Material and Methods
3. MATERIAL AND METHODS

3.1 Materials

Culture Media

Nutrient Agar (Hi-Media, Pvt. Ltd, Mumbai, India)
Mueller-Hinton Agar (Hi-Media, Pvt. Ltd, Mumbai, India)
Luria Bertani Broth (Hi-Media, Pvt. Ltd, Mumbai, India)
Peptone Water (Hi-Media, Pvt. Ltd, Mumbai, India)

Mc Farland Standard

BaCl₂ (SRL, India)
H₂SO₄ (SRL, India)

Antibiotics

Antibiotics discs used are listed below and the concentration is given in parenthesis. All the antibiotics discs were purchased from Hi-Media, Pvt. Ltd, Mumbai, India.

Cefoxitin (30µg)
Ceftriaxone (30µg)
Cefoperazone (75µg)
Cefixime (30µg)
Ceftazidime (30µg)
Cefepime (30µg)
Cefpirome (30µg)
Gentamicin (10µg)
Amikacin (10µg)
Ofloxacin (5µg)
Gatifloxacin (5µg)
Aztreonam (30µg)
Imipenem (10µg)
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PCR reagents

2X premixed Master Mixture (Fermentas Life sciences, USA)
[Consist of 0.05 u/μL Taq DNA Polymerase, reaction buffer, 4mM MgCl₂, 0.4 mM of each dNTP (dATP, dCTP, dGTP, and dTTP)]
DNase/RNase free water (Fermentas Life sciences, USA)
0.2 mL thin walled propylene tubes (Bangalore Geneti, India)

Plasmid Isolation

All the chemicals were purchased from SRL, India. λ-DNA double digested with EcoRI and HindIII was procured from Fermentas Life sciences (USA) and Ethanol from E. Merck (India).

STE (Saline + TE Buffer):

NaCl
Tris-Cl
EDTA

Sol I (pH 8.0): It can be prepared in batches of approximately 100mL, autoclaved and stored at 4°C
Glucose
Tris-Cl
EDTA

Sol II:

NaCl
SDS

Sol III:

Potassium acetate
Glacial acetic acid
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Saturated phenol:

Crystalline phenol

Hydroxyquinoline

Chloroform:isoamyl alcohol (24:1)

Ethanol

γ-DNA double digested with *EcoRI* and *HindIII*

**Agarose Gel Electrophoresis**

Agarose (Low EEO) (Bangalore Genei, India)

Ethidium Bromide (Bangalore Genei, India)

1X TAE Buffer: Chemicals were purchased from SRL, India.

[4.85 gm/L Tris·Cl, 0.35 gm/L EDTA, 1.14 mL/L Glacial acetic acid, pH 7.9]

6X Tri-Gel loading dye (Bangalore Genei, India)

High Range DNA ruler (Bangalore Genei, India)

O’Range Ruler 100 (Fermentas Life sciences, USA)

bp + 500 bp DNA Ladder
3.2 Methods

3.2.1 Clinical sample collection

A total of 14,129 samples were subjected to department of microbiology, J. N. Medical College, Aligarh Muslim University, Aligarh, for routine culture and susceptibility testing in a period of 18 months (January 2009 to June 2010). Of these Gram-negative organisms among which 1610 isolates were identified as \textit{E. coli} and 455 as \textit{K. pneumoniae} by standard microbiological techniques (Collee \textit{et al.}, 1996; Holmes & Aucken, 1998). A total of 109 \textit{E. coli} and 16 \textit{K. pneumoniae} (that were found resistant to cefoxitin) were randomly selected in order to illustrate the prevalence of AmpC \( \beta \)-lactamases and for detailed genotypic characterization. Demographic details of the patients were also noted. All these cefoxitin-resistant isolates were obtained from pus, urine, drain, semen, cervical swabs, CSF, naso-pharyngeal aspirate, vault, and abdominal fluid.

3.2.2 Antimicrobial susceptibility testing

3.2.2.1 Turbidity standard for inoculum preparation: To standardize the inoculum density for a susceptibility test, the \text{BaSO}_4 turbidity standard equivalent to 0.5 McFarland standard was used (Andrews \textit{et al.}, 2004). A 0.5 mL aliquot of 0.048 M \text{BaCl}_2 (1.175\% \text{BaCl}_2.2\text{H}_2\text{O}) was added to 99 mL of 0.18 M \text{H}_2\text{SO}_4 (1\% \text{v/v}) with constant stirring. The correct density of the 0.5 McFarland standard was verified by determination of absorbance at 625 nm with a 1 cm path length and was found to be 0.08 to 0.10. The turbidity standard was stored in aliquots of 4-6 mL and can be stored for up to six months.
3.2.2.2 **Disc diffusion susceptibility test:** Antimicrobial susceptibility testing was performed and interpreted as per CLSI (formerly NCCLS) guidelines (CLSI, 2005). Briefly, disc diffusion susceptibility test was performed on Mueller Hinton (MH) Agar. Bacterial inoculum was prepared by mixing few colonies of the test strain in the test tube containing 1.5 mL of sterile nutrient broth. The density of suspension was standardized by dilution with sterile broth to a density visually equal to 0.5 McFarland units. It results in a suspension containing approximately $1.5 \times 10^8$ CFU/mL. MH Agar plates were inoculated with a sterile cotton swab dipped in test inoculum, surplus of it was removed by rotating swab against the wall of test tube, then spreading on dried surface of MH Agar. This procedure of streaking on agar plate was repeated at least three times by rotating plate approximately 60° each time to ensure even distribution of inoculum. The antibiotics discs were applied with the help sterile forceps. The plates were inverted and placed in an incubator set at 37°C and were incubated overnight. The zone diameters were measured including the diameter of disc. Following antibiotics (all purchased from Hi-Media, Pvt. Ltd, Mumbai, India) were tested; cefoxitin (30μg), ceftriaxone (30μg), cefoperazone (75μg), cefixime (30μg), ceftazidime (30μg), cefepime (30μg), ceftizoxime (30μg), gentamicin (10μg), amikacin (10μg), ofloxacin (5μg), gatifloxacin (5μg), and aztreonam (30μg), Imipenem (10μg). *E. coli* ATCC 25922 was used as a control strain.

3.2.3 **Phenotypic detection of AmpC producers**

3.2.3.1 **Phenotypic confirmatory test:** Modified Three dimensional extract test (MTDET), as described by Shahid *et al.*, (2004), was performed to identify AmpC producers. Briefly, 10-15 mg of bacterial wet weight was scraped from culture plate and suspended in 0.5mL of peptone water in a sterile micro-centrifuge tube and incubated at 37°C for one hour. Crude enzyme extract was prepared by repeated freezing-thawing. To
ensure complete membrane lysis, the freezing-thawing may be carried out five times. Lawn culture of \textit{E. coli} ATCC 25922 was prepared on MH Agar plate and was incubated at 37°C so that plate can dry properly. Cefoxitin discs (30µg) were placed on dried MH Agar plate. With a sterile scalpel a linear trench (3cm x 1mm) was prepared in agar at a distance of 5mm from edge of the disc in an outward radial direction. 50µl of enzyme preparation was dispensed in trench and overfill was avoided. The inoculated media was incubated overnight at 37°C. Enhanced growth of the surface organism at the point where trench intersected the zone of inhibition towards the cefoxitin disc was interpreted as evidence for the presence of AmpC β-lactamase.

3.2.4 Genotypic characterization of cefoxitin-resistant isolates

3.2.4.1 DNA template preparation: Bacterial isolates were cultured on Nutrient Agar plates and were incubated overnight at 37 °C. Template DNA was prepared from freshly cultured bacterial strains by scraping 2-3 colonies and suspending them in 50 µL of molecular grade water. The bacterial suspension was then heated at 95 °C for 5 min. and then immediately cooled to 4 °C.

3.2.4.1.1 Detection of \textit{bla}_{ampc} genes: \textit{bla}_{ampc} genes were detected by PCR. as described by Feria \textit{et al.} (2002) with some modifications. Briefly, the gene of interest was amplified in a total reaction volume of 25 µl containing 0.05 µl each of primer ampC-f and ampC-r. 12.5 µL master mixture, 9.9 µL DNase/RNase free distil water. 2.5 µL of template DNA was added to 22.5 µL of master mixture. The reaction mixture was placed in MJ-mini Bio-Rad thermal cycler (Bio-Rad, USA). The PCR amplification cycle was performed with cycling conditions consisting of an initial denaturation step at 95°C for 15 min, followed by 35 cycles consisting of final denaturation step at 94°C for 60 sec, annealing at 58°C for 2 min, extension at 72°C for 3 min, and the process was completed with final
elongation step at 72°C for 10 min. Sequence of ampC-f and ampC-r primers is shown in Table 1.

3.2.4.1.2 Detection of plasmid-mediated AmpC β-lactamases: All isolates were tested by multiplex PCR for detection of family-specific plasmid-mediated AmpC β-lactamase genes by using method as described by Perez-Perez & Hanson (2002). Reaction mixture was prepared by mixing 12.5 µL master mixture, 7.44 µL DNase/RNase free distil water, 0.24 µL each of forward & reverse MOX, CIT, and DHA primers, 0.20 µL each of ACC and EBC forward & reverse primers and 0.16 µL each of forward & reverse FOX primer. 2.5 µL of template DNA was added to 22.5 µL of master mixture. Samples were placed in MJ-mini Bio-Rad thermal cycler (Bio-Rad, USA). PCR was carried out with an initial denaturation step at 94°C for 3 min, followed by 30 cycles consisting of final denaturation step at 94°C for 30 sec, annealing at 64°C for 30 sec, extension at 72°C for 1 min. PCR was completed with final elongation at 72°C for 10 min. Sequences of the primers used in AmpC multiplex PCR are shown in Table 1.

3.2.4.1.3 Characterization of specific AmpC type: Representative isolates were selected for DNA sequencing so as to define presence of specific AmpC type. The primer CIT-F (5'-TGG CCA GAA CTG ACA GGC AAA-3') and EBC-F (5'-TCG GTA -AAG CCG ATG TTG CGG-3'), as used in multiplex PCR protocol, was used as primers for sequencing of CIT and EBC amplicons respectively. The cycling conditions for amplifying CIT and EBC amplicon consist of an initial denaturation step at 95°C for 3 min, followed by 30 cycles consisting of final denaturation at 95°C for 60 sec, annealing at 55°C for 60 sec, and extension at 72°C for 60 sec, and a final elongation at 72°C for 5 min.
CIT amplicons were also amplified by using primer ISEcp1 PROM+ as forward primer in order to obtain whole ORF of \( \text{bla}_{\text{CIT}} \). Reaction mixture for PCR comprise of a total reaction volume of 25 \( \mu \text{L} \) containing 0.05 \( \mu \text{L} \) each of primers ISEcp1 PROM+ and CIT-R, 12.5 \( \mu \text{L} \) master mixture, 9.9 \( \mu \text{L} \) DNase/RNase free distil water. 2.5 \( \mu \text{L} \) of template DNA was added to 22.5 \( \mu \text{L} \) of the master mixture. The cycling conditions consist of an initial denaturation step at 95°C for 5 min, followed by 39 cycles of 95°C for 60 sec, 58°C for 60 sec, and 72°C for 60 sec, and a final elongation at 72°C for 10 min. Sequencing primers are shown in Table 1.

Sequencing was performed by the courtesy of Chromous Biotech, Ltd, Bangalore, India. Analysis of sequenced PCR amplicons was done by comparing sequence to the GenBank database of the National Centre for Biotechnology Information BLAST network.

3.2.4.2 Analysis of co-carriage of class C and Class A \( \beta \)-lactamase:

3.2.4.2.1 Detection of \( \text{bla}_{\text{CTX-M}} \), \( \text{bla}_{\text{TEM}} \), and \( \text{bla}_{\text{SHV}} \): The isolates of this study were also screened for the presence of Class A ESBLs, more specifically, \( \text{bla}_{\text{CTX-M}} \), \( \text{bla}_{\text{TEM}} \), and \( \text{bla}_{\text{SHV}} \) by the PCR protocol as described previously (Shahid, 2010). Briefly, PCR Reaction mixture was prepared by mixing 12.5 \( \mu \text{L} \) master mixture, 9.9 \( \mu \text{L} \) DNase/RNase free distil water, 0.05 \( \mu \text{L} \) each of forward and reverse primer. 2.5 \( \mu \text{L} \) of template DNA was added to 22.5 \( \mu \text{L} \) of master mixture. PCR cycling conditions consist of initial denaturation at 94°C for 7 min, followed by 35 cycles consist of 94°C for 50 sec, 50°C for 40 sec. and 72°C for 2 min. and a final elongation at 72°C for 5 min. An amplicon of 593bp was obtained indicating the presence of \( \text{bla}_{\text{CTX-M}} \).

\( \text{bla}_{\text{TEM}} \) and \( \text{bla}_{\text{SHV}} \) were detected by using the protocol as used in previous studies from our laboratory (Shahid, 2010). The cycling condition for detection of both genes were
same, comprising with an initial denaturation step at 95°C for 15 min; followed by 35 cycles comprised of final denaturation at 94°C for 1 min., annealing at 58°C for 2 min., extension at 72°C for 2 min. and final elongation at 72°C for 10 min. The amplified DNA of 936bp and 930bp were obtained for bla\textsubscript{TEM} and bla\textsubscript{SHV} respectively. Details of primers used in detection of bla\textsubscript{CTX-M}, bla\textsubscript{TEM}, and bla\textsubscript{SHV} are given in Table 1.

3.2.4.3 Characterization of mobile elements

3.2.4.3.1 Detection of Insertion sequences

3.2.4.3.1.1 Detection of ISEcpI: All 125 isolates (109 \textit{E. coli} and 16 \textit{K. pneumoniae}) were characterized for insertion sequence ISEcpI which is known to mobilize mainly bla\textsubscript{CTX-M} genes and recently it has been reported associated with various AmpC types also (Jacoby, 2009). The forward primer used was ISEcpI\textsubscript{U1} while reverse primer was P2D. Reaction mixture was prepared by mixing 0.05 µl each of primers ISEcpI\textsubscript{U1} & P2D, 12.5 µL master mixture, 9.9 µL DNase/RNase free distil water. 22.5 µL of the master mixture was aliquoted in PCR tubes and 2.5 µL of template DNA was added to it. Amplified product of ~1100 bp was obtained when PCR product was electrophoresed on 2% agarose gel. PCR cycling conditions for amplification of ISEcpI consist of an initial denaturation step at 95 °C for 3 min, followed by 30 cycles comprised of final denaturation step at 95°C for 60 sec, annealing at 55°C for 60 sec. & extension at 72°C for 1 min. and the process was completed by a final elongation step at 72°C for 10 min. Sequences of primers used in the PCR reaction is shown in Table 1.

3.2.4.3.1.2 Detection of IS26: All test isolates were then looked for another insertion sequence IS26 as diversity and its insertion in \textit{tnpA} and ISEcpI gene have been reported previously (Ensor \textit{et al.}, 2006). To determine association of IS26 with bla\textsubscript{ampC}, PCR was
performed with primer set consist of IS26-F and SHA as forward and reverse primer respectively. PCR reaction mixture was prepared in a total volume of 25 μL (0.05 μL each of primers IS26-F and SHA, 12.5 μL master mixture, 9.9 μL DNase/RNase free distil water, 22.5 μL of the master mixture and 2.5 μL of template DNA was added to it), dispensed in PCR tube and placed in MJ-mini Bio-Rad thermal cycler (Bio-Rad, USA). PCR cycling condition for amplification of IS26 comprise of an initial denaturation step at 94°C for 5 min., followed by 35 cycles consisting of final denaturation step at 94 °C for 25 sec, elongation at 52 °C for 40 sec, extension at 72 °C for 50 sec, and process was completed by a a final elongation step at 72 °C for 6 min. Amplified products of different molecular weight were observed. Primers Sequences used in the PCR reaction are shown in Table 1.

3.2.4.3.2 Characterization of integrons: cefoxitin-resistant E. coli and K. pneumoniae isolates were also looked for the presence of integrons in order to illustrate the location of antibiotic resistance genes (especially bla_ampc). Presence of integrons was demonstrated by PCR amplification, using primer set 5CS-F1 and 3CS-R as forward and reverse primers respectively. PCR reaction mixture was prepared in a total volume of 25 μL comprising of 0.05 μL each of primers 5CS-F1 and 3CS-R, 12.5 μL master mixture, 9.9 μL DNase/RNase free distil water and 2.5 μL of template DNA. The reaction mixture so prepared was dispensed in PCR tube and placed in MJ-mini Bio-Rad thermal cycler (Bio-Rad, USA). PCR cycling conditions consist of an initial denaturation step at 95°C for 3 min., followed by 30 cycles of 95 °C for 1 min, 58 °C for 1 min, 72 °C for 2 min, and a final elongation step at 72 °C for 10 min. Sequences of the primers used are shown in Table 1. Amplified products were obtained at various positions corresponding to different molecular weights.
3.2.4.3.2.1 Detection of Sul-1-type class-1 integrons: Sul-1-type class 1 integrons were detected in bacterial isolates by using primers Sul 1A and Sul 1B as forward and reverse primers, respectively. Reaction mixture for PCR comprised of 0.05 µl each of forward and reverse primer, 12.5 µL master mixture, 9.9 µL DNase/RNase free distil water and 2.5 µL of template DNA. An amplified product of 420 bp indicates presence of Sul-1 gene in the test isolate. Cycling conditions comprises of an initial denaturation step at 95 °C for 3 min., followed by 30 cycles of 95 °C for 1 min. 63 °C for 1 min. and 72 °C for 2 min., and a final elongation step at 72 °C for 10 min. Details of the primers used are shown in Table 1.

3.2.4.3.2.2 Detection of ISCR1 (ORF513): ISCR1, which was previously known as ORF513, was detected in a collection of test isolates by PCR protocol as described previously (Shahid, 2010) using ORF513 D3 and ORF513 D5 as forward and reverse primers. PCR was performed in a total reaction volume of 25 µl containing 0.05 µl each of forward and reverse primer, 12.5 µL master mixture, 9.9 µL DNase/RNase free distil water. 2.5 µL of template DNA was added to 22.5 µL of master mixture. The reaction mixture was placed in MJ-mini Bio-Rad thermal cycler (Bio-Rad, USA). PCR cycling conditions were as follows: initial denaturation at 95 °C for 3 min., followed by 35 cycles of 95 °C for 1 min, 58 °C for 1 min. 72 °C for 2 min. and final elongation at 72 °C for 10 min. An amplified product of 600bp demonstrates the presence of ORF513. Sequences of the primers used in the protocol are shown in Table 1.

3.2.5 Visualization of amplified PCR product:

PCR products were separated by electrophoresis for 2-3 h at 75 V using 1X TAE running buffer (4.85 gm/L Tris-Cl, 0.35 gm/L EDTA, 1.14 ml/L Glacial acetic acid,
pH 7.9). After electrophoresis DNA fragments were visualized and photographed by Bio-Rad Gel documentation system (Bio-Rad, USA).

3.2.6 Typing of cefoxitin-resistant isolates by RAPD-PCR:

3.2.6.1 RAPD typing: Epidemiological typing of cefoxitin-resistant isolates was done, as described previously (Shahid, 2010), so as to determine whether any specific clone is circulating in the hospital environment or to find out any relatedness/diversity in bla<sub>ampC</sub> carrying isolates. Briefly, bacterial isolates were grown on Nutrient agar plate and incubated for overnight at 37 °C. Template DNA was prepared by suspending 3-5 colonies in 50 μL molecular grade water and heating it to 95 °C for 5 min. and then immediate cooling to 4 °C. 25 pmol of primer ERIC-2b (5'-AAG TAA GTG ACT GGG GTG AGC G-3") was used. Reaction mixture was prepared by mixing 12.5 μL of master mix, 10.25 μL of distilled water, 0.25 μL of primer and 2.0 μL of template DNA. The PCR amplification was attained by placing reaction mixture containing tubes in MJ-mini Bio-Rad thermal cycler (Bio-Rad, USA). PCR cycling conditions consist of an initial denaturation step at 94°C for 2 min., followed by 35 cycles of 94°C for 1 min, 25°C for 1 min, 72°C for 4 min. and a final elongation step at 72°C for 8 min. The results were analyzed by using Bio-Rad Gel documentation system (Bio-Rad, USA) and clustering was performed by Quantity One software provided by gel documentation system. Isolates of same species were typed in same batch and isolates of different wards were compared together to identify clonal spread.

3.2.7 Plasmid analysis:

Plasmid isolation was done in all test isolates by large scale alkaline lysis method, with some modifications as described previously (Shahid <i>et al.</i>, 2003). Briefly, the bacterial
isolates were inoculated in 250 mL of Luria-Bertani Broth and incubated at 37°C for 18 hours. 2 mL of the culture was transferred into microfuge tube and was centrifuged at 10,000 rpm for 5 minutes at 4°C. Bacterial cell pellet was dissolved in 0.5 mL of STE (sucrose + TE buffer) buffer, in order to remove the cell-wall components of bacteria that may be present in medium, and were re-centrifuged at 10,000 rpm for 5 minutes at 4°C. Supernatant was discarded and the pellet was dissolved in 200 µL of ice-cold solution I (50mM glucose, 25mM Tris-Cl, and 10mM EDTA) and contents were mixed by vigorous vortexing. 400 µL of freshly prepared solution II (0.2N NaOH and 1% SDS) was then added. The tubes were stored on ice. Then 300 µL of ice-cold solution III (5M potassium acetate, glacial acetic acid, and distilled water) was added and the tubes were stored on ice for 10 minutes. Contents were centrifuged at 10,000 rpm for 5 minutes at 4°C. Supernatant will contain most of plasmid with RNA and protein as contaminant. Supernatant was transferred to a sterilized fresh tube and pellet was discarded. Equal volume of phenol:chloroform was added and the content were mixed by vortexing. The microfuge tubes were centrifuged at 10,000 rpm for 2 minutes at 4°C to separate aqueous and organic phases. Aqueous phase was removed and transferred to a fresh tube and pellet was discarded. Double-stranded DNA was precipitated by adding 2 volumes of pre-chilled ethanol, and then centrifuged at 10,000 rpm for 5 minutes at 4°C to pellet DNA. Supernatant was discarded and the tubes were inverted on paper towel so as to allow the fluid to drain away. The pellet of nucleic acid was dissolved in 40 µL of autoclaved double-distilled water. 10 µL of plasmid samples were electrophoresed in 0.8% agarose gel containing ethidium bromide. λ DNA double-digested with EcoRI and HindIII (Bangalore Genei, India) was used as molecular weight marker.
Centrifuge 2 mL of bacterial culture at 10,000 rpm for 5 min at 4°C.

Add 200μL of ice cold sol I, mix by vortexing

Add 400 μL of freshly prepared sol II

Discard supernatant

Mix the contents by inverting tubes, and store the tubes on ice

Add 300 μL of freshly prepared Sol III

Store the tubes on ice for 10 min.

Centrifuge at 10,000 rpm for 5 min at 4°C

Transfer supernatant to a fresh microfuge tube

Discard pellet

Add equal volume of phenol:chloroform, mix by vortexing

Centrifuge at 10,000 rpm for 2 min at 4°C

Transfer supernatant to a fresh microfuge tube

Precipitate the double-stranded DNA by adding two volumes of ethanol

Mix the contents by inverting tubes for 2 min.

Centrifuge at 10,000 rpm for 5 min at 4°C

Discard supernatant

Dry the pellet by inverting the tubes on paper towel

Dissolve in 40μL of autoclaved double distilled water

Fig. 4 Schematic diagram of plasmid isolation.
<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer</th>
<th>Primer sequence</th>
<th>Expected amplicon size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>bla</em>&lt;sub&gt;M&lt;/sub&gt;</td>
<td>AmpCF</td>
<td>5'-CCC CGC TTA TAG AGC AAC AA-3'</td>
<td>634 bp</td>
<td>Feria et al., 2002</td>
</tr>
<tr>
<td><em>bla</em>&lt;sub&gt;M&lt;/sub&gt;</td>
<td>AmpCF</td>
<td>5'-CTA ATG GTC GAC TTC ACA CC-3'</td>
<td>634 bp</td>
<td>Feria et al., 2002</td>
</tr>
<tr>
<td><em>bla</em>&lt;sub&gt;CIT&lt;/sub&gt;</td>
<td>MOXM</td>
<td>5'-GCT GCT CAA GGA GCA CAG GAT-3'</td>
<td>520 bp</td>
<td>Perez-Perez &amp; Hanson, 2002</td>
</tr>
<tr>
<td><em>bla</em>&lt;sub&gt;CIT&lt;/sub&gt;</td>
<td>MOXM</td>
<td>5'-CAC ATT GAC ATG GTG GTG C-3'</td>
<td>520 bp</td>
<td>Perez-Perez &amp; Hanson, 2002</td>
</tr>
<tr>
<td><em>bla</em>&lt;sub&gt;DHA&lt;/sub&gt;</td>
<td>CITM</td>
<td>5'-TGG CCA GAA CTG ACA GGC AAA-3'</td>
<td>462 bp</td>
<td>Perez-Perez &amp; Hanson, 2002</td>
</tr>
<tr>
<td><em>bla</em>&lt;sub&gt;DHA&lt;/sub&gt;</td>
<td>CITM</td>
<td>5'-TTT CTC CTG AAG GTG GGT GCC-3'</td>
<td>462 bp</td>
<td>Perez-Perez &amp; Hanson, 2002</td>
</tr>
<tr>
<td><em>bla</em>&lt;sub&gt;ACC&lt;/sub&gt;</td>
<td>ACCM</td>
<td>5'-AAC ATG CCT CTC AGC AGC CGG TTA-3'</td>
<td>346 bp</td>
<td>Perez-Perez &amp; Hanson, 2002</td>
</tr>
<tr>
<td><em>bla</em>&lt;sub&gt;ACC&lt;/sub&gt;</td>
<td>ACCM</td>
<td>5'-TTC GGC GCA ATC ATC CCT AGC-3'</td>
<td>346 bp</td>
<td>Perez-Perez &amp; Hanson, 2002</td>
</tr>
<tr>
<td><em>bla</em>&lt;sub&gt;FOX&lt;/sub&gt;</td>
<td>EBCM</td>
<td>5'-CAC ATG GGG TAT CAG GGA GAT G-3'</td>
<td>190 bp</td>
<td>Perez-Perez &amp; Hanson, 2002</td>
</tr>
<tr>
<td><em>bla</em>&lt;sub&gt;FOX&lt;/sub&gt;</td>
<td>EBCM</td>
<td>5'-TTG GTA AAG CGG ATG TCG CTA ATG-3'</td>
<td>190 bp</td>
<td>Perez-Perez &amp; Hanson, 2002</td>
</tr>
<tr>
<td>IS&lt;i&gt;&lt;/i&gt;Ep1</td>
<td>IS&lt;i&gt;&lt;/i&gt;Ep1U1</td>
<td>5'-AAA AAT GAT TGA AAG GTG ATG-3', P2D</td>
<td>1100 bp</td>
<td>Enser et al., 2006</td>
</tr>
<tr>
<td>IS&lt;i&gt;&lt;/i&gt;26</td>
<td>IS&lt;i&gt;&lt;/i&gt;26</td>
<td>5'-CAG CGC TTT TGC CTT AAT AGG-3', variable</td>
<td></td>
<td>Enser et al., 2006</td>
</tr>
<tr>
<td>IS&lt;i&gt;&lt;/i&gt;CRI</td>
<td>ORF513D3</td>
<td>5'-CTC AGC CCC TGG CAA GTT ATT-3'</td>
<td>600 bp</td>
<td>Shahid, 2010</td>
</tr>
<tr>
<td>IS&lt;i&gt;&lt;/i&gt;CRI</td>
<td>ORF513D5</td>
<td>5'-CTT TGG CCC TAG GTG CCG T-3'</td>
<td>420 bp</td>
<td>Shahid, 2010</td>
</tr>
<tr>
<td>&lt;i&gt;Sal&lt;/i&gt;-&lt;i&gt;&lt;/i&gt;i</td>
<td>SulA</td>
<td>5'-ATT CGG GAG GGG AAA CCC GGC CC-3'</td>
<td>593 bp</td>
<td>Shahid, 2010</td>
</tr>
<tr>
<td>&lt;i&gt;Sal&lt;/i&gt;-&lt;i&gt;&lt;/i&gt;i</td>
<td>SulB</td>
<td>5'-ATG TTA CGC AGC AGG GC-3'</td>
<td>variable</td>
<td>-</td>
</tr>
<tr>
<td><em>bla</em>&lt;sub&gt;C&lt;/sub&gt;&lt;sub&gt;TX-M&lt;/sub&gt;</td>
<td>CTX-MU1</td>
<td>5'-ATG TGC AGY ACC AGT AAR GTT-3'</td>
<td>593 bp</td>
<td>Shahid, 2010</td>
</tr>
<tr>
<td><em>bla</em>&lt;sub&gt;C&lt;/sub&gt;&lt;sub&gt;TX-M&lt;/sub&gt;</td>
<td>CTX-MU2</td>
<td>5'-CTG QTR AAR TAR GTS ACC AGA-3'</td>
<td>593 bp</td>
<td>Shahid, 2010</td>
</tr>
<tr>
<td><em>bla</em>&lt;sub&gt;TEM&lt;/sub&gt;</td>
<td>TEM-F</td>
<td>5'-KAC ATT AAC CTT GGT AAA TGC-3'</td>
<td>936 bp</td>
<td>Shahid, 2010</td>
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<td><em>bla</em>&lt;sub&gt;TEM&lt;/sub&gt;</td>
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<td>5'-TTT ATC CTC GGC CTT CTA AAG-3'</td>
<td>930 bp</td>
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