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ANNALS OF
Molecular and Genetic Medicine

DOI: https://dx.doi.org/10.17352/amgm

Research Article

# Expression of *blaCTX-M2* and *invA* genes of Salmonella Heidelberg isolated from poultry by Qpcr

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**Received:** 16 March, 2020
**Accepted:** 15 April, 2020
**Published:** 16 April, 2020

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**Keywords:** Antibiotic resistance; Gene expression; InvA; Salmonella

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## Summary

Salmonellosis is a disease caused by a bacterium *Salmonella*, a gram negative bacilli found in many environments, responsible for significant economic losses in poultry, and of great impact on public health. Among more than 2500 serovars, *S.* Heidelberg seems to be more invasive causing disease of greater severity than other serovars. The objective of this study was to investigate, through real-time PCR (qPCR), differences in the expression of a virulent gene (*inv*A) and an antibiotic resistance gene (*bla*CTXM-2) of *S.* Heidelberg isolated from poultry meat (slaughterhouses) and drag swabs (field). Even though all isolates showed the presence of the *inv*A gene, there were differences in the expression among the isolates, where isolates from the field showed greater expression of *inv*A compared to samples isolated from meat products. On the other hand, isolates from the slaughterhouses showed greater expression of the *bla*CTX-M2 than those isolated from field samples.

## Introduction

*Salmonella* is a leading cause of foodborne illness in humans worldwide [1]. *Salmonella enterica* subsp. *enterica* serovar Heidelberg is one of the most important serovars that cause infections in humans, resulting in mild diarrhea to severe systemic illness [2,3]. That is because *Salmonella* Heildeberg seems to be more invasive, causing more severe disease than others serovars [4].

The virulence of *Salmonella* is directly related to its ability to invade the host, to replicate within host cells, and to resist destruction by phagocytic components or plasma complement components [5]. The *Salmonella* invasiveness is encoded in genes located in Salmonella Pathogenicity Island 1 (SPI-1) and the *inv*A gene is found in this island and it is considered a marker for virulent strains of *Salmonella* spp [6]. The *inv*A gene encodes several proteins involved in the Secretion Type Three System (TTSS), which is considered a molecular device that allows the bacteria to export to the interior of the host cells some proteins called "effectors" that help the bacterium to penetrate the intestinal cell, causing changes in the cytoskeleton and hence the cell framework, events that disrupt host physiology causing the appearance of symptoms that will define a more severe disease [6,7].

There has been increasing concern over the past few years regarding the worldwide emergence of multidrug-resistant phenotypes among *Salmonella* serovars, including *S.* Heidelberg. The *bla*CTX-M gene is usually associated to cephalosporin



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resistance, an antibiotic routinealy used to treat severe cases of salmonellosis in children [8]. Since the gene *inv*A is related to pathogenesis of Salmonellosis and gene blaCTX-M to antimicrobial resistance, we hypothetized that the expression of both genes were different depending on the location of the isolation (field or slaughterhouse). Thus, the main objective of this study was to investigate, through real-time PCR (qPCR), differences on the expression of *inv*A and *bla*CTXM-2 genes of *S.* Heidelberg isolated in the field (environmental swabs) and slaughterhouses (meat products).

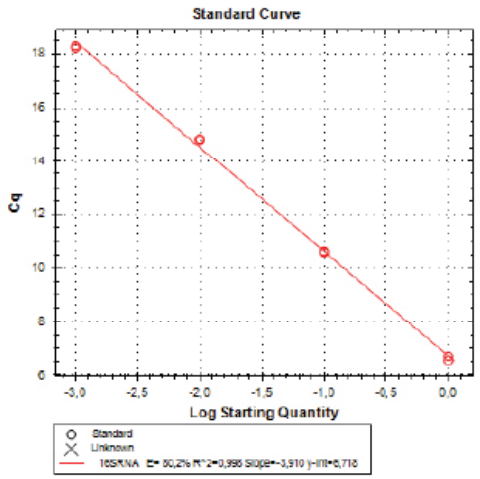


**Figure 1:** Standard calibration curve for the reference gene (16SRNAr) after mRNA extraction of *S.* Heidelberg.

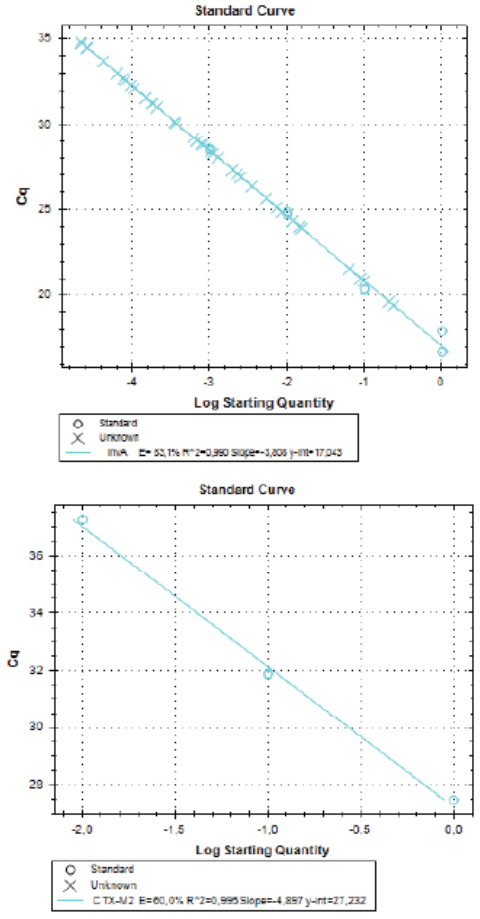


**Figure 2:** Calibration standard curve for *inv*A and *bla*CTX-M2 genes after mRNA extraction of *S.* Heidelberg.

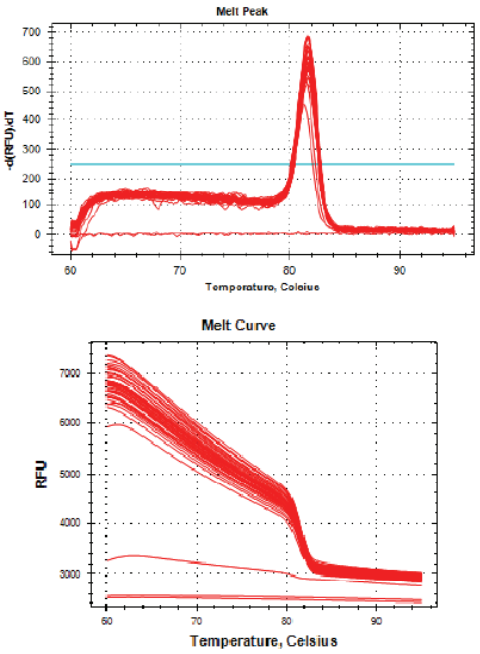


**Figure 3:** Melting peak and melting temperature of the gene 16SRNAr of *S.* Heidelberg used as reference gene.

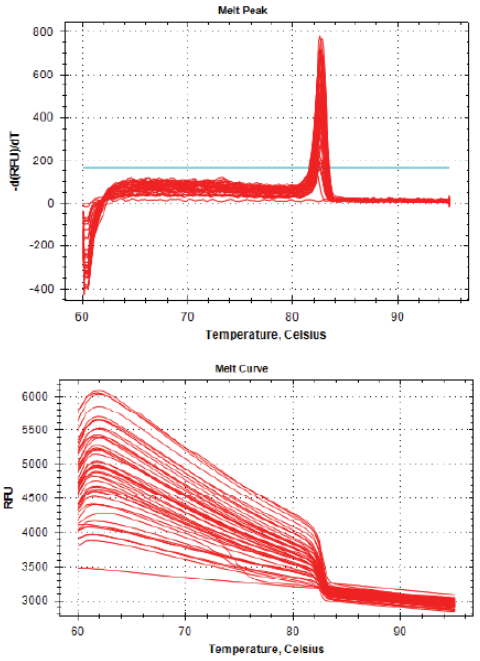


**Figure 4:** Melting peak and mealting temperature of the gene *inv*A.

## Results

### Real-time PCR (qPCR)

Real-time PCR was performed for 16SRNAr, *inv*A and *bla*CTX-M2 genes. The gene 16SRNAr was used as a control gene, and a straight line was obtained for calibration with a correlation coefficient ($R^2$) of 0.995 and reaction Efficiency (E)



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of 80.2% (Figure 1). As for the genes *inv*A and *bla*CTX-M2, the $R^2$ values were 0.990 and 0.995, respectively and E values were 83.1% and 60%, respectively (Figure 2). The analyses of the melting curve and melting temperature for the 16SRNAr gene, *inv*A and *bla*CTX-M2 can be seen in Figure 3-5, respectively. A single peak for 16SRNAr and *inv*A genes was identified, leading to the conclusion that there was no formation of nonspecific products, unlike *bla*CTX-M2 gene that showed several peaks known as "shoulders".

Table 2 shows the average values of Cq (quantification cycle) and gene expression for *inv*A and *bla*CTX-M2 genes for each *S*. Heidelberg isolate. All isolates of *S*. Heidelberg studied expressed *inv*A gene. However, the amount of expression of the type III secretion system varied among isolates. Different results were observed for *bla*CTX-M2 gene, where some isolates did not show the enzyme responsible for cefalosporin resistance. The sample number 55 (gene expression= 1:00), isolated from drag swab, was used as standard sample for the calculation of the expression of the other isolates. Among the isolates from slaugtherhouses (meat products), the sample identified as ID 53 showed *inv*A gene expression 3.42 times greater than the control sample. Some samples from the field (drag swabs, ID= 58) showed 66.88 times higher expression than the control and it was considered the samples with greater potential of virulence.

Expression of *bla*CTX-M2 gene was higher in samples from the slaughterhouse compared to field samples (ID 54 expression was 74.84 times higher than the control sample). Field isolate (ID 56) showed gene expression 24.26 times higher than the control and were considered the sample with larger amounts of beta-lactamase enzyme expressed by *bla*CTX-M2. In general, *inv*A expression was 2.53 times higher in samples collected from the field compared to samples from the slaughterhouse (Figure 6). On the other hand, Figure 7 shows that the expression of *bla*CTX-M2 gene was 4.94 higher in samples collected at the slaughterhouse compared to field samples.

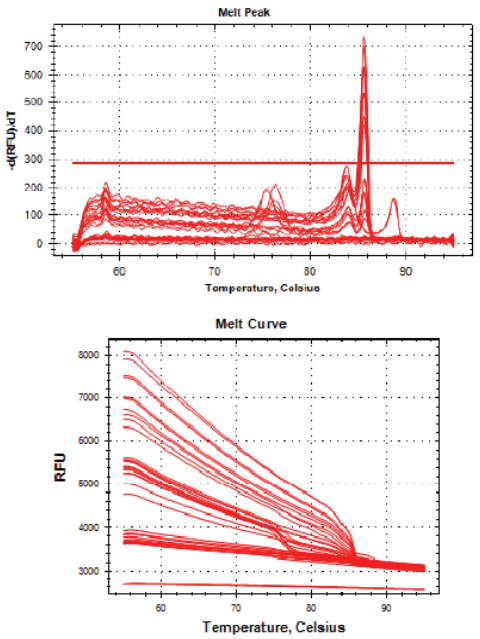


**Figure 5:** Melting peak and melting temperature of the gene *bla*CTX-M2.

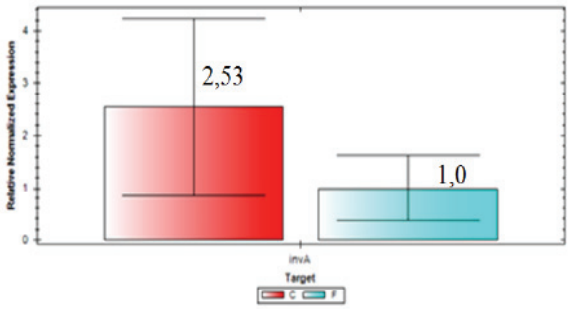


**Figure 6:** Normalized relative *inv*A expression of *S*. Heidelberg isolated from the field (C) and slaughterhouse (F).

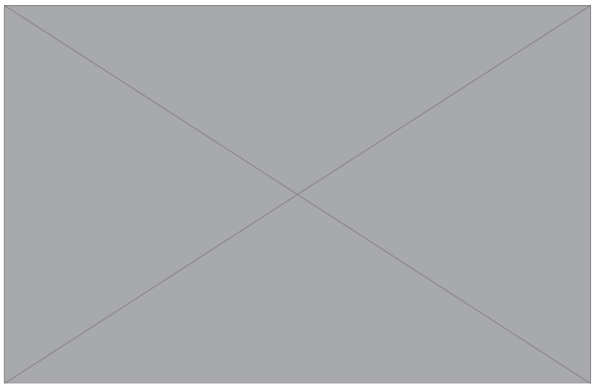

**Figure 7:** Relative normalized *bla*CTX-M2 expression of *S*. Heidelberg isolated from the field (C) and slaughterhouse (F).

## Discussion

It is possible to conclude, based on our results, that all isolates of *S*. Heidelberg analyzed were able to express the gene *inv*A and that there were differences in the expression, with greater level of expression in those samples isolated from drag swabs.

Since all isolates used in these study were previously tested and considered resistants to ceftiofur, our findings regarding the expression of *inv*A allow us to infer that the consumption of a product of poultry origin with this virulent strain of *Salmonella* could lead to human infection of difficult treatment [9], despite the fact that β-lactamase *bla*CTX-M2 has not been identified by qPCR technique, since other enzymes might be responsible for the resistance .

One reason for this observed variation may be due to the great diversity of resistance genes to antibiotics encoding β-lactamases in members of the *Enterobacteriaceae* family [10]. According to Singh, Batish and Grover [11], the other reason for the variation in the expression of *bla*CTX-M2 gene observed may be due to the fact that some strains lost the plasmid containing the resistance gene during storage.

The isolated ID 103 of *S*. Heildelberg from drag swab was



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shown to be even more virulent (48.10) (Table 1) and have been isolated from drag swab (field) can also become a problem for public health, as well as virulent showed expression of the gene for the enzyme *bla*CTX-M2 (1.21) (Figure 2). The two isolates, ID 103 and ID 53 would be excellent candidates for further *in vivo* tests of pathogenicity. The selection of resistant bacteria may result in a more virulent, since the presence of resistance of pathogenic bacteria can lead to a delay in the administration of antimicrobial therapy may be insufficient to eliminate them [12].

**Table 1:** Identification (ID) of the isolates of *S.* Heidelberg, source, origin (slaughterhouse and field), and the average values of Cq, and gene expression for *inv*A and *bla*CTX-M2.

| | | *invA* | | *bla*CTX-M2 | |
|---|---|---|---|---|---|
| ID | Source | Gene expression | Cq medium | Gene expression | Cq medium |
| Slaughterhouse | | | | | |
| 52 | poultry meat | 0.10 | 31.16 | 0.191 | 31.39 |
| 53 | poultry meat | 3.42 | 23.97 | 0.000 | 33.15 |
| 54 | poultry meat | 0.41 | 27.01 | 74.842 | 24.37 |
| 62 | poultry meat | 0.08 | 32.00 | 0.000 | 30.09 |
| 69 | poultry meat | 0.02 | 30.59 | 0.000 | 31.66 |
| 70 | poultry meat | 0.01 | 33.15 | 15.487 | 27.03 |
| 79 | poultry meat | 2.47 | 25.28 | 0.374 | 33.10 |
| Field | | | | | |
| 55* | drag swab | 1.00 | 24.10 | 1.00 | 29.35 |
| 56 | drag swab | 0.01 | 30.28 | 24.26 | 23.90 |
| 57 | drag swab | 0.01 | 34.48 | 0.36 | 33.33 |
| 58 | drag swab | 66.88 | 20.26 | 8.41 | 28.99 |
| 77 | drag swab | 0.73 | 24.86 | 0.05 | 33.79 |
| 80 | drag swab | 0.65 | 28.16 | 0.00 | 39.91 |
| 81 | drag swab | 1.36 | 26.86 | 0.15 | 31.81 |
| 82 | drag swab | 0.04 | 34.77 | 5.08 | 31.67 |
| 91 | dead bird | 3.49 | 29.15 | 0.00 | 39.49 |
| 103 | drag swab | 48.10 | 21.30 | 1.21 | 32.22 |
| 104** | drag swab | 0.24 | 30.15 | 0.55 | 33.41 |
| Average | | 7.17 | 28.19 | 7.33 | 31.59 |
| Standard deviation | | 18.10 | 4.18 | 17.58 | 4.04 |
| Coefficient of variation | | 2.53 | 0.15 | 2.40 | 0.13 |

* Standard sample used to calibrate gene expression.

** All samples were isolated in 2013 in the state of Paraná, except ID 104 which was isolated in 2012.

Often the fact that the isolates have shown one phenotypical resistance but did not express the *bla*CTX-M2 enzyme can be related to the presence of other β-lactamases also responsible for resistance to cephalosporins, such as *bla*CMY-2 and *bla*TEM. The resistance of β-lactamase mediated extended spectrum *bla*CMY-2 gene is a major mechanism of resistance to cephalosporins among strains of *Salmonella enterica* [12,13]. Resistance to β-lactam antibiotics, such as ceftriaxone, is correlated to an increase in the expression level of this enzyme [14]. In addition to express the *inv*A gene, the ID 62 and 69 isolates of meat products. They showed no expression level of *bla*CTX-M2 gene (Table 1).

Among the isolates from meat products, isolated ID 54 and 70 showed the highest levels of gene expression of *bla*CTX-M2 enzyme, this tells us that contamination by these bacteria might have impaired treatment, as well as the presence of the gene resistance, also showed that high doses of antibiotics are necessary to eliminate the bacteria, however *in vivo* analysis should be conducted in the future to confirm the relationship of the expression of *bla*CTX-M2 enzyme and resistance to ceftiofur, with possible failures in the treatment of salmonellosis. This scenario illustrates the big problem of antimicrobial resistance to public health, since all isolates were from meat products, showing the ease of transmission of these bacteria to humans by the consumption of contaminated poultry-derived products. Once infected with ESBL producing bacteria resistant to antibiotics that are commonly used for the treatment of humans, becomes more difficult and expensive therapy is often necessary hospitalizations for longer periods, it is possible that the drug is not enough to eliminate over there.

It is worth noting that the biggest problem of these superbugs are cases of complications in susceptible patients, such as those immunosuppressed. The selection of antibiotic-resistant pathogens, such as ceftiofur that have the same active principle of ceftriaxone, emphasizes once again the importance of the control of these pathogens, as these can be considered a potential source of resistant bacteria that can be transmitted to the bacteria in living beings. And if this should happen it increases the possibility of failures in the treatment of salmonellosis in children. In Figure 5 it is possible to observe a peak melting with abnormal peaks, which is called "shoulder" and is probably a consequence of the pair unspecific primers used, since the optimal size of the PCR product generated by qPCR is 80-150 bp and 486 bp not, size found. The sample 55 isolated from the ID field (drag swab) as well as having the potential for virulence (1.0), also demonstrated the expression of *bla*CTX-M2 enzyme (1.0) (Table 1), this was the standard sample used for calibration to calculate gene expression.

Increased resistance to broad-spectrum cephalosporins (ceftiofur and ceftriaxone) from *Salmonella* spp isolates is of significant interest to public health. This is due to the fact that ceftriaxone is an important drug of choice for the treatment of children with severe salmonellosis. Therefore, it is possible a guess that the consumption of a product of poultry origin contaminated with a bacterium resistant to ceftiofur or even contact with an animal that is infected with this bacterium



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**Table 2:** List of the genes, primer sequences, fragment size, primer concentration, number of the access in the Gene Bank and literatura reference.

| Gene | Primers | Fragment (pb) | Concentration (µM) | Access Gene Bank | Reference |
|---|---|---|---|---|---|
| *inv*A | F- GTGAAATTATCGCCACGTTCGGGCAAc | 284 | 0.15 | U43239 | Rahn et al., 1992 |
| | R-TCATCGCACCGTCAAAGGAAC | | | | |
| 16SRNAr | F-CAGAAGAAGCACCGGCTAACTC | 87 | 0.3 | X80681 | Botteldoom et al., 2006 |
| | R-GCGCTTTACGCCCCAGTAATT | | | | |
| *bla*CTX-M-2 | F-GGCGTTGCGCTGATTAACAC | 486 | 0.1 | X92507 | Chen et al., 2004 |
| | R-TTGCCCTTAAGCCACGTCAC | | | | |

could cross-resist to ceftriaxone. As a result, the use of this agent antimicrobials in food animals is under increased scrutiny for being a potential agent responsible for the emergence and spread of resistance to ceftriaxone in *Salmonella* spp and other enteric pathogens [9,15]. It is important to highlight the importance of sanitary and hygienic measures in the public health system, as well as in animal husbandry production system. Thus, it is evident the need for a health program and biosecurity measures to prevent colonization and infection of animals in order to limit the spread of this epidemic bacterium [16,17].

## Conclusion

All of *S*. Heidelberg isolates analyzed are virulent, but we observed a variation between isolates from slaughterhouses and field. By comparing the two organic groups, field isolates showed a higher expression of *inv*A. On the other hand, by analyzing the expression of *bla*CTX-M2 gene it was verified that isolates from slaughterhouses showed higher gene expression, and the resistance to ceftiofur could be explained by the presence of other β-lactamases and mechanisms. Further research should be conducted to better understand the mechanisms and the expression levels of these β-lactamases in the search for a solution that mitigates the prevalence of drug resistant.

## Experimental procedures

### RNA extraction

Molecular biology techniques were performed in the Laboratory of Molecular Biology, Immunology and Microbiology (LABMIM) of the State University of Santa Catarina (UDESC) in the West Center of High Education (CEO) in Chapecó city, Santa Catarina State, Southern Brazil. The samples submitted to RNA extraction were 18 isolates of *S*. Heidelberg obtained from a previous studies, well known ceftiofur resistants through the technique MIC (Minimum Inhibitory Concentration).

It should be noted that all samples were isolated in Paraná State in 2013, except the ID 104 which was isolated in 2012. First, the samples were removed from the freezer and grown in Brain Heart Infusion (BHI) for 24 hours at 37°C, followed by Brilliant Green agar for more 24 hours at 37°C. For RNA extraction, 3 to 5 colonies were isolated and inoculated again in BHI broth for 24 hours at 37°C up to a concentration of approximately $1.0\times10^9$ CFU/mL. RNA was extracted using the PureLink® RNA Mini Kit (Ambion, Life Technologies, Carlsbad, USA) and stored in liquid nitrogen (-80°C). The quality of the RNA was estimated by the OD 260/280 ratio, where ratio of 1.8 was considered optimal, indicating RNA free of proteins and other chromophores.

### Reverse transcriptase

In order to sinthetize cDNA from the RNA, a Reverse Transcriptase kit (Applied Biosystems, Foster, USA) with high capacity was used. Firstly, the RNA was treated with deoxyribonuclease I enzyme - Amplification Grade (Invitrogen Life Technologies, Carlsbad).

The preparation of the RT mastermix was made up using 300 to 700 ng of RNA (10 µL), 2 µL buffer, 0.8 µL of dNTP mix (100 mM), 2 primers, 1 µL enzyme Reverse Transcriptase (RT), and nuclease-free water up to a total volume of 20 µL for each reaction. The cDNA was treated with the enzyme inhibitor RNaseOUT Recombinant Ribonuclcease™ (Invitrogen Life Technologies, Carlsbad, USA). The cDNA was stored at -15°C until use in the qPCR reaction. The preparation of the cDNA was performed in a termiciclador T100 (Bio-Rad). A Minus Reverse Transcriptase control (MRT) was used as negative control in order to assess any amount of DNA contamination in the RNA preparation.

### Real-time PCR

The analysis of gene expression linked to the resistance of cephalosporins (*bla*CTX-M2) and the virulence gene *inv*A was performed using real-time PCR (qPCR). As reference gene the 16SRNAr gene was used. The concentration of the primer fragment size (bp), author and reference used to assess gene expression are described in Table 2.

The qPCR reactions were performed using the kit SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, USA) according to the manufacturer's instructions. Amplification conditions for *inv*A and 16SRNAr genes were obtained as described by Singh and Mustafa (2013)[10] and these were: 95°C for 10 minutes, 40 cycles of denaturation 95°C for 15 seconds, annealing and extension 60°C for 45 seconds.

Amplification conditions for *bla*CTX-M2 gene was obtained as described by Chen, et al., [18-23], which were: 95°C for 10 minutes, 40 cycles of denaturation 95°C for 30 seconds, annealing at 55 °C for 1 minute and extension at 72 °C for 1 minute and the final step was 72°C for 7 minutes. For all three genes, after 40 cycles of amplification all samples were subjected to analysis of the dissociation curve (melting curve) to confirm the absence of non-specific products and primer dimers. The samples were subjected to a gradual temperature increase of 0.1°C for 5 seconds, from 60°C up to 95°C. To determine the efficiency of the reaction and the dissociation



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curve reactions were optimized for the four genes evaluated. To this end, there was a pool with all samples followed by serial dilutions (pure cDNA, 1:10, 1:100 and 1:1000). Each sample was done in duplicate in specific qPCR optical plates with 96 wells, sealed with optical adhesive film, and amplified in Real Time CFX96 thermocycler (Bio-Rad). Amplification results were analyzed using the Bio-Rad CFX Manager software . A No Template Control (NTC) to omit any RNA template was used as a negative control for extraneous nucleic acid contamination.

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