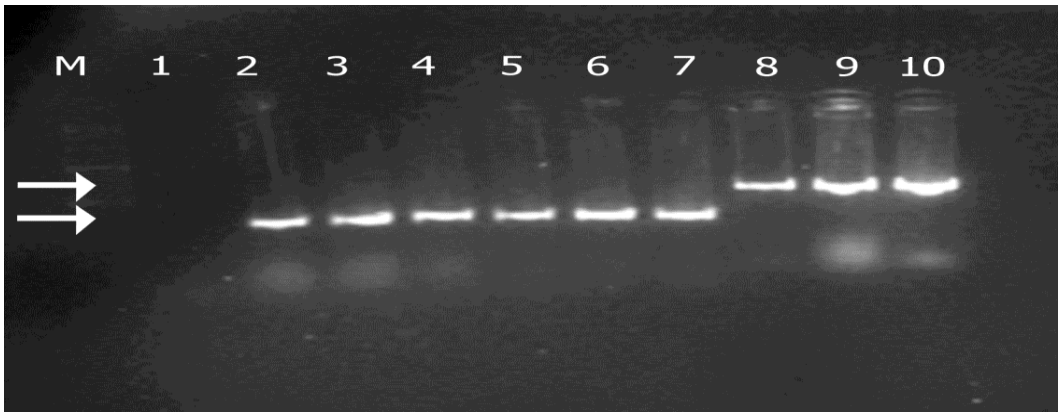

Figure 4: lane (2-7) 310 pb PCR amplification product of 16S rDNA which was specific for *E. faecalis* were identified in all *E. faecalis* isolates and lane 8,9,10 (956 bp) PCR amplification product of 16S rDNA which was specific for *P. aeruginosa* also were identified in all *P. aeruginosa isolates*, in 1.5% agarose gel electrophoresis. M (100 pb) DNA ladder.1, negative control (no DNA template).

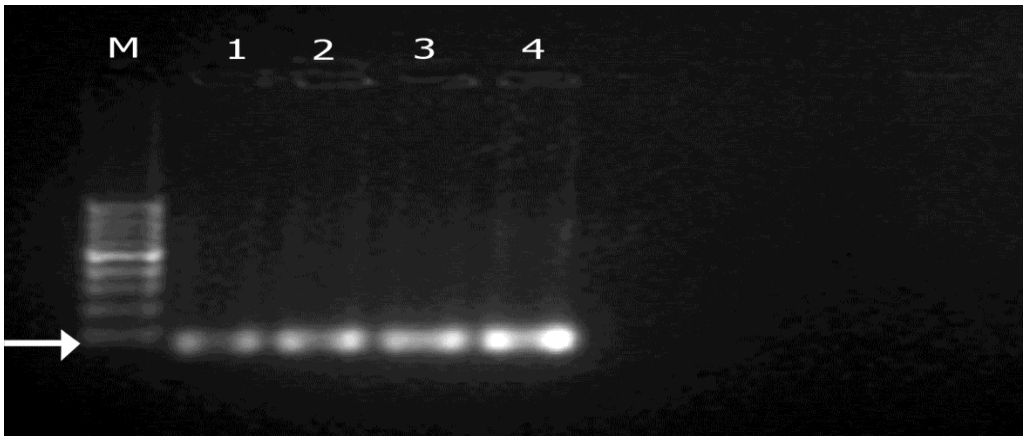

Figure 5: (1-4) 70 bp PCR amplification product of 16S rDNA which was specific for *Staphylococcus aureus* were identified in all *Staphylococcus aureus* isolates in 1.5% agarose gel electrophoresis. M (100 bp) DNA ladder

DISCUSSION

This study diagnosed CAB based on blood cultures drawn directly from the PC port or from the dialysis blood tubing coming from the PC. This result is similar to Saad (1999). Our result also reported a predominance of Gram-positive organisms (90.3%) in contrasts with the findings of Saad (1999). The wide variety of both Gram-positive and Gram-negative infections seen, is similar to the spectrum of organisms reported by Landry *et al*., (2010) who found that a predominance of Gram-positive organisms (87.7%). Also this study has reported a predominance of Gram-positive coagulase- negative staphylococci (59.7%). This result goes in line with the result reported by Taylor *et al*., (2004) who found the most microbial aetiology of CAB in haemodialysis patients was coagulase-negative staphylococci (45%). In this study, both VITEK 2 compact system and PCR assay with primers targeted to the 16S rRNA gene identification to the species level, reliably distinguished one species from each other. With VITEK 2 compact system sensitivity as 100% for *P. aeruginosa, Staphylococcus aureus and Enterococcus faecalis* and sensitivity was 97.2% and 85.7% for *Staphylococcus epidermidis* and *Enterococcus faecium* respectively compared to 16SrRNA based PCR confirmation.

In an era of bacterial resistance, a careful and correct selection of antibiotics is important to increase the chance of successful treatment and to reduce the rate of bacterial resistance. Prior to this study, the empirical antibiotics for HD CRBSIs in our centres were intravenous Vancomycin and gentamicin. Based on the present study, it's noted that all Gram-negative bacteria were sensitive to Amikacin. Unfortunately, Gentamicin resistance was noted among Gram-negative organisms. Our results also realized that coagulase-negative Staphylococcus was the most common Gram-positive organisms and they were sensitive to Vancomycin. Thus, following this study results, empirical antibiotics for HD CRBSIs in our centre might switch to intravenous Vancomycin and Amikacin.

Bacterial resistance has become a worldwide problem as a result of the abuse of antibiotic drugs and the transfer of

resistance genes between bacteria. This result revealed that the class 1 intI gene was detected in 87.1% (54/62) of isolates and the class 2 intI gene was not found in this study, this result is similar to the result obtained by Zhao *et al*., (2011).

Researchers thought that the occurrence rate of integrons in Gram-negative bacteria was higher than in Gram-positive bacteria (Zhao *et al*., 2011). This simulates the result in this study in which occurrence rate of integrons in Gram-negative bacteria was 100% while the occurrence rate of integrons in Gram-positive bacteria was 85.7%.

The present study found a significant association between integron carriage and higher rates of resistance to oxacillin. Also the integron harboring isolates were significantly more resistant to erythromycin, clindamycin ciprofloxacin and rifampicin. this result is similar to the result obtained by Zhao *et al*., (2011) who reported that Class 1 integrons including genes that encode aminoglycoside resistance and β-lactam resistance.

In this study, there was an inverse association between resistance to levofloxacin, moxifloxacin, benzylpenicillin, oxacillin, gentamicin, tetracycline and trimethoprim/sulfamethoxazole with integron carriage, where integron negative isolates were more resistant to these antibiotics.

CONCLUSION

PCR assay with primers targeted to the 16S rRNA gene identification to the species level, reliably distinguished one species from each other. Class 1 intI gene was detected in 87.1% (54/62) of isolates with occurrence rate of 100% in Gram-negative bacteria and 85.7% in Gram-positive bacteria while class 2 intI gene was not found during this study.

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