

First report of isolation and antibiotic susceptibility pattern of *Raoultella electrica* from table eggs in Jaipur, India

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Abstract

Raoultella electrica, a Gram-negative, non-spore-forming, rod-shaped facultative anaerobe, was identified during a regular investigation of bacterial contamination in table eggs in the winter season. A total of 165 hen's eggs were collected in the winter season from 15 different areas of the city of Jaipur, India. Gram-negative *Enterobacteriales* were isolated on selective and differential media by the conventional plate method and were further identified by several biochemical tests and 16S rRNA gene sequencing. Commonly prescribed antibiotics for enteric infection were used for antibiotic susceptibility testing. For isolated microorganisms, different resistance patterns were found against the different antibiotics used ($p < 0.01$). The multiple antibiotic resistance index of bacterial isolates ranged from 0.10 to 0.60. *R. electrica* strain IGB/NBRC 109676/KCTC 32430 was isolated for the first time from commercial chicken's eggs.

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Introduction

The genus *Raoultella* comprises Gram-negative, aerobic, non-motile, capsulated rods and was recently assigned to the family *Enterobacteriaceae*. The genus was named after French bacteriologist Didier Raoult. The genus *Raoultella* was previously designated under the genus *Klebsiella* and has recently been declared to be distinct [1] on the basis of molecular characterization and some specific biochemical activities such as carbon source intake, as *Klebsiella* never uses histamine as a carbon source or grows at a lower temperature (except for *K. oxytoca*) [2,3].

On the basis of genomic similarities and differences, this genus is grouped into four species: *Raoultella ornithinolytica*, *Raoultella planticola*, *Raoultella terrigena* and *Raoultella electrica*.

Raoultella species can generally be found in the natural environment (soil, plants and water). Some strains of the *Raoultella* species may also be present in the intestinal and upper respiratory tracts [4]. Most of the *Raoultella* spp. (*R. ornithinolytica*, *R. planticola*, *R. terrigena*) are opportunistic pathogens that frequently cause pneumonia, infections of the biliary tract and bacteraemia in immunocompromised patients. Of all these species, *Raoultella ornithinolytica* and *Raoultella planticola* have mostly been reported as human pathogens [5–7], and *Raoultella terrigena* has been reported to be a rare opportunistic causative agent [8]. However, no available literature has related the pathogenicity of *R. electrica* to human or animals. Kimura et al. [9] isolated a novel *Raoultella* spp. from the anodic biofilms of a glucose-fed microbial fuel cell and proposed it as *R. electrica* strain IGB.

Eggs are consumed worldwide and are considered an important part of a healthy diet. The hen's egg is one of the most nutritious foods of animal origin, as it contains a high amount of protein, lipids, vitamins and minerals [10]. However, these nutrient substances make the egg's environment favourable for the growth of microorganisms, resulting in egg contamination.

A basic reason for egg contamination may be that the egg emerges from the hen's body through the cloaca, from which faeces is also excreted. The faecal material may thus adhere to the egg's surface, and contamination may occur in the different egg contents through shell penetration by microorganisms and different environmental factors such as temperature and humidity, which help bacterial penetration and increase the frequency of contamination [11].

As a result of improper handling, storage and environmental factors, marketed eggs may be contaminated; strong chances exist for new microbial species to infect chicken eggs. It is thus necessary to investigate and identify the bacterial microflora present on eggs and to examine these pathogens against different antimicrobial agents so as to determine the sensitivity level of a particular antibiotic for the proper clinical treatment of infections caused by consuming infected eggs and egg products.

Materials and methods

Sample collection and sample enrichment

A total of 165 hen's eggs were purchased from roadside vendors and dairies of 15 different sites of Jaipur city, India, during the 2015 winter season. The sampled eggs from different sites, which were collected in sterile plastic bags, were taken to the laboratory and processed within 6 to 8 hours of collection.

The sampled eggs were processed for the isolation of microorganisms from eggshell, albumin and yolk content. For the isolation of microflora from the eggshell, a swab technique (sterilized cotton swabs dipped in autoclaved buffered peptone water) was used for sampling. The eggshell was swabbed, and the cotton swabs were inoculated in 10 mL BPW (Buffered peptone water) in screw-cap tubes and incubated for 24 hours at 37°C [12]. For egg albumin samples, the outer surface of the egg was sterilized by wiping it with 70% ethanol, and it was opened from the air sac area of the egg [13]. The albumin part of five selected eggs was removed and homogenized. The homogenized albumin samples were serially diluted in normal saline (0.9% NaCl) and incubated at 37°C for 24 hours [14]. The same methodology as that used for albumin sampling was used for egg yolk samples.

Isolation and characterization

The prepared and incubated eggshell, egg albumin and egg yolk samples were used for the isolation of Gram-negative *Enterobacteriales* by the conventional plate method [15]. Selective and differential media were used for the isolation of enterobacteria: MacConkey agar, European Molecular Biology Laboratory agar, *Salmonella*–*Shigella* agar, bismuth sulphite agar and xylose–lysine deoxycholate agar (Hi-media, Mumbai, India).

Isolated microorganisms were then characterized through biochemical tests such as oxidase, catalase, casein hydrolysis, starch hydrolysis, carbohydrate fermentation, indole, MRVP (Methyl Red & Vogues-Proskauer), citrate utilization, urease and Kligler iron agar tests.

Antibiotic susceptibility test

The antibiotic susceptibility test was performed by the disc diffusion method [16–18]. The turbidity of bacterial culture broth was matched with 0.5 McFarland turbidity standard. Commonly prescribed antibiotics such as ceftiofuran, azithromycin, amoxicillin, gentamicin, cefixime, levofloxacin, vancomycin, ciprofloxacin, tetracycline and amoxiclav (Hi-media) for patients with gastrointestinal infection were used for the antibiotic susceptibility test. Results thus obtained were interpreted as per Clinical and Laboratory Standards Institute standards. The results were later analyzed by SPSS 16.0 software (IBM SPSS, Chicago, IL, USA). The multiple antibiotic resistance (MAR) index was also calculated, as the MAR index provides useful information for the evaluation of health risk [19,20].

16S rRNA gene sequencing

Seven isolates (with MAR index values of 0.5 or more) from the total isolates were subjected to 16S rRNA gene sequencing. These selected isolates were cultured in nutrient broth media and incubated overnight at 37°C. After incubation, 1.5 mL of broth sample was centrifuged and the respective bacterial pellets processed for genomic DNA extraction following the combined protocol of Weisburg et al. [21] and Wilson [22] with slight modifications.

The 16S rRNA gene was amplified in a thermal cycler (PCR) following the protocol of He (<http://www.bio-protocol.org/bio101/e53>) using universal 16S rRNA gene primers: forward, 27F: AGAGTTTGATCCTGGCTCAG, and reverse, 1492R: TACGTTACCTTGTTACGACTT (Eurofins Genomics India, Bangalore, India). The PCR was initiated with denaturation of DNA at 95°C for 2 minutes, and subsequently the number of cycles (denaturation at 94°C for 30 seconds, annealing at 54°C for 40 seconds, extension at 72°C for 90 seconds) was set to 35; the final extension was performed at 72°C for 10 minutes. The amplified PCR amplicons were then electrophorized at 1500 bp under ultraviolet light.

The amplified and purified 16S rRNA genes of selected isolates were subjected to automated DNA sequencing. Sequence data were generated by the BDT 3.1 cycle sequencing kit on an ABI 3730xl Genetic Analyzer (Eurofins Genomics India). The sequences were then phylogenetically analyzed by BLAST, Clustal W, PHYLIP 3.695 and TreeView 1.6.6 [23–25].

Results

Of the 165 sampled eggs, 48 (comprising 38 samples of eggshell, four samples of albumin and six samples of yolk) were found to be contaminated with microorganisms. The egg samples that showed significant growth on nutrient agar were then characterized by Gram staining, and 40 isolates were found to be Gram negative. These Gram-negative bacterial isolates were later isolated on selective-differential media, and out of 40 Gram-negative isolates, only 23 showed significant growth on selective-differential media. These 23 isolates were then characterized by biochemical tests. During this regular examination of bacterial contamination in table eggs, some isolates showed similarity to *Raoultella* according to their biochemical activities.

The antibiotic sensitivity test showed that all isolates were sensitive to the antibiotics gentamicin and levofloxacin; about 94% of the isolates were sensitive to ciprofloxacin and 88% to azithromycin. However, amoxicillin and cefixime showed the highest number of resistant isolates, at 90% and 84%, respectively (Table 1).

Molecular characterization of the bacterial isolates with high MAR index value (0.5 or higher) was performed. The isolates were identified as *Escherichia hermannii*, *Escherichia vulneris*, *Salmonella enterica* subsp. *enterica* serovar Typhimurium, *Providencia rettgeri*, *Shigella boydii* and *R. electrica*, and the 16S rRNA gene sequences of these isolates were submitted to the GenBank database (Table 2).

Discussion

The results of growth on nutrient agar revealed that eggshell is the most frequently contaminated egg part compared to albumin and yolk contents. This finding is in accordance with the results of Adesiyun et al. [26] and Arathy et al. [27].

The resistance frequencies of gentamicin, levofloxacin, azithromycin, ciprofloxacin, amoxicillin and cefixime observed in our study were in accordance with previously published results

TABLE 2. Identification and GenBank accession numbers of selected bacterial isolates

Sample ID	Identified as:	GenBank accession no.
KLR ^W 2	<i>Escherichia hermannii</i> strain CIP 103176	KY658464
SMS ^W 4	<i>Raoultella electrica</i> strain IGB	KY658465
AGR ^W 5	<i>Salmonella enterica</i> subsp. <i>enterica</i> serovar Typhimurium strain ATCC 13311	KY657585
DLR ^W 1	<i>Providencia rettgeri</i> strain DSM 4542	KY657588
DLR ^W 3	<i>Shigella boydii</i> strain P288	KY657589
AJR ^W 1	<i>Escherichia vulneris</i> strain ATCC 33821	KY657586
AJR ^W 4	<i>Escherichia vulneris</i> strain ATCC 33821	KY657587

[28–31] but were different from other published results [32,33]. On the basis of the results obtained, it can be concluded that the antibiotics gentamicin, levofloxacin, ciprofloxacin and azithromycin worked more competently than the other antibiotics used. Different resistance patterns were observed against different antibiotics (Table 3). The MAR index of the isolates (which ranged from 0.1 to 0.6) also revealed the contamination level and health risks at the sample collection sites [34,35].

According to the present study, the eggs available in the market of Jaipur, India, carry a load of different microorganisms, thus showing the poor hygiene level and poor sanitary conditions present for egg storage and transportation. The presence of coliforms and enterobacterial populations are generally used to measure the quality of food and the hygiene level. All the identified bacterial isolates in our study are generally considered to be pathogenic and are often associated with life-threatening diseases, except *R. electrica*, which has not been isolated from eggs in any previous reported studies to date.

R. electrica has been isolated only from anodic biofilms of a glucose-fed microbial fuel cell [9], with no pathogenicity described. In the present study, for the first time, *R. electrica* has been isolated from a roadside vendor of SMS (Sawai Man Singh) sampling site, a hospital-dominated area. Because the sampling site was near a hospital, there is a possibility that the bacterium could be transferred from the hospital zone to the eggs, which roadside vendors keep in the open. Because the egg can easily be contaminated by pathogenic, nonpathogenic or opportunistic pathogenic bacterial species if not properly stored, and because these contaminated eggs are unsafe for consumers if eaten raw,

TABLE 1. Results of antibiotic susceptibility testing

Antibiotic	Sensitive (%)	Intermediate (%)	Resistant (%)	p (group)
Azithromycin	88.4 ± 1.45	2.89 ± 2.89	8.69 ± 2.51	0.0023
Gentamicin	100	0	0	
Tetracycline	68.11 ± 1.45	10.14 ± 1.45	21.73 ± 2.5	
Vancomycin	27.53 ± 1.45	10.14 ± 1.45	62.31 ± 1.45	
Amoxicillin	5.79 ± 1.45	4.33 ± 2.50	89.85 ± 1.45	
Amoxiclav	44.92 ± 1.45	1.44 ± 1.44	52.17 ± 2.51	
Cefoxitin	26.08 ± 2.51	5.79 ± 1.45	68.11 ± 2.9	
Cefixime	11.59 ± 1.45	4.33 ± 2.5	84.05 ± 1.45	
Ciprofloxacin	94.2 ± 1.45	2.89 ± 1.44	2.89 ± 1.44	
Levofloxacin	100	0	0	

Data are presented as mean ± standard error; n = 3.

TABLE 3. Resistance profiles of isolated microorganisms

Isolate	Resistance profile
<i>Escherichia hermannii</i> strain CIP 103176	CFM AMX CX TE AMC VA
<i>Raoultella electrica</i> strain IGB	CFM AMX CX TE AMC VA
<i>Providencia rettgeri</i> strain DSM 4542	CFM AMX CX AMC VA
<i>Shigella boydii</i> strain P288	CFM AMX CX AMC VA
<i>Salmonella enterica</i> subsp. <i>enterica</i> serovar Typhimurium strain ATCC 13311	CFM AMX CX AMC VA
<i>Escherichia vulneris</i> strain ATCC 33821	CFM AMX CX AMC VA
<i>Escherichia vulneris</i> strain ATCC 33821	CFM AMX CX AMC VA

CFM, Cefixime; AMX, Amoxicillin; CX, Cifoxitin; TE, Tetracyclin; AMC, Amoxiclav; VA, Vancomycin.

there is a need to educate the public about the proper handling and storage of eggs and to increase the market's hygiene level.

The *R. electrica* isolated in this study was found to be resistant to different antibiotics generally used against gastrointestinal infections. Resistance was observed against cefixime, cefoxitin, amoxicillin, amoxiclav, tetracycline and vancomycin, but the microorganism was sensitive to gentamicin, azithromycin, levofloxacin and ciprofloxacin.

The present study provides basic data that confirm that some new microbial species include microorganisms isolated from consumable eggs. To our knowledge, our study is the first to isolate and identify *R. electrica* from table eggs in Jaipur city, India. Our study shows the possibilities of *Raoultella* infection in commercial eggs as well as in poultry industry. Further investigation regarding the entrance and consequences of *R. electrica* in poultry, especially in eggs, will be done later.

Conclusion

We conclude that egg samples collected from different areas of Jaipur city were found to be contaminated with bacterial isolates of family *Enterobacteriaceae*. The isolates were identified as *Escherichia hermannii*, *Escherichia vulneris*, *Salmonella enterica* serovar Typhimurium, *Shigella boydii*, *R. electrica* and *Providencia rettgeri*. Most of these isolates were found to be pathogenic. However, a new microbial species, *R. electrica*, was for the first time isolated from marketed eggs. Thus, we suggest that commercially sold eggs be occasionally monitored to identify the bacterial species related to egg contamination. Our study may also be helpful for generating the baseline data for the emergence of multidrug-resistant bacteria related to egg contamination. Because the prevalence of occurrence of antibiotic resistance is increasing, the use of antibiotics for the treatment of infection must be limited in the poultry industry so as to reduce the development of antibiotic resistance in microorganisms.

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Conflict of interest

None declared.

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