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# Isolation and characterization of *Enterobacter* from egg yolk

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**Abstract**

The present study describes different approaches that lead to the identification of *Enterobacter* from commercially sold eggs in Chennai, Tamil Nadu, India. Morphological and biochemical methods were applied to categorize a Gram negative, catalase positive, oxidase negative, ampicillin and cephalosporin resistant, erythromycin susceptible, tetracycline mildly resistant isolate into either *Bacillus* or *Enterobacter* genera. DNA Sequencing was performed using the 16s rRNA primers. The resulting sequences were subjected to a BLASTn search in the NCBI database that yielded a 99% genome similarity with *Enterobacter xiangfangensis* and *Enterobacter hormaechei*. Further, on the genomic pipeline of Center for Genomic Epidemiology (CGE), the bacterium was identified as *Enterobacter hormaechei*. A bacterial smear of the same was prepared and it's m/z values were generated by subjecting to MALDI-TOF to identify its genus and species as *Enterobacter cloacae* and *Enterobacter kobei* as the first and second best hit respectively. The study explores suitability of the diverse methods of bacterial identification and highlights the need of a consensus approach.

Eggs are high nutrition food as they consist of proteins, essential amino acids, vitamin A, riboflavin, folic acid, vitamin B6, and vitamin B12, minerals such as iron, calcium, phosphorus and potassium[5]. This is also the reason that they are often contaminated separately or concurrently by different enteric pathogens like *E. coli* and *Salmonella* species[21]. Increased embryo mortality, low hatchability and early chick mortality are major determinants in the pathology of poultry production. Illness in humans is also widespread due to consumption of these microbes. The prevalence of pathogenic strains in eggs is a subject of high concern, where tolerance is dependent on the acceptable load of the organisms[3,11].

The egg shell and albumin act as physical and chemical barriers respectively and

therefore exhibit antimicrobial activity[9]. Despite these natural defense mechanisms, microbial contamination of eggs is rampant and leads to food poisoning in humans[1]. The intensity of the food poisoning depends on the count and type of bacteria. Eggs are frequently contaminated by Gram-positive bacteria due to their greater tolerance to desiccation[6]. In order to mitigate food poisoning, it is imperative to identify the micro-organisms prevalent in the egg. In the present study, we report the incidence and prevalence of *Enterobacter hormaechei* in the yolk of boiled egg. Our results pose a compelling reason to streamline the process of microbial identification with special reference to emerging pathogens such as *Enterobacter*.

*Bacterial Isolation :*

Eggs were commercially sourced in Chennai, Tamil Nadu, India. Egg was hard boiled in water (50 °C) and yolk separated. Ten grams of minced egg yolk was subjected to 3 fold dilutions with sterilized distilled water and every dilution was plated over MRS Agar (Sigma Aldrich, USA). The spread plates were incubated at 37°C for 24 h. Colonies were isolated and repeatedly streaked to obtain pure cultures. Sub-culturing was done at regular intervals in nutrient broth and glycerol stock was prepared for selected streak isolates. The isolates were maintained in 20 % glycerol stock solution till further use.

*Gram staining :*

A thin smear of the isolate was heat-fixed on a clean slide. Two drops of 0.5 % crystal violet were added to the smear for 1 minute, washed with water and stained with Gram's iodine solution for 1 minute. The slide was flooded with ethanol for 30 seconds and 2 drops of 2 % safranin was added for 1 minute, rinsed again with tap water and blotted dry using a filter paper. Slide was examined under a light microscope. *Pseudomonas aeruginosa* and *Streptococcus mutans* were used as Gram negative and Gram-positive controls respectively.

*Catalase test :*

A drop of 3 % (v/v) $H_2O_2$ was placed on a clean microscopic slide and mixed thoroughly with loop-full of bacterial culture. The slide was observed to produce gas bubbles. *Pseudomonas aeruginosa* and *Streptococcus mutans* were used as a positive and negative control, respectively.

*Cytochrome oxidase test :*

Cytochrome oxidase strips (Sigma Aldrich, USA) were saturated with a solution of N,N-dimethyl-1,4-phenylene diamine and placed in a sterile glass slide. An overnight culture of isolate was smeared on to the strip. *Pseudomonas aeruginosa* and *Escherichia coli* were used as a positive and negative control.

*Carbohydrate utilization test :*

The organism was tested for utilization of 12 sugars (Lactose, Xylose, Maltose, Fructose, Dextrose, Galactose, Raffinose, Trehalose, Melibiose, Sucrose, L-Arabinose, Mannose) present in the strip (KB009A, Hi-Media). The isolate was transferred to nutrient broth and maintained till a $\geq$ 0.5 O.D. was obtained according to manufacturer's protocol.

Each well of the kit was then inoculated with 50 µL of the inoculums and maintained in at 35 ± 2°C and incubated for 20 h. To understand the results, change in colour of medium was interpreted according to manufacturers' chart.

*Motility test :*

Vaseline was applied on the four corners of the cover slip with a toothpick. A drop of bacterial culture was placed on the centre of the cover slip and inverted over the concavity glass slide. The motility was examined by first focusing on the drop under low power objective then switched to high power objective. *Escherichia coli* and *Lactobacillus acidophilus* were used as a positive and negative control.

*Temperature tolerance :*

The isolate was tested with a range of temperatures, ranging from 40 °C to 80 °C to record its heat tolerance. One mL of culture was diluted with 9 mL of sterile broth in a test tube and placed in water-baths maintained at each specific temperature for a period of 1 h. The culture was brought to room temperature and then inoculated in a conical flask for 24 h. Growth of bacterial cells was enumerated by measuring absorption in a UV Spectrophotometer ($OD_{600}$). Parallelly, 1 ml was taken and spread plating was done to check the growth. For control, plating was done without thermal treatment.

*Colony morphology of the isolates :*

The colony morphologies of isolates such as size, surface, elevation, margin, form, colour, opacity was observed, and the results were recorded.

*Antibiotic susceptibility :*

Fresh overnight culture of the isolates was spread on Muller Hinton (MH) agar plates and antibiotic discs (Ampicillin - 10µg/disc, tetracycline - 10µg/disc, erythromycin - 10µg/disc) were placed on the surface using sterile forceps. The plates were incubated at 37 °C for 24 h. The zone of inhibition was measured inclusive of the diameter (6 mm) of the discs. Results were expressed as sensitive, S (≥ 20 mm); intermediate sensitive, I (15-19 mm) and resistant, R (≤ 14 mm)[17].

*Growth curve :*

A 1 % culture was maintained in 100 mL MRS broth under sterile conditions at 37°C for 48 h. Optical density was measured in a UV Spectrophotometer ($OD_{600}$) after a 3 h time interval.

*Maintenance of isolates in glycerol stock :*

In a 2 mL cryovial, 500 µL of the overnight culture was vortexed with 500µL of 20 % glycerol and stored at 4 °C initially for 4 h and then in deep freezer at -20 °C. The cells were checked for revival by inoculating in MRS broth.

*DNA isolation and whole genome sequencing :*

Isolation of bacterial genomic DNA was performed using the CTAB method (DNeasy Qiagen kit) as per manufacturer's instructions. To amplify the V3-V4 region of 16S rDNA fragments in bacteria, a 25 µL PCR

(polymerase chain reaction) mixture was prepared using 2.5 µL of microbial DNA (5 ng/µL), 10.5 µL of ReadyMix™ Taq PCR Reaction Mix (Sigma Aldrich) that included Taq DNA polymerase, dNTPs and reaction buffer, 5 µL of specific V3 Forward primer (5' CCTACGGGNBGCASCAG 3') and 5 µL of V4 Reverse primer (5' GACTACNV-GGGTATCTAATCC 3') and water to make up the volume. The thermal profile of the PCR was set to 95 °C for 3 min, 25 cycles of 95 °C for 30 s, 56 °C for 30 s and 72 °C for 45 s and a final elongation step at 72 °C for 10 min. The amplified product was checked on 1 % agarose gel and gel purification was done to remove non-specific amplifications. The PCR reaction was repeated to increase the quantity of the template region upto 50 µL. Five ng of amplified product was used for library preparation using NEBNext Ultra DNA library preparation kit. The library quantification and quality estimation were done in Agilent 2200 TapeStation and the prepared library was sequenced in Illumina HiSeq 2500 with 2 * 250 cycles chemistry. The sequenced raw data was trimmed and assembled using the Galaxy program (http://galaxyproject.org/).

*MALDI-TOF :*

Prior to MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization - Time of Flight detector) mass spectroscopy analysis, the isolates were cultured for 24 h on nutrient agar medium at 28 °C. The cells were plated, and single colonies were allowed to grow using streak plate method. The direct transfer protocol was followed to study the mass spectrum of the isolates. A bacterial smear (approximately 0.1 mg colonies) was transferred to the MALDI target spot. After air drying the smear at room temperature (28 ± 2 °C), sample spots were overlaid with 1µL of matrix solution (10 mg/mL a-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid). Analysis was performed on Microflex LT bench-top mass spectrometer (Bruker Daltonics, USA) following manufacturer's instructions. Machine was initially calibrated using known bacterial strains *Lactobacillus reuteri*, *Lactobacillus acidophilus*, *Escherichia coli*, *Streptococcus mutans* and *Pseudomonas aeruginosa*. Calibration, measurement (m/z) and sample identification were achieved by the pre-installed software which runs on the basis of peptide mass fingerprint matching with Bruker taxonomy database (v 3.3.1).

In the present study, physiological, biochemical and molecular analyses were conducted to identify the organism on the basis of methods described in Bergey's Manual of Systemic Bacteriology[13]. Universal primers were used to amplify 16s rRNA of the isolated bacterium and subjected to sequencing. Bioinformatics analysis of the sequence was done using BLASTn (Basic Local Alignment Search Tool, nucleotide sequences similarity search, NCBI) and the genomic pipeline of the CGE (Center for Genomic Epidemiology) server. MALDI-TOF mass spectroscopy analysis of the isolated colonies was performed on the peptides produced from the protein digests and their respective m/z was identified through peptide mass finger printing analysis. The data obtained from the study depicts considerable differences in the results predicted by the different softwares used in the study.

Good manufacturing practices (GMP)

are obligatory practices that allow critical adherence to quality and safety of food products. Despite these compliance measures, pathogenic bacteria still enter into human food chain, causing diseases. Identification of pathogenic microorganisms is the first step towards such disease mitigation. Enterobacteriaceae are gram-negative, non–spore-forming, facultative anaerobes that ferment glucose and other sugars, reduce nitrate to nitrite, and produce catalase, except *Plesiomonas,* which do not produce oxidase[14]. Most of them are motile by virtue of peritrichous flagella. In the present study, we report the recurring presence of a single isolate in boiled egg yolks. To determine the taxa of the isolated organism, classical methods were employed by subjecting the culture to various tests as described in section 2. All the isolates were observed to be motile Gram-negative rod-shaped singles, pairs, and chains in red colour under high power objective (Figure 1a). The colonies were convex, creamy white, opaque, entire, and irregularly round and rough with smooth edges (Figure 1b and 1c).

Further, the isolated bacterium was tested for its catalase activity. Catalase is an enzyme synthesized by living organisms, including bacteria that mediate the breakdown of hydrogen peroxide into oxygen and water. As $H_2O_2$ is formed during aerobic metabolism, microbes that are capable of growing in aerobic conditions can only liberate it. Bacteria that cannot produce catalase are strict anaerobes or facultative anaerobes such as *Streptococci,* capable of only fermenting but do not respire using oxygen[20]. In the present study, a release of oxygen was observed as bubbles on the slide of the bacteria isolated from the egg yolk with 3% $H_2O_2$ confirming the isolates to be catalase positive. Cytochrome oxidase is a vital enzyme in the bacterial electron transport chain that catalyzes the oxidation of cytochrome c during the reduction of oxygen for water formation. In the present study, N, N-dimethyl-1,4-phenylene diamine was used as an artificial electron donor for cytochrome c, for its oxidation by cytochrome c oxidase into a purple-coloured indophenol. The bacterial isolate upon incubation with cytochrome oxidase, remained reduced and colourless suggesting that the isolate is oxidase negative (Figure 2).

Carbohydrate fermentation is well adapted for the identification of pathogenic anaerobic bacteria. Speciation of isolated bacteria was studied by the detection of acid and gas produced due to fermentation leading to decreased pH followed by a change in colour[12]. The carbohydrate fermentation study of the yolk isolate is Lactose (+), Xylose (+), Maltose (-), Fructose (+), Dextrose (-), Galactose (+), Raffinose (+), Trehalose (+), Melibiose (+), Sucrose (-), L-Arabinose (+) and Mannose (+) respectively. Temperature tolerance of the bacterium isolated from the egg yolk has been tested by exposing to a series of temperatures from 40 °C to 80 °C with duration of 24 h to 120 h. Since bacteria cannot thermoregulate, their internal temperatures are almost equal to the environmental temperature. Temperatures that are below optimum will decrease the enzyme activity, eventually slowing down the metabolism, whereas exposure of bacteria to high temperatures will lead to the denaturation of carrier proteins, enzymes and lead to cell death. A bacterial growth curve relates growth

to temperature where the optimal temperature favours peak growth. In the present study, maximum growth of the bacterium isolated from the egg yolk has been observed in the temperatures ranging between 40 °C to 60 °C for 48 h to 120 h, suggesting that the bacterium might be a mesophile, capable of tolerating moderately high temperatures for long durations (Table-1).

Table-1. Growth of the organism recorded at higher temperatures. Legend "-" denotes the absence of growth, "+": denotes the slow growth and "++" denotes a rapid growth

| Duration of incu-bation | Temperature | | | | |
|---|---|---|---|---|---|
| | 40 °C | 50 °C | 60 °C | 70 °C | 80 °C |
| 24 h | + | + | + | + | + |
| 48 h | ++ | ++ | ++ | + | + |
| 72 h | ++ | ++ | ++ | + | - |
| 96 h | ++ | ++ | ++ | - | - |
| 120 h | ++ | ++ | ++ | - | - |

The ability of the bacteria to adapt to the fluctuations in the temperatures is attributed to the lipids, chaperones and heat shock proteins that are synthesized by the bacteria during their exposure to high temperatures, which help in proper folding of proteins and prevent their denaturation[2].

The emergence of antibiotic resistance and its rapid spread among pathogenic bacterial isolates are considered as grave threats to the public health worldwide. During the last few decades, multidrug-resistant Gram-negative bacterial strains such as *Acinetobacter baumannii*, *E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa,* and Gram-positive methicillin-resistant *Staphylococcus aureus* (MRSA) were increasingly reported in hospital settings due to extensive and inadequate dose regimen of antibiotics[8,16]. Rapid emergence of multidrug-resistant bacteria is a peril to global public health due to the very limited number of available options for antibiotics[4]. The susceptibility of isolated bacterium was assayed against 4 antibiotics cephalosporin, ampicillin, tetracycline and erythromycin. By measuring the zone of inhibition on a lawn of a pure culture, the isolated bacterium was classified to be sensitive S (≥ 20 mm) to cephalosporin and ampicillin, intermediate sensitive I (15-19 mm) to tetracycline and resistant R (≤ 14 mm) to erythromycin (Fig. 3 and Table-2).

In the antibiotic susceptibility study, the isolates were seen susceptible to erythromycin and resistant to cephalosporin, ampicillin and tetracycline. This could be due to the production of carbapenamase with versatile hydrolytic capabilities as observed in Gram-negative multidrug-resistant bacteria. Carbapenamases are frequently reported in Enterobacteriaceae and are involved in the inhibition of β-lactam antibiotics[7]. Ever since the identification of carbapenamase genes IMP-1 in *Pseudomonas aeruginosa,* OXA-23 in *Acinetobacter baumannii* and KPC-1 in *Klebsiella pneumoniae* it has been identified that the global distribution of the drug-resistant carbapenamase genes is a grave challenge[18].

Genome of the isolate was studied for classification and species identification. The 16S rRNA gene is a powerful tool to achieve this because it consists of highly conserved nucleotide sequences, interspersed with

Table-2. Zone of Inhibition visualized on the bacterial lawn with various concentrations of antibiotics

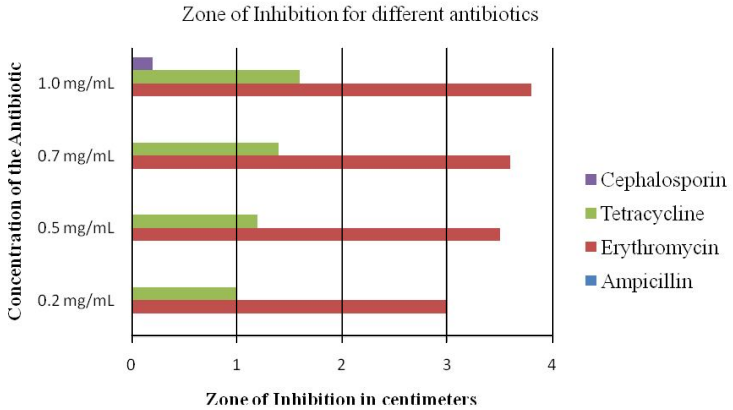
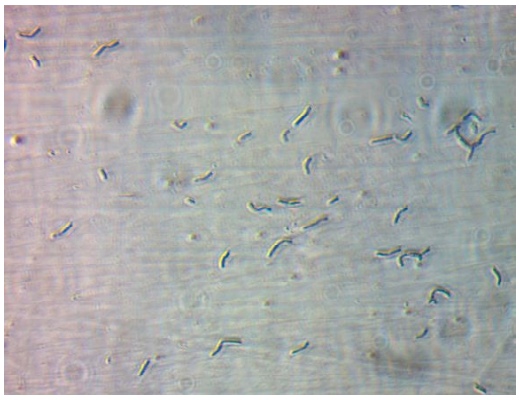



Table-3. Sequence similarity data matrix from BLASTn(NCBI) with strains of *Enterobacter hormaechei* and *Enterobacter xiangfangensis*

| Description Strain Number of the *Enterobacter hormaechei* 16S ribosomal RNA gene, partial sequence | Maximum Score | Total score | Query Cover | E- value | Percentage Identity | Accession |
|---|---|---|---|---|---|---|
| 0173 | 1277 | 1277 | 99% | 0.0 | 98.44 | KP236303.1 |
| LMA108 | 1131 | 1131 | 87% | 0.0 | 98.57 | KX856282.1 |
| LMA123 | 776 | 776 | 60% | 0.0 | 98.52 | KX856297.1 |
| HPCAQ10CR8 | 736 | 736 | 47% | 0.0 | 99.27 | JQ512965.1 |
| DescriptionStrain Number of the *Enterobacter xiangfangensis* 16S ribosomal RNA gene, partial sequence | Maximum Score | Total score | Query Cover | E- value | Percentage Identity | Accession |
| PUFST126 | 1587 | 1587 | 100% | 0.0 | 99.99 | MG371998.1 |

Table-4. Resistance results from CGE server

| Template | Score | Expec-ted | Temp-late length | Temp late identity | Template coverage | Query identity | Query cover-age | Depth | Q value | p Value |
|---|---|---|---|---|---|---|---|---|---|---|
| blaTEM-116_1_AY 425988 | 5685 | 0 | 861 | 99.88 | 100.12 | 99.77 | 99.88 | 7.15 | 5684.40 | 1.0e-26 |
| blaTEM-185_1_JF 795538 | 134 | 1 | 861 | 24.16 | 29.27 | 82.54 | 341.67 | 0.29 | 128.47 | 1.0e-26 |
| blaTEM-197_1_HQ 877606 | 220 | 1 | 861 | 27.99 | 29.04 | 96.40 | 344.40 | 0.29 | 214.51 | 1.0e-26 |
| blaTEM-205_1_KC 900516 | 112 | 1 | 858 | 15.62 | 17.37 | 89.93 | 575.84 | 0.1 | 106.49 | 1.0e-24 |
| blaTEM-220_1_KM 998962 | 191 | 1 | 861 | 27.29 | 29.62 | 92.16 | 337.65 | 0.30 | 185.49 | 1.0e-26 |



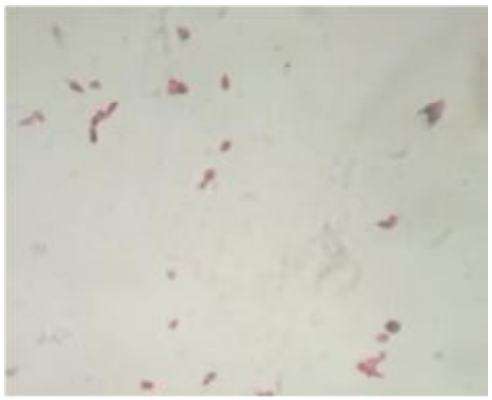


Figure 1a. Single isolates of the bacterium observed under 40x oil immersion light microscope; and under Gram stain

Figure 1b. Opaque and creamy white colonies of the bacterial isolate

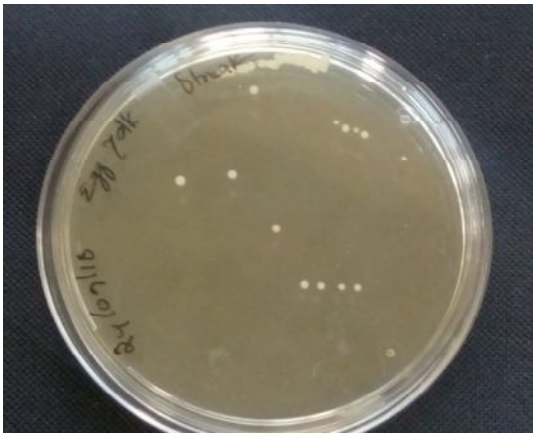


Figure 1c. Isolated single colonies of the isolate showing entire and convex morphology

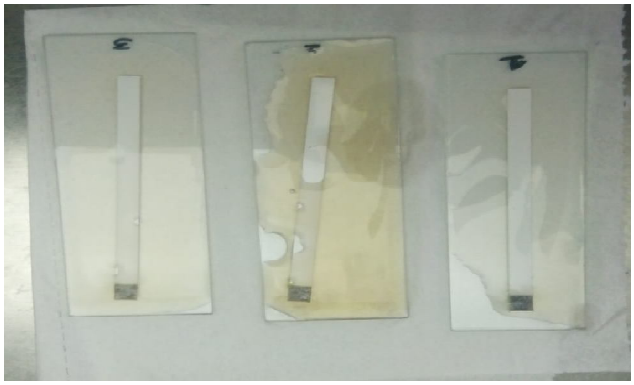


Fig. 2. Bacterial Isolate replicates under incubation with cytochrome oxidase test strips. No colour indicated that the isolates are oxidase negative

Figure 3. Discs showing the zone of inhibition upon treatment with cephalosporin, ampicillin, tetracycline and erythromycin

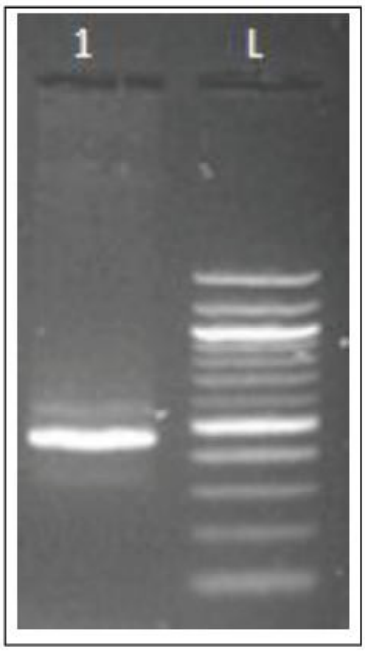



Figure 4. PCR amplification of a 16s rRNA using V3 and V4 primers and separation on an agarose gel Lane 1 indicates the amplified product and Lane 2 indicates the marker.

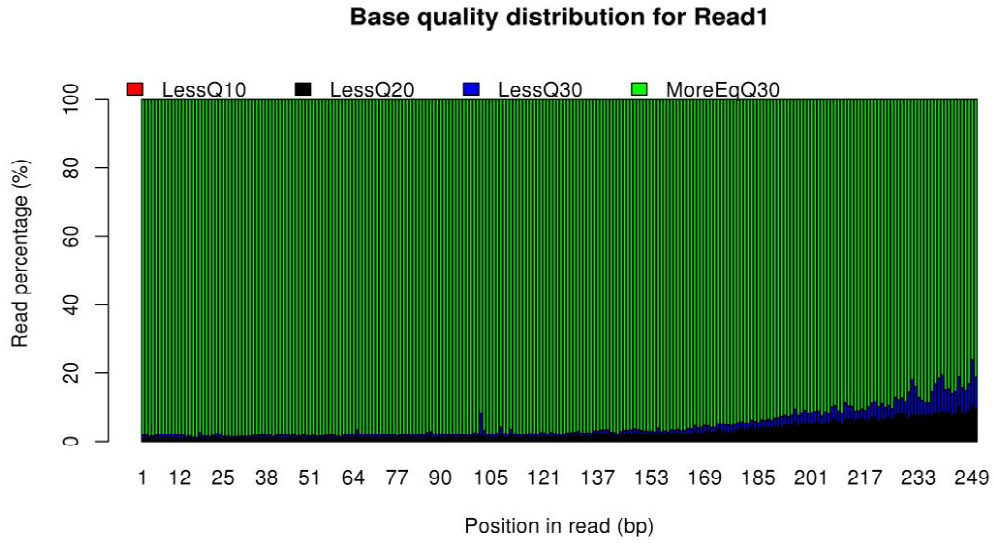



Figure 5. Raw Read summary of the isolated bacterium 16srRNA with Phred Quality Score distribution (%)

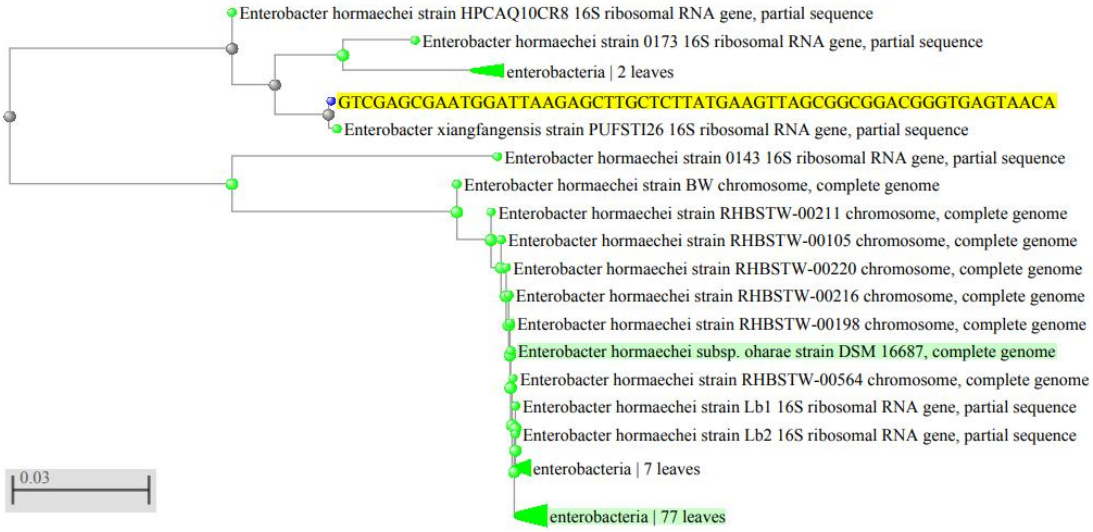
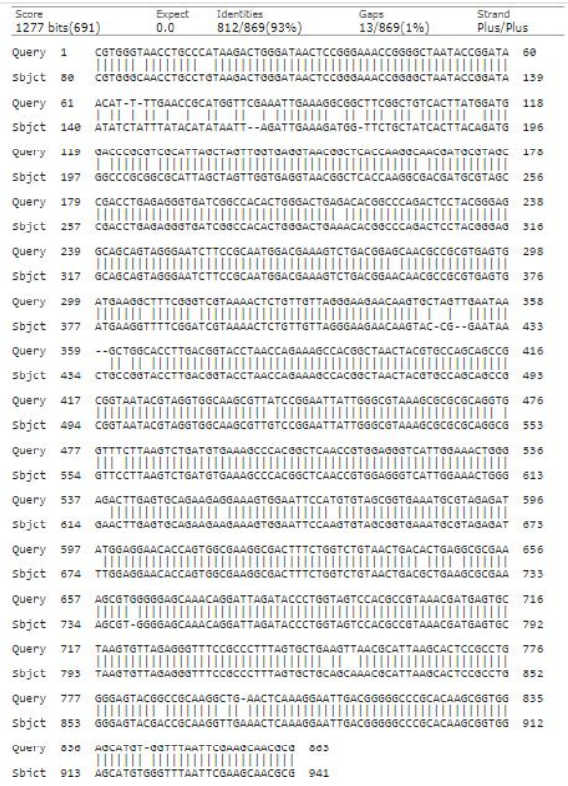



Figure 6. Distance matrix obtained from NCBI BLASTn search



| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 1277 bits(691) | 0.0 | 812/869(93%) | 13/869(1%) | Plus/Plus |

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Sbjct  80   CGTGGGCAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATA  139

Query  61   ACAT-T-TTGAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATG  118
Sbjct  140  ATATCTATTTATACATATAATT--AGATTGAAAGATGG-TTCTGCTATCACTTACAGATG  196

Query  119  GACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGC  178
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Query  417  CGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTG  476
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Sbjct  793  TAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAGCAAACGCATTAAGCACTCCGCCTG  852

Query  777  GGGAGTACGGCCGCAAGGCTG-AACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGG  835
Sbjct  853  GGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGG  912

Query  836  AGCATGT-GGTTTAATTCGAAGCAACGCG  863
Sbjct  913  AGCATGTGGGTTTAATTCGAAGCAACGCG  941
```

Figure 7a. Sequence similarity with partial sequence of 16S ribosomal RNA of *Enterobacter hormaechei* strain 0173 (Sequence ID: KP236303.1; Length: 1111)


Figure 7b. Sequence similarity with partial sequence of 16S ribosomal RNA of *Enterobacter hormaechei* strain LMA108 (Sequence ID: KX856282.1; Length: 818)

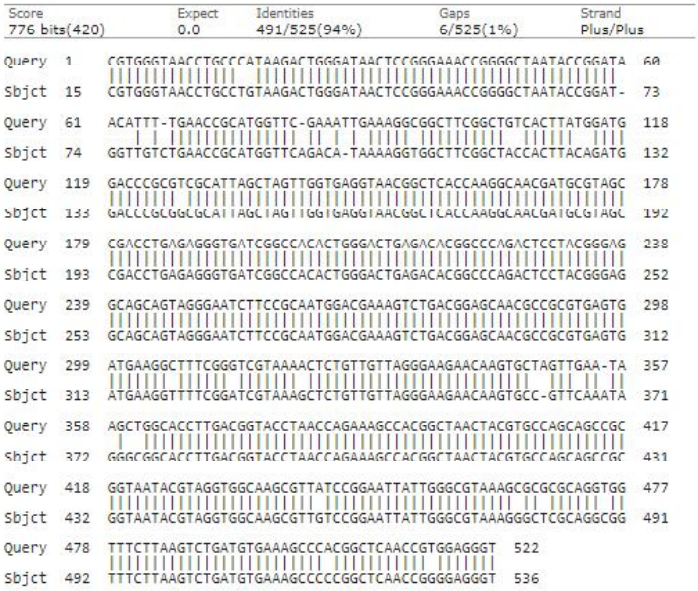


Figure 7c. Sequence similarity with partial sequence of 16S ribosomal RNA of *Enterobacter hormaechei* strain LMA123 (Sequence ID: KX856297.1; Length: 536)

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 736 bits(398) | 0.0 | 406/409(99%) | 3/409(0%) | Plus/Plus |

```
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Query  578  CGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTA  637
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Query  638  ACTGACACTGAGGCGCGAAAGCGTGGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCA  697
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Query  698  CGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAAC  757
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  239  CGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAAC  298

Query  758  GCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTG-AACTCAAAGGAATTGACGG  816
            |||||||||||||||||||||||||||||||||||||||| |||||||||||||||||||
Sbjct  299  GCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGG  358

Query  817  GGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAA  865
            ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  359  GGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAA  407
```

Figure 7d. Sequence similarity with partial sequence of 16S ribosomal RNA of *Enterobacter hormaechei* strain HPCAQ10CR8 (Sequence ID: JQ512965.1; Length: 791)

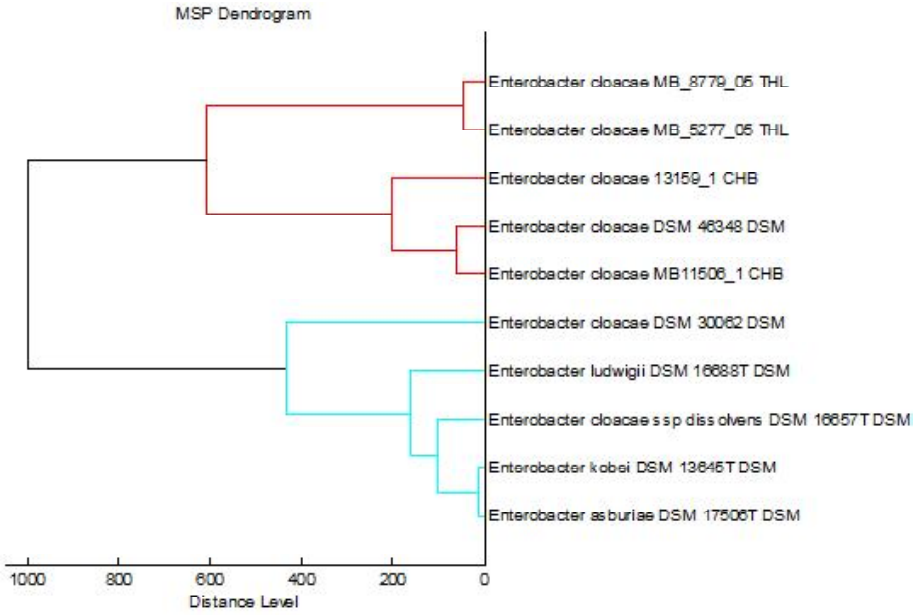


Figure 8. Dendrogram obtained by cluster analysis of MALDI-TOF MS spectra of five Enterobacter representatives.

variable regions that are genus- or species-specific[10]. PCR amplification of the 16s rRNA region using V3 and V4 primers yielded a product of size 3 Kb (Fig. 4).

The product was sequenced on a Illumina HiSeq2500 sequencer and depicted 6,60,941 total paired end reads each with a sequence length of 250 bp and an average Phred score of 36.25, indicating a greater probability of a correct base read with 99% accuracy. More than 80% of the total reads have generated a Phred score > Q30, indicating that the error-probability is <= 0.001 (Fig. 5).

The GC content of the reads ranged from 30 - 60 % (average of 55.71 %). The base composition of the sample was 21.82 % A, 28.16 % C, 27.55 % G and 22.1 % T respectively. The sequences were submitted (SUB6295766) to NCBI SRA archive database and are available with the accession number SAMN12726439 and experiment number SRX6908940 under the project ID PRJNA564919.

Microbial profiling includes identification of bacteria on the basis of sequence homology and estimation of their percentage environmental[15]. The RNA sequences of egg yolk isolated bacteria were submitted to nucleotide BLAST in NCBI database using BLASTn search (Query ID - 1c165050) (Fig. 6), and the matching hits have shown 99.27 % identity with *Enterobacter hormaechii* and *Enterobacter xiangfangensis* (Table-3 and Fig. 7).

The server from Center for Genomic Epidemiology has also identified the genome of the organism as *Enterobacter hormaechei* sub sp. *stegwartii*. The genomic pipeline was able to identify the antibiotic resistance genes in the microbe (Table-4).

For increasing the accuracy and applicability of identifying the bacterial isolate from egg yok, its 16s rRNA was subjected to identification using MALDI-TOF. With three replicate tests,16s rRNA peptides of the isolate were submitted to the MALDI-TOF analysis, and were identified to be *Enterobacter cloacae* and *Enterobacter asburiae* with a score value of 1.84 as best matches of the test organism and *Enterobacter kobei* with a score value of 1.81 as the second best match (Fig. 8).

MALDI-TOF is an alternative method for the identification of 16S rRNA due to its favorable speed and applications. Sung *et al*.[19] used 16s rRNA to identify three of *Aeromonas* species - *A. hydrophila*, *A. caviae*, and *A. veroniii*.

Through this study, bacterium isolated from the egg yolk was identified to be a species of *Enterobacter* with a high possibility of *E. hormaechei.* Identification was attempted in three different ways – classical methods, NCBI BLASTn and the more recent MALDI-TOF. While the classical methods for species level identification are arduous, PCR based BLASTn and mass spectra based MALDI-TOF were able to identify the genus and species within few seconds. The results obtained from modern methods are acceptable up to genus level. Species level identification disagreed in these methods and therefore poses as an important need of the hour. This study highlights the importance of establishing a database consensus at a time when mapping of bacterial evolution and prevalence has emerged as an important task in all countries. This study highlights the importance of GMP in poultry industry and the resilient way in

which pathogenic and antibiotic resistant bacteria are still ending up in our food chain. Food mapping and emerging pathogen mapping shall be helpful to prevent contamination of foods.

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***Conflict of Interest :***

The authors declare no competing interests.

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