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Identification and Characterization of Pathogenic Enterobacterial Isolates Responsible for Egg Contamination

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Abstract: This study aims at the isolation and identification of pathogenic enterobacteria responsible for egg contamination in poultry farms by using different biochemical tests and molecular characterization i.e. 16s rRNA gene analysis. A total of 90 eggs were collected from three different Poultry farms in Jaipur city, India. Micro-organisms were isolated by using differential medium i.e. MacConkey agar, EMB agar and characterized by using different biochemical tests like catalase test, indole test, methyl-red and voges-proskauer test (MR-VP), citrate utilization test, H_2S production, Urease test, Gas production, Glucose and Lactose fermentation etc. On the basis of biochemical analysis selected isolates were subjected to 16s rRNA gene analysis, as 16s rRNA gene analysis is a powerful technique for bacterial taxonomy and identification. Both the biochemical and molecular analysis revealed that most of the isolates belong to family *Enterobacteriaceae* and were identified as *Escherichia coli* O157:H7str. EC4115, *Staphylococcus epidermidis* ATCC 14990 and *Pluralibacter gergoviae*. The study concludes that the eggs and egg products are contaminated with pathogenic microbes and may cause diseases if consumed raw or uncooked. Thus, there is a serious need to pay attention in increasing the hygienic level of commercial eggs so as to prevent the occurrence of prevalence of microbial contamination in eggs.

Key words: Egg, poultry farm, *Enterobacteria*, *Enterobacteriaceae*, 16s rRNA

INTRODUCTION

The hen's egg is one of the most nutritious foods of animal origin, as it contains high amount of proteins, lipids, vitamins and minerals (Rzedzicki and Stepien-Pysniak, 2009). The presence of these nutrient substances in the eggs creates an appropriate environment for the development of bacterial microflora, including pathogenic bacteria, thus causing contamination in eggs which makes them a potential source of pathogens participating in the etiology of food borne diseases in humans (Stepien-Pysniak, 2010; Al-Bahry *et al*., 2012).

Egg has two main natural defense systems (Gautron and Nys, 2006), first is the eggshell (which includes the cuticle and membranes), that act as a physical barrier against bacterial penetration and second is a chemical barrier (composed of proteins) that exhibits anti-microbial activity found in the albumin part. In spite of the presence of natural defense systems the eggs can be contaminated/infected with different micro-organisms (Abdullah, 2010). One of the reasons for egg contamination may be that the egg emerges out from the hen's body through the same way from where faeces is excreted, so the faecal material may adhere on the egg surface and contamination occur through shell penetration by micro-flora (Ansah *et al*., 2009). The shell membrane of the egg contaminates more frequently than albumin and yolk part as bacteria grow fast on the shell membrane because of the presence of iron (Sabarinath *et al*., 2009).The egg shell contamination may result from deposition of fecal material on the shell from oviduct, egg crates, packing and storage, clothes and hands of poultry workers, dust, transporting, marketing and weather conditions (Al-Bahry *et al*., 2012). The frequency of egg contamination is increasing rapidly because of the absence of standard structures, drainage system and sanitation system in the poultry farms and other environmental factors such as temperature and humidity helps in bacterial penetration (Ansah *et al*., 2009).

Although freshly laid eggs are generally sterile, however they may constitute, if contaminated, a public health hazard, leading to losses from economic point of view through spoilage. An important factor influencing quantitative bacterial contamination of eggs is the temperature at which they are stored, because the safety of eggs depends on the number of bacterial cells on the shells and in the content of the egg and on the rate at which they multiply within it.

The symptoms and severity of food poisoning resulting from consumption of contaminated eggs depends not only on the number of bacterial cells in the contents or on the shells of the eggs, but also on the type of bacteria (Bradshaw *et al*., 1990). The microflora of the eggshell is dominated by Gram-positive bacteria, whereas Gram-negative bacteria are best equipped to overcome the

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antimicrobial defenses of the egg content. Earlier studies have shown that egg contamination is mostly caused by the enterobacteria belonging to the family *Enterobacteriaceae* which includes coliforms, fecal coliforms, *E. coli* (Holt *et al*., 2000). The Enterobacteria are heterogeneous group of gram-negative rods whose natural habitat is the intestinal tract of humans and animals and the family *Enterobacteriaceae* includes many genera like *Enterobacter*, *Escherichia*, *Klebsiella*, *Salmonella*, *Serratia*, *Shigella*, *Proteus* and others. Some enteric organisms, eg, *Escherichia coli*, are part of the normal flora and incidentally cause disease, while others, the *Salmonella* and *Shigella* are regularly pathogenic for humans (Stepien-Pysniak, 2010).

According to Abbas *et al*. (2015) the egg shell is a significant reservoir of food borne pathogens such as *Salmonella typhimurium*. Microbial contamination of egg has important outcome to the poultry industry and illness from contaminated egg is a serious public health problem around the world, as the egg can act as a vector in the transmission of food poisoning organism. Regarding the increasing consumption of egg and its products and cases of gastroenteritis in hospitals are also very frequent, so it is necessary to investigate occurrence and prevalence of egg contamination at poultry and market level.

So, the present study is undertaken to isolate, identify and characterize the pathogenic Enterobacteria responsible for the egg contamination in different poultry farms of Jaipur city using biochemical and molecular techniques. Molecular techniques are used for species level identification of bacterial isolates. DNA based molecular techniques are highly specific (such as 16S rRNA gene sequencing) and plays a pivotal role in the accurate identification of bacterial isolates (Woo *et al*., 2008).

MATERIALS AND METHODS

Sample collection: A total of 90 egg samples (30 eggs from each site) were collected from different Poultry farms i.e. Site 1: Ajmer road (AJR), Site 2: Agra road (AGR), Site 3: Delhi road (DLR) in Jaipur, India. Egg samples were randomly taken from the production area of poultry farms in sterile plastic bags and directed to the laboratory for further experimentation.

Sample processing: The egg samples were processed for the isolation of micro-organisms from egg shell and inner part within six hours of collection. For egg-shell sampling, five eggs were selected on the basis of their outer appearance like crack in eggshell, blood spots or presence of fecal material on the egg surface etc. Sterile cotton swabs dipped in sterile buffered peptone water (BPW) was used to swab the entire surface area of the eggshell. The swabs were directly inoculated into 10 ml BPW in screw-capped bottles and incubated at 37°C for 24 h (Harsha *et al*., 2011). The same eggs from which shell sample was taken will be used for interior egg content sampling. For egg-albumin and yolk sampling, the outer surface of the eggs were firstly disinfected (by wiping with surgical gauze soaked in 70% ethanol) and opened around the air sac area. All the albumin part of five eggs was taken out and homogenized to form one sample. The homogenized albumin samples were serial diluted in normal saline till 10^{-4} dilution and then incubated at 37°C for 24 h. Same procedure as for albumin was used for egg-yolk sample processing also for the isolation of micro-organisms.

Isolation and characterization: The incubated egg-shell, egg-albumin and egg-yolk samples were then used for the isolation and characterization of micro-flora. Bacterial isolation was performed by the conventional plating method. Qualitative determination of Gram-negative microorganisms, with particular consideration given to the family Enterobacteriaceae, was carried out on selective and differential media (i.e. MacConkey agar and EMB agar) at a temperature of 37°C for 24-48 h (Sabarinath *et al*., 2009). For the identification of bacterial isolates gram's staining was used and the isolates that were reported to be Gram-negative were used for further processing. The samples which showed positive results on MacConkey agar and EMB agar were used for further identification and characterization by performing different biochemical tests like catalase test, amylase production test, casein hydrolysis, indole test, methyl-red and voges-proskauer test (MR-VP), citrate utilization test, H_2S production test, Urease test (Breed *et al*., 1957).

Genomic DNA isolation: Out of the total samples, only four samples were subjected for 16s rRNA gene analysis due to similarity in biochemical test results, so as to avoid duplication of the similar results. The selected isolates were cultured in nutrient broth media and incubated overnight at 37°C. After incubation 1.5 ml of broth sample was withdrawn aseptically and centrifuged at 6000 rpm for 10 min and the respective bacterial pellets were processed for genomic DNA extraction using GeNei™ Genomic DNA Extraction kit, Cat# 616102800011730, following the given protocol. The purity of the extracted DNA was confirmed by running 2.5 µg DNA separately from each sample on gel electrophoresis set at 50V for 45 min at 25°C. The resultant DNA bands were observed using UV-Transilluminator (Bio-rad).

PCR amplification of 16S rRNA gene: 16S rRNA gene fragments of DNA samples were amplified by using universal 16S rRNA gene primers (forward primer 8F: AGAGTTTGATCCTGGCTGAG and reverse primer 1492R: ACGGCTACCTTGTTACGACTT). The PCR amplification was carried out in Thermal cycler with 25 µl of final reaction volume containing -7.5 µl ddH_2O, 12.5 µl 2X

PCR master mix (GeNei™ PCR amplification kit), 1.0 µl forward primer 8F, 1.0 µl reverse primer 1492R and 3.0 µl diluted DNA (30 ng/µl). The PCR was initiated with denaturation of DNA at 95°C for 2 min and subsequently the number of cycles (denaturation at 94°C for 30 sec, annealing at 52°C for 30 sec, extension at 72°C for 90 sec) were set to 30 and the final extension was performed at 72°C for 10 min. 5 µl from the resulting PCR amplicons of DNA samples were separately mixed with 1 µl of 5X gel loading dye and electrophoresis was carried out on 1.2% agarose gel containing ethidium bromide (EtBr) (0.1 µg/ml) at constant electric field of 5V/cm for 30 min in 0.5X TAE buffer. The amplified PCR product 16S rRNA gene fragments of DNA samples were confirmed at 1500bp as compact single band. DNA was visualized separately under UV-light using gel documentation system (Bio-Rad).

Sequencing and analysis of 16S rRNA gene: The purified 16S rRNA genes of selected isolates were subjected to automated DNA sequencing. Sequence data was generated by primer walk using BDTv3.1 chemistry on ABI 3730xl (Xcelris Labs Limited, Ahmedabad) and were then used to identify the bacterial isolates with BLASTN analysis using the NCBI GenBank nucleotide database and the homology to the closest bacterial organisms with maximum similarity ranging from 96-100% was obtained. The best first fifteen to twenty 16S rRNA gene sequences in BLASTN result were chosen for the analysis of homology match using Neighbor-Joining method (Saitou and Nei, 1987) and aligned using multiple sequence alignment software, Clustal W. These alignment results were used to construct phylogenetic tree using PHYLIP 3.695, TREE VIEW 1.6.6 and MEGA6 software tool (Tamura *et al*., 2013).

RESULTS AND DISCUSSION

Isolation and biochemical characterization: The results obtained on nutrient agar plates showed that all the egg shell samples, 50% of albumin samples and 10% of yolk samples showed growth (positive results). Samples which showed growth on nutrient agar plates were then identified by gram's staining. The results of Gram's staining showed that 85% isolates were Gram negative whereas remaining isolates were gram positive bacteria. The results obtained on MacConkey and EMB agar showed that only 70% isolates showed growth on both MacConkey and EMB agar medium. The results of different biochemical tests performed showed that all the isolates were positive for Catalase test, whereas all the isolates were negative for Urease utilization and VP tests. 70% isolates were positive for indole, 90% isolates for MR, 10% isolates for citrate utilization and H_2S production tests.

The results obtained showed that most of the isolates were of the family Enterobacteriaceae (Table 1) (Bailey and Scott, 1978). The presence of members of family Enterobacteriaceae in table eggs have been reported by several investigators (Jones *et al*., 2004; Adesiyum *et al*., 2005; Musgrove *et al*., 2008). Pathogenic bacteria like *Diplococcus* spp., *Escherichia coli*, *Salmonella* spp., *Shigella* spp., *Klebsiella* spp., *Proteus* spp. And *Staphylococcus aureus* have also been isolated from the shells and inner contents of commercial hen's eggs (El-Prince and Enas, 1988; Etches, 1992; Kim, 2007). Mahdavi *et al*. (2012) reported that the most frequent isolates from eggs in Isfahan, Iran were *Buttiauxella agrestis*, *Cedecea lapagei*, *Cedecea davisae*, *E. coli*, *Enterobacter erogenes*, *Erwinia herbicola*, *Klebsiella pneumonia* and *Psedumonas aeruginosa*. Musgrove *et al*. (2004) also confirmed that the bacteria most frequently isolated from eggs are Gram-negative bacteria such as *Enterobacter* spp., *E. coli* and *Klebsiella* spp. These microbes were isolated infrequently, mainly from eggshell surfaces, irrespective of storage conditions or the source of the eggs. Suresh *et al*. (2006) reported that 6.1% of eggshells and 1.8% of egg inner contents showed the presence of *Salmonella* in South India. According to Olivier *et al*. (2009) the egg shell gets contaminated through contact with contaminated surfaces such as nest, dust, feed, during transportation and storage containers, workers and animals, so it is necessary to refrigerate the eggs during transportation and storage.

DNA isolation and PCR amplification: Out of the total isolates obtained, finally four isolates were subjected to 16s rRNA gene analysis due to similarity in biochemical test results. The genomic DNA of selected isolates AJR3, AGR2, AGR4 and DLR5 were extracted and confirmed by agarose gel electrophoresis resulting in the single band of high molecular weight DNA as observed under UV illuminator (Fig. 1). These isolated genomic DNA were further quantified by Nanodrop spectrophotometer as ranging from 450-550 ng/µl.

The 16S rRNA genes of isolates AJR3, AGR2, AGR4 and DLR5 were amplified by PCR and the resulting amplified gene were electrophorized and visualized as a compact band of expected 1500bp DNA using gel documentation system (Fig. 2).

Sequence alignments and phylogenetic inference: Bacteria can be classified based on phylogeny. A phylogenetic tree can be derived from the comparison with 16S rRNA or other gene sequences (Pui *et al*., 2011). In the present study, the gene sequence data obtained from Xcelris Labs for isolates AJR3, AGR2, AGR4 and DLR5 were analyzed with BLASTN search tool using nucleotide database of NCBI GenBank for the

Table 1: Biochemical response of selected Bacterial isolates

Biochemical tests	AJR3	AGR2	AGR4	DLR5	Genre which may be present
Catalase test	+	+	+	+	*Citrobacter, Enterobacter, Escherichia, Proteus, Salmonella, Shigella, Yersinia* etc.
Glucose fermentation	+	+	+	+	*Citrobacter, Enterobacter, Escherichia, Salmonella, Proteus* etc.
Lactose fermentation	-	-	+	-	For positive results-*Citrobacter*, Escherichia, Shigella, Serratia etc. For negative results-*Edwardsiella, Salmonella, Proteus, Providencia, Yersinia* etc.
Gas production	-	-	-	-	*Citrobacter, Enterobacter, Escherichia, Salmonella, Proteus, Yersinia* etc.
Indole production	+	+	-	+	For positive results-*Citrobacter, Edwardsiella, Escherichia, Proteus, Providencia and Shigella etc.* For negative results-*Arizona, Enterobacter, Klebsiella, Salmonella and Yersinia* etc.
MR reaction	+	+	+	+	For positive results-*Citrobacter, Edwardsiella, Escherichia, Proteus, Providencia, Salmonella, Shigella and Yersinia* etc. For negative results-*Enterobacter, Serratia and Yersinia etc*
VP reaction	-	-	-	-	*Citrobacter,Edwardsiella, Escherichia, Proteus, Providencia, Salmonella, Shigella and Yersinia* etc.
Citrate use	-	-	-	-	For positive results-*Arizona, Citrobacter, Enterobacter, Klebsiella, Proteus, Providencia,Serratia and Yersinia* etc. For negative results-*Enterobacter, Escherichia, Salmonella, Serratia,Shigella and Yersinia* etc.
Urease test	-	-	-	-	*Arizona, Enterobacter, Escherichia, Providencia Salmonella, Shigella and Yersinia*
H_2S production	-	-	-	+	For positive results-*Arizona, Escherichia, Proteus and Salmonella* etc. For negative results-*Citrobacter,Enterobacter, Escherichia, Klebsiella, Providencia, Serratia, Shigella and Yersinia* etc.

*(+) = positive test, (-) = negative test

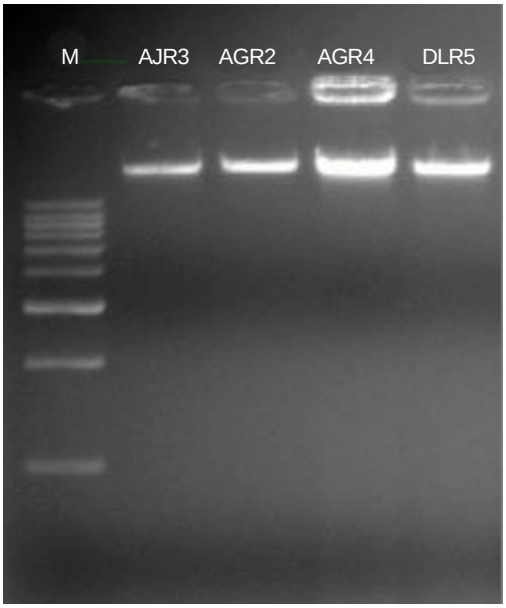

Fig. 1: Visualization of isolated genomic DNA under UV illuminator

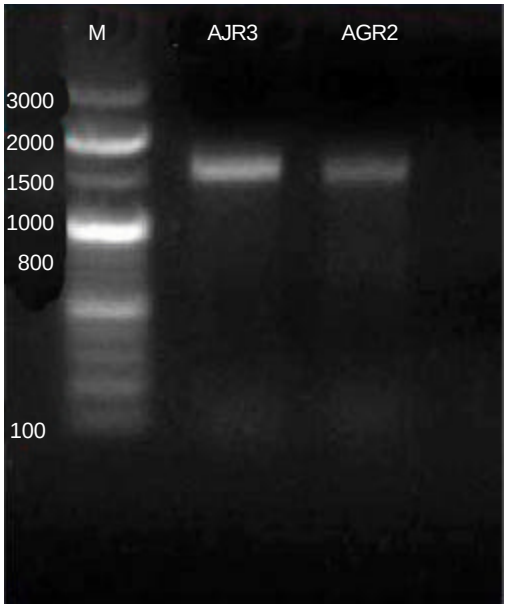

Fig. 2: Visualization of PCR amplified16SrRNA gene under gel documentation system

identification of bacterial isolates. The best homology microorganisms were selected. The homologous 16S rRNA gene sequences of the selected strains were obtained from the nucleotide databases of NCBI with

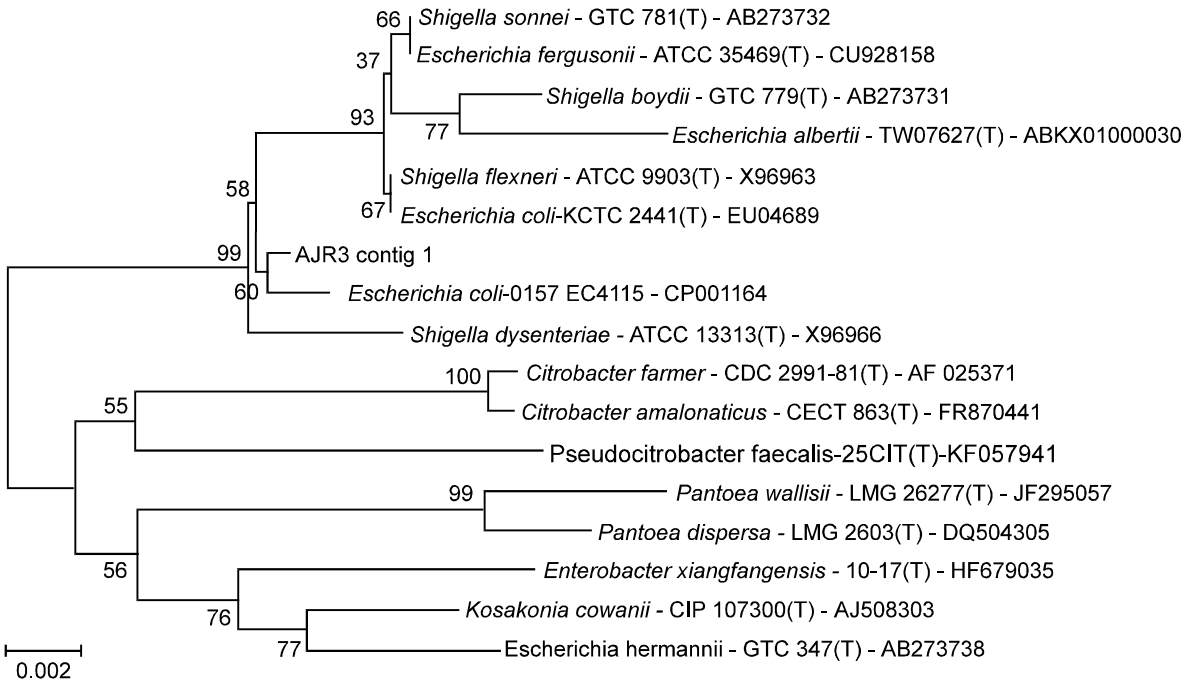

Fig. 3: Phylogenetic tree of AJR3 with the selected best homologous known bacterial strains

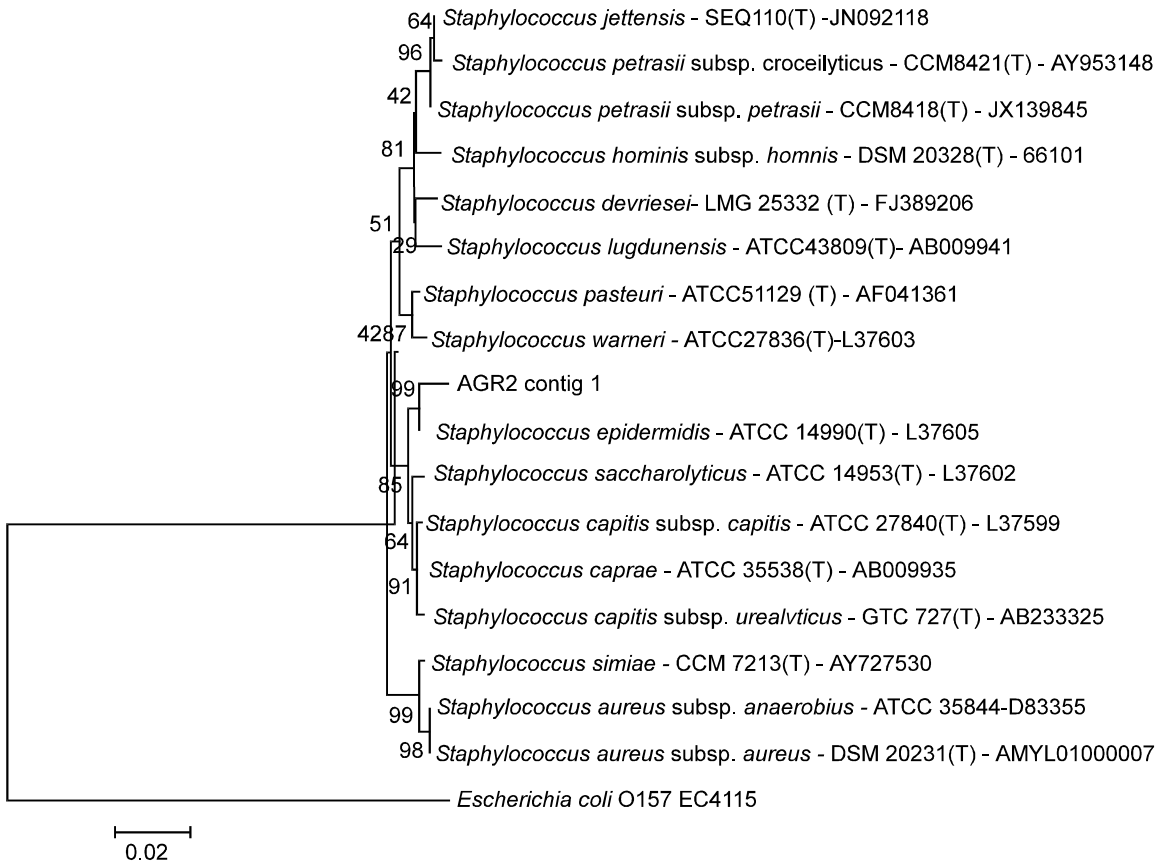

Fig. 4(a): Phylogenetic tree of AGR2 with the selected best homologous known bacterial strains

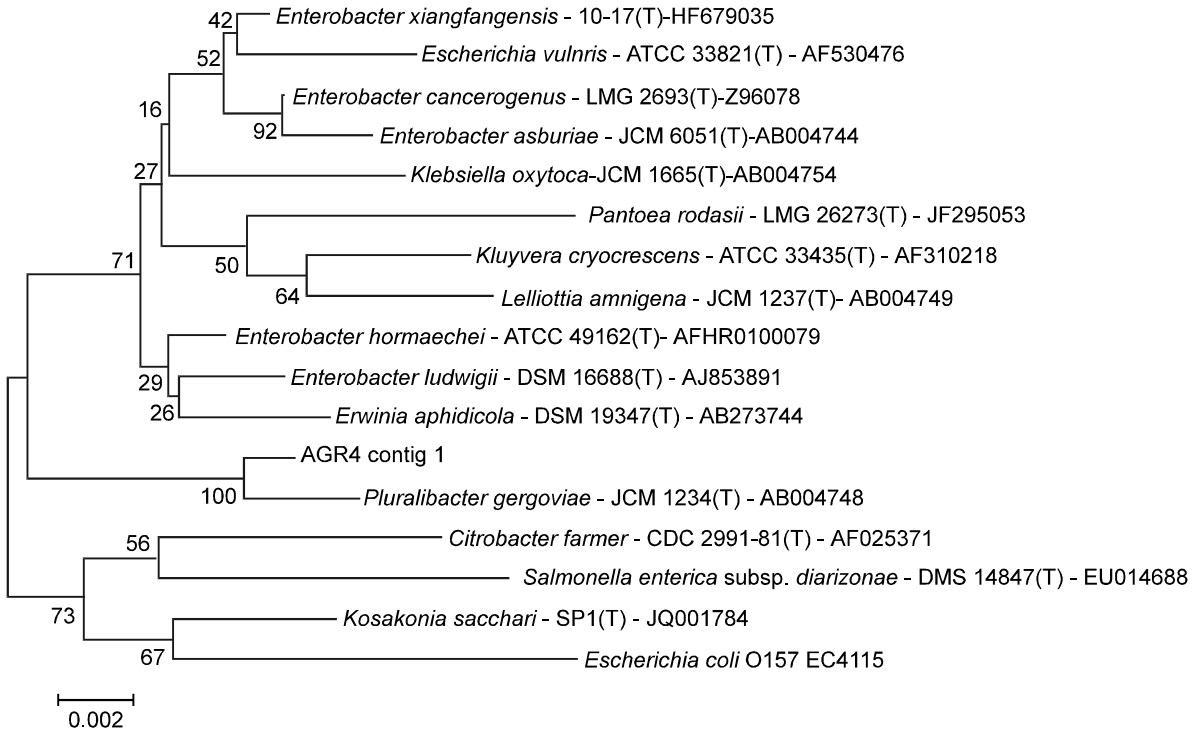

Fig. 4(b): Phylogenetic tree of AGR4 with the selected best homologous known bacterial strains

respect to the isolates. The selected homology sequences of 16S rRNA genes were aligned, respectively using multiple sequence alignment tool ClustalW and the aligned results were processed and the phylogenetic tree was generated through PHYLIP 3.695, TREE VIEW 1.6.6 and MEGA6 software tool as shown in the Fig. 3, 4a, 4b and 5.

Based on the existing database in Genebank, all the isolates obtained showed similarity with pathogenic bacteria. The isolates of AJR3 and DLR5 shared 99-100% similarity with *Escherichia coli* O157:H7str. *EC4115*, AGR2 shared 99% similarity with *Staphylococcus epidermidis* ATCC 14990 and AGR4 showed 100% similarity to *Pluralibacter gergoviae* (previously designated as *Enterobacter gergoviae*).

E. coli are gram negative rods, facultative an aerobic bacteria of microorganisms called coliforms, belonging to family *enterobacteriaceae* that is usually found in the digestive system (Intestinal tract) of humans and animals and transmitted through faecal contamination. *E. coli* can cause diarrheal disease in humans, referred to as diarrhea genic *E. coli*. *Escherichia coli* O157:H7is a subset of pathogenic *E. coli* and causes a distinctive and sometimes deadly disease. *E. coli* is a significant cause of diarrhea in developing countries and localities of poor sanitation (Hartland and Leong, 2013). *Staphylococcus epidermidis* is a common bacterium of the skin, mucous membranes, axillae, head and nares of humans and other mammals (Otto, 2009), so the infection of *S. epidermidis* in eggs could be due to surface contact with workers or animals in poultry farms and also during transportation and storage. *S. epidermidis* can cause severe infections in the immune-compromised persons and can cause lethal septicaemia and meningitis (Schaenfelder *et al*., 2010). *Pluralibacter gergoviae* is most frequently implicated with pneumonia (Michael, 1998).

Cortes *et al*. (2004) also reported that 45% of eggs were contaminated with *E. coli*. *E. coli* are known to contaminate the surface of egg while mechanical process can spread the bacteria through eggs and meat. Ahmed and Shimamoto (2014) detected *E. coli* O157:H7 in Egyptian beef, chicken meat, raw milk and cheese. Abdullah (2010) studied the degree of contamination of table egg with bacteria of genus *Staphylococcus*, *E. coli* and *Salmonella* according to source of eggs and indicated a relatively high degree of contamination of table egg with *Staphylococcus* bacteria and Enterobacteriaceae both in yolk and on egg shell. Przybylska (2000) reported that food poisoning and food-borne infection following consumption of eggs or dishes containing eggs are usually caused by *Salmonella*, as well as *Staphylococcus aureus*, *Escherichia coli* and other coli bacilli.

The results obtained in this study showed that there was no contamination by *Salmonella* spp., as *Salmonella* was not isolated and this suggested that all the egg samples taken were *Salmonella* free. This may be

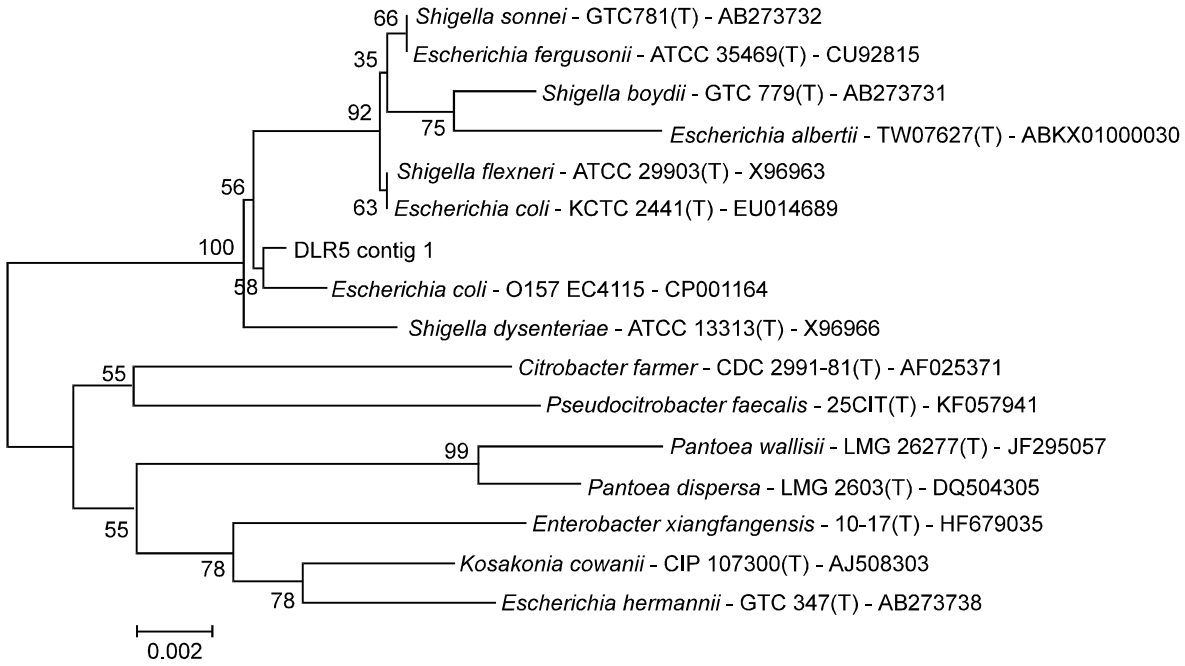

Fig. 5: Phylogenetic tree of DLR5 with the selected best homologous known bacterial strain

attributed to the fact that poultry farmers practice strict medication and care at poultry farms. Coliforms and Enterobacteriaceae populations can be used as a measure of food quality and sanitary processing conditions (Carter and Cole, 1990). The presence of these pathogenic bacteria in eggs isolated from different poultry farms in the present study indicates the poor sanitary conditions in the poultry farms. The *Staphylococcus*, *Pluralibacter* and *E. coli* which were isolated from the samples are often implicated with fecal contamination. These could be of great health concern since species of these bacteria cause illness and these contaminated eggs if consumed raw or semi-raw may be responsible for sporadic or epidemic diseases.

Although the enteric bacteria isolated from egg samples in the present study are not universally considered to be pathogens, but these may present a health risk for consumers using raw or undercooked eggs in their diet. WHO predicts that serious problems with opportunistic infections may appear in the 21st Century. For this reason it is necessary to educate the public about good sanitary practices in handling eggs and preparing them for consumption.

Conclusion: The present study, thus, concludes that the egg samples collected from poultry farms in Jaipur city were found to be contaminated with bacteria such as *Escherichia coli*, *Staphylococcus epidermidis* and *Pluralibacter gergoviae* which were compared to their similarity to some pathogenic bacteria, according to the gene sequences obtained from NCBI. So the consumption of these eggs may be harmful to the consumers and may become the source of food borne diseases, if consumed raw or uncooked. The consequence of these risks in human can vary from mild to fatal illness. Thus, it becomes necessary to control the transmission/infection of these micro-floras in poultry farms and to set up proper hygiene and sanitation system at poultry farm and market level so as to decrease the occurrence and prevalence of contamination of eggs. And this can be done by hand hygiene agreement, environmental decontamination in poultry farms and prophylactic antibiotic treatments.

Note: The 16S rRNA gene sequences of isolates AJR3, AGR2, AGR4 and DLR5 were submitted to GenBank database using Bankit submission tool of National Center for Biotechnology Information (NCBI), USA. The GenBank accession numbers were assigned to the submitted sequences of AJR3, AGR2, AGR4 and DLR5 as KT799652, KT735050, KT835655 and KT799653, respectively.

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