4.1. **Study duration and settings:**

The study was conducted during the period from August 2010 to January 2013 on samples from patients admitted in Kasturba Medical college hospital Attavar and Ambedkar circle, Mangalore.

4.2. **Sample size calculation:**

On the basis that 24% of the hospital isolates are haemolytic (in accordance with results of previous studies)\(^3\)\(^-\)\(^7\),

A sample size of 300 was calculated using the formula:

\[
n = \frac{Z^2 \alpha^2 p(1-p)}{L^2} \quad \text{(with 80% power)}
\]

**Study design:** Descriptive study.

**Sampling methods:** Convenience sampling.

4.3. **Patients and participants:**

The study population included hospitalized patients of all age groups whose extraintestinal clinical samples grew *E. coli* and those subjects who had received antimicrobial drugs during the past one month, who had asymptomatic UTI, polymicrobial infections and those who were discharged without treatment with antimicrobial drugs were excluded.

4.4. **Clinical Details:**

The clinical details like evidence of haemolysis, systemic inflammatory response syndrome and organ failure features of HUS and Thrombocytopenic purpura (TTP), symptoms of pneumonia, ARDS, presence of diabetes, patient’s immune status, symptoms of UTI, WBC count, TC, Sodium potassium level, patient under dialysis or in ventilator, urine output, creatinine level were collected in a student’s proforma. Details of antibiotics used and clinical outcome were collected, APACHE II scores were calculated for all patients based on the available data to quantify clinical virulence and severity of the infection. Patients follow up were done for 1 year period.

*Invasive infection:* Blood stream infection with feature of MODS, systemic inflammatory response syndrome were consider as invasive infection.
4.5. Isolation and Identification:

Three hundred strains of *Escherichia coli* were isolated from specimen such as clean catch midstream urine, blood, wound swab, pus, CSF, ascitic fluid and intravascular devices, using standard sterile procedures from the study population.

The isolates were identified based on colony morphology MacConkey’s agar, Gram staining and by standard biochemical tests. Blood isolates were identified using automated biochemical system Vitek 2 (bioMerieux).

4.6. Phenotypic characterization:

4.6a. Haemolysin production:

Production of haemolysin were detected by growing the different strains in LB medium overnight (37º C) and dropping 50 µL of this culture on a Petri dish containing sheep blood agar. The culture were incubated at 37º C overnight and haemolysin production were verified by the presence of a clear hemolytic halo around the colony.

4.6b. Sorbital MacConkey Agar:

Isolated strains were cultured on Sorbital MacConkey agar and incubate at 37º C overnight. Non sorbital fermenting *E.coli* were identified by the appearance of colorless colony.

4.6c. Biofilm production:

The capacity to form biofilms was assayed in microtitre plates essentially as described by O’Toole & Kolter. Briefly, cells were initially grown for 18 h in Trypticase soy broth (TSB) at 37º C. Subsequently, culture were diluted 1 in 100 with fresh TSB and 200 µl were inoculated into 96 well polystyrene microtitre plates and incubated for 18 h at 37º C. After incubation, content of each well was gently removed by tapping the plates. The wells were washed four times with 200 µl of phosphate buffer saline (PBS Ph 7.2) to remove free floating planktonic bacteria. Biofilms formed by adherent organisms in plate were fixed with Bovin fixative and stained with crystal violet. Excess stain was rinsed off by thorough washing with deionized water and plates were kept for drying. Optical density (OD) of stained adherent bacteria were determined with a ELISA reader at wave length of 630 nm. These OD values were considered as an index of bacteria adhering to surface and
forming biofilms. Interpretation: mean OD values <0.120 consider as a non biofilm producers and mean OD values >0.240 consider as a biofilm producers.

4.6d. Antimicrobial susceptibility testing:

Antimicrobial agents were tested by the Kirby-Bauer disk diffusion method in accordance with CLSI guidelines. The antibiotic discs used were: ampicillin 10 μg, amoxicillin 20 μg, piperacillin/Tazob 100/10 μg, amoxicillin clavulanic acid 20 μg/10 μg, ceftriaxone 30 μg, ceftazidime 30 μg, cephepime 30 μg, cefoperazone/sulbact 75/30 μg, aztreonam 30 μg, imipenem 10 μg, amikacin 30 μg, gentamicin 30 μg, ciprofloxacin 5 μg, Trimethoprim/ sulfamethoxazole 75 μg.

4.6d.1. Phenotypic testing for ESBLs:

Isolates were tested for ESBL via the combination disc methods using cefotaxime (30μg) and ceftazidime (30μg) and discs of cefotaxime–plus-clavulanate (30μg+10μg) and ceftazidime–plus-clavulanate (30μg+10μg). A ≥ 5 mm increase in diameter of the inhibition zone of the cefotaxime–plus-clavulanate and ceftazidime–plus-clavulanate disc, when compared to the cefotaxime and ceftazidime disc alone, was interpreted as phenotypic evidence of ESBL production.

4.6d.2. Detection of AmpC type β lactamases:

AmpC β- lactamase production was measured using cefoxitin (zone diameter <18mm was considered AmpC positive.) The AmpC disc test for plasmid mediated AmpC β- lactamase detection was carried out according to the procedure previously described.

A lawn culture of E.coli ATCC 25922 was prepared on Mueller - Hinton agar plate. Sterile discs (6 mm) moistened with sterile saline (20μl) and inoculated with several colonies of test organism were taken .The inoculated discs were then placed besides a cefoxitin disc (almost touching) on the inoculated plate. The plates were incubated overnight at 35º C, aerobically. A positive test appears indicated as flattening or indentation of cefoxitin Inhibitory Zone in the vicinity of the test disc. A negative test had an undistorted zone.
Control strains were included for all ESBL and AmpC β-lactamase detection protocols.

4.6d.3. Detection of carbapenemase production:

Plates of Mueller-Hinton agar were inoculated with suspensions of the test strains adjusted to turbidities equivalent to 0.5 McFarland standards. A sat of discs (HiMedia) of IPM, MRP and ETP (10µg each) were applied to the surface of the agar, plates were incubated overnight at 35°C aerobically, and diameters of zone of inhibition (≥23 mm indicated sensitivity, 20 to 22 mm indicated intermediate resistance and ≤19 mm indicated resistance) were recorded. Carbapenemase production was further confirmed by modified Hodge test (MHT) using the 3 antibiotics, namely – Imipenem, Meropenem & Ertapenem.322

4.6d.4. Detection of metallo-β-lactamase producers:

Identification of MBL activity was performed by two methods: a carbapenem – EDTA combined disk method and the MBL E-test (HiMedia). A known MBL producing isolate were used as a positive control for all tests.

4.6d.4a. Combined disk test: The Imipenem+EDTA, Meropenem+EDTA and Ertapenem+EDTA combined disk method was performed as described previously.322 Test strain suspensions adjusted to 0.5 McFarland standard were inoculated on Mueller–Hinton agar plates. Two IMP (10µg) disks (HiMedia) were placed on each agar plate, and 10µL of a 0.5 M EDTA solution (pH 8.0) was added to one of the IPM disks. After incubation over night at 35°C the inhibition zones of the IPM disks with and without EDTA were compared. A ≥7 mm increase in the zone diameter for IPM in the presence of EDTA was interpreted as a positive test result, same procedure was also followed for Meropenem and Ertapenem.

4.6d.4b. MBL E-test: The double sided seven–dilution range of IPM (4 to 256 µg/mL and IPM (1 to 64 µg/mL) in combination with a fixed concentration of EDTA (IPM-EDTA E-test, HiMedia) was used to detect MBL production and MIC of IPM to the test isolates and was performed according to the recommendations of the
If the ratio of the value obtained for IPM : the value of IPM+EDTA was ≥ 8 or if a zone was observed on the side coated with IPM + EDTA but absent on the side coated with IPM alone for any test isolate, it was interpreted as positive for MBL.

4.7. Genotypic characterization:

4.7.1. Preparation of Template DNA:

500µl of sterile distilled water was taken in a micro centrifuge tube. Into it 4-5 freshly sub-cultured identical colonies of the isolates were inoculated. This was heated in a water bath at 95°C for 10 minutes and then centrifuged at 10,000 rpm for 10 minutes. The supernatant containing bacterial DNA was used as template for PCR.

4.7.2. Phylogenetic Grouping:

Phylogenetic group of the E.coli isolates was determined based on chuA and yjaA and DNA fragment TSPE4.C2 genes, according to the procedure previously described. The sequences of the primers and the thermocycling conditions used were as follows;

\[
\text{ChuA.F : } 5' \text{ GAC GAA CCA ACG GTC AGG AT 3'} \\
\text{ChuA.R : } 5' \text{TGC CGC CAG TAC CAA AGA CA 3'} \\
\text{YjaA.F : } 5' \text{TGA AGT GTC AGG AGA CGC TG 3'} \\
\text{YjaA.R : } 5' \text{ATG GAG AAT GCG TTC CTC AAC 3'} \\
\text{TspE4C2.F : } 5' \text{GAG TAA TGT CGG GGC ATT CA 3'} \\
\text{TspE4C2.R : } 5' \text{CGC GCC AAC AAA GTA TTA CG 3'}
\]

Template DNA was amplified by Multiplex PCR with the use of oligonucleotide primers obtained from Sigma Aldrich Pvt. Ltd. India. The PCR was performed in a final reaction volume of 50µl containing 750 Mm Tris-HCl, 200mM \((\text{NH}_4)_2\text{SO}_4\), 2.5mM MgCl\(_2\) 0.2mM each dNTP, 0.4µM of each primers, 1 U of Taq DNA polymerase and 5µl template DNA. An Eppendorf thermocycler was used for amplification. The program for amplification included a step of initial denaturation at
94\(^\circ\) C for 5 min, followed by 30 cycles of 94\(^\circ\) for 30 sec, 55\(^\circ\) for 30 sec and 72\(^\circ\) for 30 sec and a final extension step at 72\(^\circ\) for 7 min.

The PCR products were loaded in 2% wt/vol agarose gel prepared in Tris-borate –EDTA buffer at 120V for 1 hour and detected by ethidium bromide staining after electrophoresis.

4.7.3. Detection of virulence factor (VF) genes by multiplex PCR assay:
Three sets of multiplex PCR were developed to detect following genes:

**Set 1:** PCR screening for \textit{papC, cnf1& neuC} was performed according to the procedure previously described. \(^{279}\)

\[
\begin{align*}
papC F & = 5'\text{GTGGCAGTATGAGTAATGACCGTTA} & 3' \\
papC R & = 5'\text{ATATCCCTTCTGCAGGGATGCAATA3'} \\
cnf1 F & = 5'\text{AAGATGGAGTTTCCTATGCAGGAG} & 3' \\
cnf1 R & = 5'\text{CATTCAGAGTCCTGCCCTCATTATT} & 3' \\
NeuC F & = 5'\text{AGGTGAAAAGCCTGGTAGTGTG3'} \\
NeuC R & = 5'\text{GGTGGTACATCCCGGGATGTC3'}
\end{align*}
\]

Template DNA was amplified by Multiplex PCR with the use of oligonucleotide primers obtained from Sigma Aldrich Pvt. Ltd. India. The PCR was performed in a final reaction volume of 50µl containing 750 Mm Tris-HCl, 200mM (NH\(_4\))\(_2\)SO\(_4\), 2.5mM MgCl\(_2\), 0.2mM each dNTP, 0.4µM of papC primers and 0.6µM of cnf1 and neuC primers, 1 U of Taq DNA polymerase and 4µl template DNA. An Eppendorf thermocycler was used for amplification. The program for amplification included a step of initial denaturation at 95\(^\circ\) C for 3 min, followed by 25 cycles of 94\(^\circ\) for 30 sec, 61\(^\circ\) for 30 sec and 68\(^\circ\) for 3 min and a final extension step at 72\(^\circ\) for 3 min.

The PCR products were loaded in 2% wt/vol agarose gel prepared in Tris-borate –EDTA buffer at 120V for 1 hour and detected by ethidium bromide staining after electrophoresis.
Material and Methods

Set 2: PCR screening for *hlyA, fimH* & *iutA* was performed according to the procedure previously described.\(^\text{279}\)

\[
\begin{align*}
\text{*hlyA F:} & \quad 5'\text{AACAAGGATAAGCACTGGTTCTGGCT} \ 3' \\
\text{*hlyA R:} & \quad 5'\text{ACCATAAAGCGGTCATTCCCCTGCA} \ 3' \\
\text{*fimH F:} & \quad 5'\text{TGCAGAACGGATAAGCGGTGG} \ 3' \\
\text{*fimH R:} & \quad 5'\text{GCAGTCACCTGCCCCTCCGGTA} \ 3' \\
\text{*iutA F:} & \quad 5'\text{ATCGGCTGGACATCATGGGAAC} \ 3' \\
\text{*iutA R:} & \quad 5'\text{CGCATTTACCGTCGGGACG} \ 3'
\end{align*}
\]

Template DNA was amplified by Multiplex PCR with the use of oligonucleotide primers obtained from Sigma Aldrich Pvt. Ltd. India. The PCR was performed in a final reaction volume of 50μl containing 750 Mm Tris-HCl, 200mM (NH\(_4\))\(_2\)SO\(_4\), 2.5mM MgCl\(_2\), 0.2mM each dNTP, 0.6μM of *hlyA* primers and 0.3μM of *iutA* and *fimH* primers, 1 U of Taq DNA polymerase and 4μl template DNA. An Eppendorf thermocycler was used for amplification. The program for amplification included a step of initial denaturation at 95\(^\circ\) C for 3 min, followed by 25 cycles of 94\(^\circ\) for 30 sec, 61\(^\circ\) for 30 sec and 68\(^\circ\) for 3 min and a final extension step at 72\(^\circ\) for 3 min.

The PCR products were loaded in 2% wt/vol agarose gel prepared in Tris-borate –EDTA buffer at 120V for 1 hour and detected by ethidium bromide staining after electrophoresis.

Set 3: PCR screening for *stx1* & *stx2* was performed according to the procedure previously described.\(^\text{324}\)

\[
\begin{align*}
\text{*STX1 F:} & \quad 5'\text{ACA CTG GAT GAT CTC AGT GG} \ 3' \\
\text{*STX1 R:} & \quad 5'\text{CTG AAT CCC CCT CCA TTA TG} - 3' \\
\text{*STX2 F:} & \quad 5'\text{CCA TGA CAA CGG ACA GCA GTT-3'} \\
\text{*STX2 R:} & \quad 5'\text{CCT GTC AAC TGA GCA GCA CTT TG} \ 3'
\end{align*}
\]

Template DNA was amplified by Multiplex PCR with the use of oligonucleotide primers obtained from Sigma Aldrich Pvt. Ltd. India. The PCR was
Material and Methods

performed in a final reaction volume of 50µl containing 750 Mm Tris-HCl, 200mM (NH₄)₂SO₄, 2.5mM MgCl₂ 0.2mM each dNTP, 0.4µM of each primers, 1 U of Taq DNA polymerase and 5µl template DNA. The program for amplification included a step of initial denaturation at 95⁰C for 5 min, followed by 25 cycles of 95⁰ for 1 min, 58⁰ for 30 sec and 72⁰ for 1.5 min and a final extension step at 72⁰ for 5 min.

The PCR products were loaded in 2% wt/vol agarose gel prepared in Tris-borate –EDTA buffer at 120V for 1 hour and detected by ethidium bromide staining after electrophoresis.

4.7.4. Molecular detection of β-lactamase Genes:

4.7.4a. ESBL genes:

PCR screening for TEM, SHV & CTXM was performed with minor modifications, according to the procedure as described earlier.³²⁵,²⁹⁷

**CTXM F:**  5’TTTGCAGATGTGCAGTACCAGTAA3’
**CTXM R:**  5’CGATATCGTTGTTGTTGCTGCAAA3’

**TEM F:**  5’ATGAGTATTCAACATTTCCGTG3’
**TEM R:**  5’TTACCAATGCTTAATCAGTGAG3’

**SHV F:**  5’CGGTCAGCGAAAAACACCT3’
**SHV R:**  5’TCCCGCAGATAATTACACCAC3’

Template DNA was amplified by Multiplex PCR with the use of oligonucleotide primers obtained from Sigma Aldrich Pvt. Ltd. India. The PCR was performed in a final reaction volume of 50µl containing 750 Mm Tris-HCl, 200mM (NH₄)₂SO₄, 2.5mM MgCl₂ 0.2mM each dNTP, 0.5 µM of each primers, 1.5 U of Taq DNA polymerase and 5µl template DNA. An Eppendorf thermocycler was used for amplification. The program for amplification included a step of initial denaturation at 95⁰ for 3 min followed by 30 cycles of 95⁰ for 1 min, 58⁰ for 1 min and 72⁰ for 1 min and a final extension step at 72⁰ for 7 min. The PCR products were loaded in 2% wt/vol agarose gel prepared in Tris-borate –EDTA buffer at 120V for 1 hour and detected by ethidium bromide staining after electrophoresis.
Material and Methods

**CTX-M15:**

PCR screening for CTX-M15 was performed with minor modification according to the procedure as described earlier.\(^{326}\)

**CTXM15 F:** 5’CACACGTGGAATTTAGGGACT3’

**CTXM15 R:** 5’GCCGTCTAAGGCGATAAACA3’

Template DNA was amplified by Multiplex PCR with the use of oligonucleotide primers obtained from Sigma Aldrich Pvt. Ltd. India. The PCR was performed in a final reaction volume of 50µl containing 750 Mm Tris-HCl, 200mM (NH\(_4\))\(_2\)SO\(_4\), 2.5mM MgCl\(_2\), 0.2mM each dNTP, 0.4 µM of each primers, 1.5 U of Taq DNA polymerase and 5µl template DNA. An Eppendorf thermocycler was used for amplification. The program for amplification included a step of initial denaturation at 95\(^{\circ}\) for 3 min followed by 30 cycles of 95\(^{\circ}\) for 1 min, 50\(^{\circ}\) for 1 min and 72\(^{\circ}\) for 1 min and a final extension step at 72\(^{\circ}\) for 7 min.

The PCR products were loaded in 2% wt/vol agarose gel prepared in Tris-borate –EDTA buffer at 120V for 1 hour and detected by ethidium bromide staining after electrophoresis.

**4.7.4b Plasmid mediated AmpC genes:**

PCR screening for AmpC genes was performed with minor modification according to the procedure as described by Perez-Perez et al.\(^{245}\)

**MOXM F:** 5’GCTGCTCAAGGACACAGGAT3’

**MOXMR:** 5’CACATTGACATAGGTGTGGTGC3’

**CITM F:** 5’TGGCCAGAACCTGACAGGCAAA3’

**CITMR:** 5’TTTCTCTGAACGTGGCTGGC3’

**DHAMF:** 5’AACTTTTCACAGGTGTGCTGGG3’

**DHAMF:** 5’CCGTACGCATACTGGCTTTGC3’

**ACCMF:** 5’AACAGGCTCAGGAGCCGTTA3’

**ACCMR:** 5’TTCGCCGCAATCATCCCTAGC3’

**EBCMF:** 5’TCGGTAAAGCCGATGTTGCGG3’
Material and Methods

**EBCMR:** 5’CTTCCACTGCGGCTGCCAGTT 3’

**FOXMF:** 5’AACATGGGGTATCAGGGAGATG 3’

**FOXMR:** 5’CAAAGCGCGTAACCGGATTGG 3’

Template DNA was amplified by Multiplex PCR with the use of oligonucleotide primers obtained from Sigma Aldrich Pvt.Ltd. India. The PCR was performed in a final reaction volume of 50µl containing 750 Mm Tris-HCl, 50mM KCl, 1.5mM MgCl₂, 0.2mM each dNTP, 0.6 µM primers MOXM, CITM, DHAM, 0.5µM primers ACCM, EBCM and 0.4µM primers FOX; 1.25U of Taq DNA polymerase and 2 µl template DNA. An Eppendorf thermocycler was used for amplification. The program for amplification included a step of initial denaturation at 94°C for 3 min followed by 25 cycles of 94°C for 30 sec, 64°C for 30 sec and 72°C for 1 min and a final extension step at 72°C for 7 min. The PCR products were loaded in 2% wt/vol agarose gel prepared in Tris-borate –EDTA buffer at 120V for 1 hour and detected by ethidium bromide staining after electrophoresis.

**4.7.4c.MBLgene:**

PCR screening for NDM-1 (New Delhi metallo-β-lactamase) gene was performed with minor modification according to the procedure as described by Nordmann et al.\textsuperscript{327}

**NDM1 F:** 5’GGTTTGGCGATCTGGTTTTC3’

**NDM1R:** 5’CGGAATGGCTCATCAGCATC3’

Template DNA was amplified by Multiplex PCR with the use of oligonucleotide primers obtained from Sigma Aldrich Pvt. Ltd. India. The PCR was performed in a final reaction volume of 50µl containing 750 Mm Tris-HCl, 200mM (NH₄)₂SO₄, 2.5mM MgCl₂ 0.2mM each dNTP, 0.2 µM of each primers, 1 U of Taq DNA polymerase and 5µl template DNA. An Eppendorf thermocycler was used for amplification. The program for amplification included a step of initial denaturation at 94°C for 3 min followed by 36 cycles of 94°C for 30sec, 52°C for 40 sec and 72°C for 50 sec and a final extension step at 72°C for 5 min.
The PCR products were loaded in 2% wt/vol agarose gel prepared in Tris-borate–EDTA buffer at 120V for 1 hour and detected by ethidium bromide staining after electrophoresis.

4.8. Statistical analysis:

The statistical analysis was performed by using SPSS, version 17.0. Correlation of numerical data with drug resistance and virulence was done using Pearson’s correlation coefficient and all categorical data were correlated by chi-square test. Association of specific genotype with phenotype and clinical outcome were done by chi-square test. *P* < 0.05 was considered statistically significant.