

Isolation, Identification and Resistance of *Salmonella* spp. in Eggs for Human Consumption

ABSTRACT

Aim: The present investigation aims to construct a comparative analysis based on isolation, identification and observation of resistance of *Salmonella* spp. in eggs for human consumption.

Study Design: This is an experimental study.

Place and Duration of Study: The present study was conducted in the Microbiology laboratory of Department Microbiology, St. Xavier's College (Autonomous), Kolkata (West Bengal, India), from January to March, 2020.

Methodology: In this study, a total of 48 poultry (chicken) eggs from local and super markets (24 samples from each market) were examined to study the diversity of microorganisms in eggshell surface as well as in the internal contents with the specific objective to isolate *Salmonella* spp. and to study their resistance against a range of antibiotics- Cefotaxime (30 mcg), Levofloxacin (5 mcg), Aztreonam (30 mcg), Imipenem (10 mcg), Amikacin (30 mcg) and Ceftazidime (30 mcg).

Results: Microscopic and biochemical observations revealed the presence of *Salmonella* spp. in 68% egg samples. About 22% of the identified *Salmonella* spp. cultures were resistant to the antibiotics used in the study. Further, the total and the outer membrane proteins of specific isolated *Salmonella* spp. were isolated, few of which exhibit antibiotic resistance. More than 50% of the cultures exhibited biofilm formation which is a clear indication of the potential risk that it may pose after egg consumption. A 33 kDa and a 36 kDa protein was isolated from the resistant cultures and analysed by SDS-PAGE that could serve as promising vaccine development agents and the level of toxicity was checked on Human Cheek Cells (HCCs), of which 15% caused morphological distortions in the cells.

Conclusion: The study was conducted to isolate, identify and observe the antibiotic resistance pattern of *Salmonella* spp. from eggs obtained from local and supermarkets. The study clearly indicates the prevalence of antibiotic resistant *Salmonella* spp. contamination in eggs that may be detrimental for human consumption. Thus, appropriate measures should be adopted by vendors and poultry farm workers to ensure supply of egg samples suitable for human consumption. From the research and development point of view, the results hold a promising prospect to develop potential therapeutics and vaccine candidates against selected *Salmonella* spp. to reduce the incidence of food-borne infections in humans.

Keywords: *Salmonella*, antibiotics, resistance, pathogenic, vaccine, food-borne infections.

1. INTRODUCTION

Food-borne diseases are an ever-expanding health problem on a global scale. *Salmonella* is most commonly associated with foodborne illnesses resulting in food poisoning^[1]. Infection mostly results due to ingestion of food tainted with bacteria, however, there are other ways of transmission^[2]. Among the pathogenic bacteria, typhoidal serotype and non-typhoidal *Salmonella* serotype are the root cause of hospitalization and deaths worldwide^[3]. *Salmonella* has long been characterized as an essential zoonotic pathogen. The most important route of transmission of zoonotic pathogens from animals to human is via food products of animal origin such as poultry, dairy, etc. In recent years, diseases related to *Salmonella* infection have been reported by several studies conducted in different parts of the world. Most of the infections caused by *Salmonella* give rise to asymptomatic or self-limited disease; nonetheless, immune-compromised individuals require antibiotic treatment. Recently, multi-drug resistant (MDR) cultures have emerged due to the considerable use of antibiotics in humans. Previous studies report moderate to high resistance of *Salmonella*, isolated from poultry eggs, to a wide range of antibiotics such as ampicillin, oxacillin, cephalothin, florphenicol chloramphenicol, tetracycline, neomycin and cotrimazole^{[4][5]}. The casualty rate in individuals infected with resistant *Salmonella* is 21 times greater than that infected with susceptible cultures^[6]. Majority of the outbreaks of Salmonellosis in humans have been linked with the consumption of eggs. As per reports from WHO, cases of Salmonellosis have risen significantly.

Eggs are among the most nutritious and economical foods on the planet. In any case, there is a colossal heap of microorganisms that are found in and on the eggs, which makes the handling of eggs and preparation of associated products essential. This happens during the handling of fecal material as well as when dust and dirt in nests and layered cages contaminate the eggs while it is being laid. Therefore, it is necessary to investigate the microbial contamination in eggs in order to enhance their microbial safety for human consumption. The mode of contamination includes vertical and horizontal transmission^[7]. Through vertical transmission, bacteria are introduced from infected reproductive tissues to eggs prior to shell formation. Horizontal transmission usually occurs from faecal contamination on the eggshell as the eggs are released via the cloaca, where the excretion of faeces also takes place. Be that as it may, the vertical type of transmission is for the most part connected with pathogenic microorganisms. The species of *Salmonella* can contaminate the inside of eggs before the shells are formed. Egg shells may also get contaminated with *Salmonella* from poultry droppings (poop) or the zone where they are laid. As per reports from WHO, production modules devised in previous researches predicts the occurrence of *Salmonella* contaminated eggs. Reports suggest that the probability of humans getting infected from the pathogen and develop a disease is dose dependent and this dose depends on the growth of *Salmonella* between the time the egg was laid and then prepared later on^[8]. Scarcity of data on the increased occurrences of *Salmonella* species associated with eggs in the poultry is a major health concern. Though attempts have been made everywhere, the present scenario of this pathogen in eggs is still very much obscure. Therefore, this study was aimed to determine the presence, distribution and antibiotic sensitivity of *Salmonella* in poultry (chicken) eggs to assess and address this issue globally. In our study, a total of 48 poultry (chicken) eggs from local and super markets (24 samples from each market) were examined to study the diversity of microorganisms in eggshell surface as well as in the internal contents. The isolated microorganisms were identified and characterised and subsequently checked for their potential pathogenicity (if any). The suspected pathogenic microorganisms were screened and several assays were performed to check the various levels of toxicity. The risk of contamination by microorganisms, and for the most part by *Salmonella*, is a major concern during egg production and preparation of associated egg products. Many eggs sold in the market are cleaned (treated) utilizing various different synthetics during commercial egg processing. These chemicals though have been selected keeping in mind the fact that the cuticle (outer layer of protection) is not damaged are playing a significant role in modifying the biochemical properties of the concerned microflora. In our study, we isolated the microorganisms from the egg shell surface as well as from the contents procured from different sources which includes poultry eggs from local market (untreated) and supermarket (treated). The microorganisms were characterised, using a combination of culture based and non-culture-based methods. The isolated microorganisms were studied both qualitatively and quantitatively. The suspected isolated microorganisms were then identified using several basic and specific techniques including Gram characterization, specific biochemical assays and protein characterization studies to check for the presence of any potential toxin. To study the same, total protein was isolated and analyzed using SDS gel electrophoresis. A 33 kDa and a 36 kDa protein was isolated and analysed that could serve as potential toxins whose effects were studied on Human Cheek Cells (HCC). The HCCs after suitable incubation with the suspected pathogen were observed under a suitable microscope and the results were inferred. Hence, in this study, an effort has been made to characterize potential food-borne pathogens from eggs and their characteristics have been studied. It will help us not only to assess the potential pathogenic microorganisms present in eggs but at the same time give us an insight of their potential roles to devise specific strategies to

combat them and make eggs safer for human consumption. Our study aimed to examine the contamination levels of food borne pathogens in eggs from different sources.

2. MATERIALS AND METHODS

2.1. Sample Collection

The study was conducted to determine the presence, distribution and antibiotic sensitivity of *Salmonella* in poultry (chicken) eggs. The eggs were collected from different sources namely a) Local Market and b) Supermarket. UV treated egg (local market) and boiled egg (control) were also taken resulting in total four types of egg samples. Eggs were collected randomly from different local vendors and supermarkets in Kolkata city over a period of 1 week. A total of 48 eggs were analyzed for the presence of *Salmonella* in eggshell and egg content. Eggs were collected in sterile polythene bags and transferred by refrigerated containers to laboratory for bacteriological investigation^[9].

2.2. Isolation of microbes (preferably *Salmonella*) from eggs

Both eggshells and internal contents of the eggs were taken as samples. Buffered peptone water (BPW) was used as solvent for the egg samples. BPW was prepared by adding Peptone (10 g), NaCl (5 g), K₂HPO₄ (3.5 g), KH₂PO₄ (1.5 g) to 1000mL of water. For working with eggshells, each sample of egg was immersed in 10 ml of BPW for 2 minutes and then removed. The egg-shell rinse was immediately stored in sterile container. For working with internal contents, the eggs were dipped in 70% ethanol for 2 minutes to prevent internal contents contamination from egg shell surface. Each egg was then broken aseptically and homogenized thoroughly (Remi CM-101 cyclomixer) for 30 seconds. A 2 ml of aliquot of the internal content was then transferred to 8ml of BPW. 100 µl of samples were plated on selective media MacConkey Agar and plates were observed for growth after incubation for 24 hours at 37°C. Rappaport-Vassiliadis soya peptone broth (RVS broth) was used as an enrichment growth medium for the isolation of *Salmonella* species. It was prepared by adding Soya peptone (5 g), NaCl (8 g), KH₂PO₄ (0.6 g), K₂HPO₄ (0.4 g), MgCl₂·6H₂O (30 g) and Malachite green (0.04 g) to 1000 ml of water. This RSV media was incubated with the egg samples to isolate *Salmonella* spp. present in the sample. The isolated bacterial cultures were grown and maintained on enrichment media Brilliant Green Agar (BGA)^[9, 10].

2.3. Identification of microorganisms

A) The isolated organism was grown on BGA and Rappaport-Vassiliadis soya broth which are differential media for *Salmonella* spp. and shows some special characteristics confirming the presence of the desired organism. B) Gram staining was also done alongside to observe the morphological characteristics of the organism. C) The organism was then checked for pellicle formation in LB broth. Pellicle formation is a qualitative indication of the *Salmonella* culture being pathogenic and resistant to antibiotics^[10].

2.4. Antibiotic sensitivity test

Antibiotic sensitivity test was performed on eight different cultures isolated, following the Kirby-Bauer Method^[11]. Antibiotic hexa-ring (Hexa G-minus 5, HIMEDIA) was taken having 6 different types of discs of antibiotics- Cefotaxime (30 mcg), Levofloxacin (5 mcg), Aztreonam (30 mcg), Imipenem (10 mcg), Amikacin (30 mcg) and Ceftazidime (30 mcg). It was carefully kept on the spread culture plates and was incubated at 37°C for 24 hours. Next day the plates were observed for antibiotic sensitivity of the bacteria by checking for the zone of inhibition (if any).

2.5. Hemolysis: Effect on Red Blood Cells (RBCs)

This test was performed to check the capability of the isolated bacterial cultures to breakdown red blood cells. Blood agar (enriched media) plates (HiMedia) which contain sheep blood along with nutrient agar contents was used for this purpose. 48-hours old bacterial culture broth was used from which 100 µl it is streaked on the blood agar plates and kept for incubation at 37°C for 24 hours. After incubation, the plates were checked for the presence of zone of hemolysis (discoloured zone) indicative of active hemolysis^[12].

2.6. Biofilm assay

The colony morphology was determined as described by ^[13] with some modifications. The isolated stock bacterial cultures were grown on Nutrient agar (NA) plates incubated at 37°C overnight. The isolated colonies of organism were transferred from plates to 5 ml of Luria Bertani (LB) broth containing tryptone (10 g), yeast extract (5 g) and NaCl (10 g). The organisms were allowed to grow in a shaking incubator at 37°C for 6 hours. Each bacterial isolate was plated (3 µl) on LB agar without sodium chloride supplemented with Congo red (40 µg/ml) and Coomassie Brilliant Blue (20 µg/ml). The inoculated plates were incubated at 37°C for 96 hours and colonies were observed macroscopically.

2.7. Protein Analysis-Total protein isolation

Whole cell lysates of *Salmonella* for SDS PAGE analysis was prepared essentially (as described by ^[14]). One colony was transferred from MacConkey agar plate to 100 ml of Tryptic Soy Broth and incubated overnight at 37°C. Subsequently, the broth culture was centrifuged at 15000 rpm for 15 min at 4°C. The sediment was resuspended in 10 ml of Phosphate Buffer Solution (PBS, pH 7.2). One ml of the suspension was transferred into 1.5 ml microcentrifuge tubes and centrifuged at 15000 rpm for 15 min at 4°C. The sediment was suspended in 10 µl of 10% SDS and an equal volume of loading buffer [0.125M Tris (hydroxymethyl) aminomethane, 4% SDS, 10% 2-mercaptoethanol, 0.2% bromophenol blue] was added. After vigorous shaking by vortex, the prepared samples were boiled for 10 min at 100°C, centrifuged for 1 min (15000 rpm at 20°C) and the supernatants were stored at -20°C until use. SDS-PAGE was carried out and the gel was analysed for any protein followed by analysis of the results obtained.

2.8. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were heated with 1X SDS-loading buffer (50mM Tris- Cl; pH-6.8), 2.5% β-mercaptoethanol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol at 95°C for 10 minutes to bring out the extract. The samples were then centrifuged at 10000 rpm for 2 minutes at 4°C and then resolved by SDS-PAGE. Samples were run in SDS-gel running buffer using an electrophoresis cell. SDS-PAGE was run at 40mA and 280 volts to resolve the components (BIORAD gel apparatus was used)^[14]. The first well was loaded with the molecular weight marker (Thermo Fisher).

2.9. Toxin isolation

Cultures were grown in 15 ml of CAYE medium ^[15] at 37°C with shaking for 20 hrs (200 rev/min). Subsequently, the cultures were centrifuged at 10000 rev/min for 8 min at 4°C. The cell pellet was washed once with 5 ml of PBS (NaCl – 8 g/L, KCl – 0.2 g/L, Na₂HPO₄ – 1.44 g/L and KH₂PO₄ – 0.24 g/L in 1000 ml water). After this, the suspension was centrifuged again and resuspended in 3 ml of PBS. Each washed cell preparation was sonicated for two separate 4 min periods. After sonication, cell suspensions were centrifuged at 12500 rev/min for 20 mins at 4°C. This isolated toxin from the different cultures were used in cell toxicity assay. Human Cheek (squamous epithelial) Cells were used in this investigation.

2.10 Toxicity assay on Human Cheek Cells (HCCs)

A sterile cotton swab was taken and was gently scraped on the inside of the mouth. Then the cotton swab was smeared on the centre of the microscope slide for 2-3 seconds. To this, 20-50 µl of toxin was added and kept undisturbed for 2-5 mins. A drop of 0.5% methylene blue was added and a coverslip was placed on top. It was observed under the microscope (400X magnification). Simultaneously, a control slide was made containing cheek cells in saline only with stain^[16].

3. RESULTS AND DISCUSSION

3.1. Isolation and Identification of *Salmonella* from eggs

100 µl of samples were plated on selective media MacConkey Agar plates and incubated for 24 hours at 37°C and observed for growth the next day. Transparent and colourless colonies with no zone of bile salt precipitation were observed (from local egg wash, local egg contents and supermarket egg wash) and inferred to belong to *Salmonella* species. The suspected pathogenic cultures of *Salmonella* were labelled. Isolated colonies were then transferred to RVS broth used as an enrichment media for the growth of *Salmonella* species. The isolated *Salmonella* cultures were maintained on selective media Brilliant Green Agar (BGA) (Figure 1) to obtain pure culture and was used for further experiments. Gram staining was performed to observe the morphological characteristics of the suspected organism. Gram negative rod-shaped cells of the isolated *Salmonella* culture suspected to be a food-borne pathogen were observed microscopically (under 40X) (Figure 2-a). The organism was checked for pellicle formation in LB broth indicative of the *Salmonella* culture being pathogenic and resistant to antibiotics (Figure 2-b). Microscopic and biochemical observations revealed the presence of *Salmonella* spp. in 68% egg samples. Compared to the market samples, the incidence of *Salmonella* was lower in boiled and UV treated egg samples, which was slightly lower (12%) or consistent (20%) with another report of *Salmonella* isolation from eggshells (34%) [31]. In this study, *Salmonella* isolates were recovered not only from eggshells, but also from internal content. Previous studies revealed that under normal conditions of storage and moisture, *Salmonella* contaminating eggshells could migrate to the egg content [17], which might result in human infections.

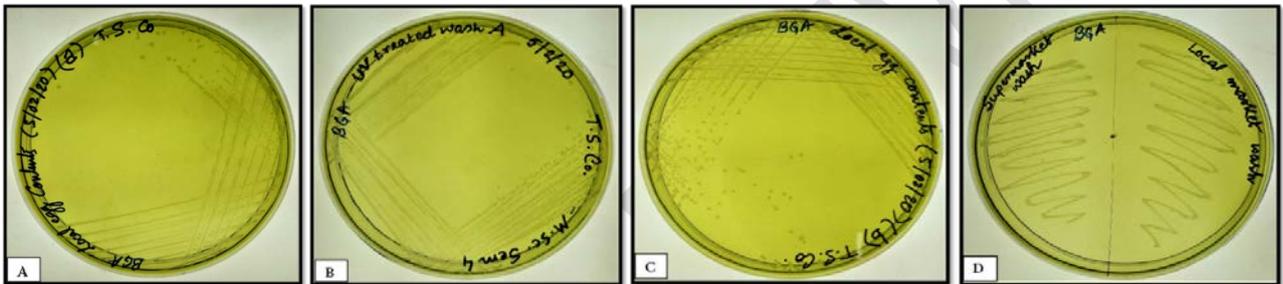


Figure 1: Brilliant green Agar (BGA) plates (A-D) showing the growth of suspected *Salmonella* colonies (a and b) from local and supermarket eggs after incubation for 24 hours at 37°C; Key: (A- Local egg contents suspected colony b, B- UV treated local egg wash suspected colony a, C- Local egg contents suspected colony b, D- Supermarket egg wash suspected colony a and Local egg wash suspected colony b).

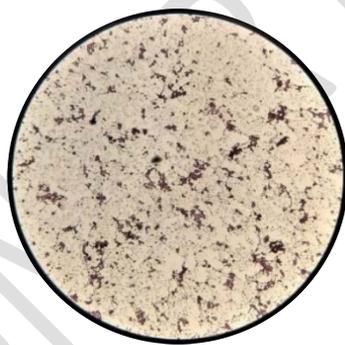


Figure 2: Figure 2a- Gram staining showing the presence of Gram-negative rod-shaped cells of the isolated *Salmonella* culture (400X magnification); Figure 2b- Arrow indicating the pellicle formation in LB shown by the suspected *Salmonella* species.

3.2. Antibiotic Resistance

The antibiotic sensitivity towards the different antibiotics were observed, namely- Cefotaxime (30 mcg), Levofloxacin (5 mcg), Aztreonam (30 mcg), Imipenem (10 mcg), Amikacin (30 mcg) and Ceftazidime (30 mcg). Results were interpreted as per the standardized reference procedures of Clinical Laboratory Standards Institute (CLSI) [18]. The suspected colonies isolated from UV treated local egg wash (colony a), local egg wash (colony b), and local egg contents (colonies a and b) were found to be mild to

moderately susceptible to all the six antibiotics. The suspected colony isolated from supermarket egg wash (colony a) was observed to be resistant towards all the antibiotics that were used in this investigation (Figure 3). Previous studies report moderate to high resistance of *Salmonella*, isolated from poultry eggs, to a wide range of antibiotics such as ampicillin, oxacillin, cephalothin, florphenicol chloramphenicol, tetracycline, neomycin and cotrimazole^{[4][5]}. Hence, these higher group of antibiotics were monitored for antibiotic sensitivity test but about 22% of the identified *Salmonella* spp. cultures were resistant to the antibiotics used in the study. Thus, it can be inferred that this culture can pose serious health implications by consuming eggs that are not properly cooked or boiled.

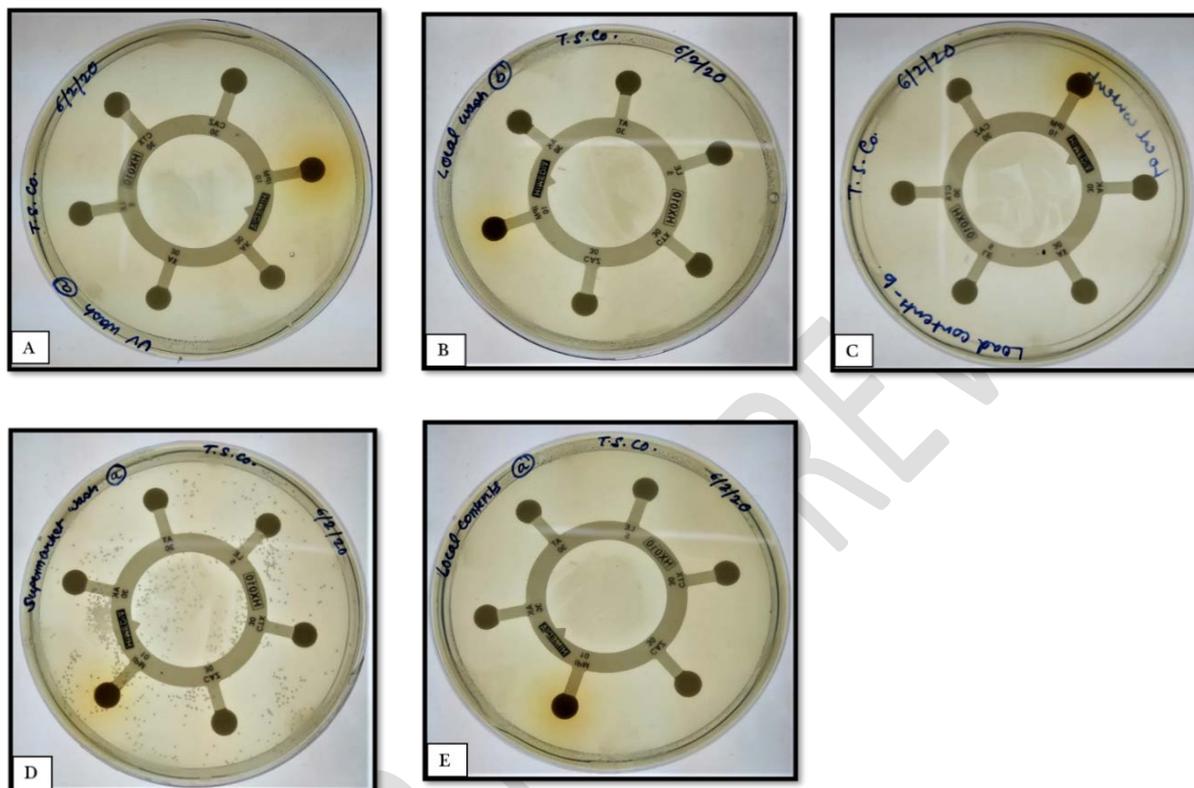


Figure 3: Antibiotic Sensitivity Assay plates (A-E) showing sensitivity of the egg microflora (local and supermarket egg shell surface and contents). Key: (A- UV treated Local egg wash suspected colony b- sensitive, B- Local egg contents suspected colony b- sensitive, C- Local egg contents suspected colony b- sensitive, D- Supermarket egg wash suspected colony a- Resistant and E- Local egg contents suspected colony b- sensitive.

Table 1: Table showing the Zone of Inhibition values (in nearest millimeter) observed for the bacterial isolates against the six antibiotics (Figure 3, A-E).

Antibiotic Sensitivity Assay plates	Antibiotic Concentration (μg)					
	Cefotaxime (30)	Levofloxacin (5)	Aztreonam (30)	Imipenem (10)	Amikacin (30)	Ceftazidime (30)
A	20 \pm 0.50	17 \pm 0.50	19 \pm 0.80	19 \pm 0.35	15 \pm 0.75	21 \pm 0.20
B	22 \pm 0.15	23 \pm 0.40	26 \pm 0.75	23 \pm 0.45	24 \pm 0.30	25 \pm 0.50

C	23±0.75	21±0.50	22±0.35	20±0.20	21±0.15	23±0.10
D	0	0	0	0	0	0
E	18±0.30	16±0.45	17±0.10	15±0.15	12±0.20	17±0.75

3.3. Hemolysis

The Blood agar plates streaked with suspected *Salmonella* culture (48 hours old culture) after incubation for 24 hours at 37°C were observed by checking for presence of zone of hemolysis (discoloured zone). The suspected colonies isolated from UV treated wash (colony a and b), Supermarket egg wash (colony a and b), Local egg contents (a and b) and Local egg market wash (colony a) did not exhibit hemolysis (gamma-hemolysis). The suspected colony isolated from Local egg market wash (colony b) exhibited hemolysis (beta-hemolysis) indicating its possible deleterious effect that it can confer by breakdown of red blood cells. The cultures isolated from boiled egg were streaked as negative control (Figure 4). Previous reports also demonstrate the pathogenicity of specific *Salmonella* strains that have hemolysis ability. Evidence from earlier studies implicates the detrimental role of hemolysis in the impairment of a variety of host defense mechanisms, including macrophage dysfunction and impairment of adaptive immune response. Hemolysis stimulates the induction of heme-oxygenase-1 in bone marrow, which impairs the ability of developing neutrophils to induce a competitive oxidative burst^[12]. Consequently, impaired neutrophils become a new niche for replication of intracellular bacteria resulting in various health risks.

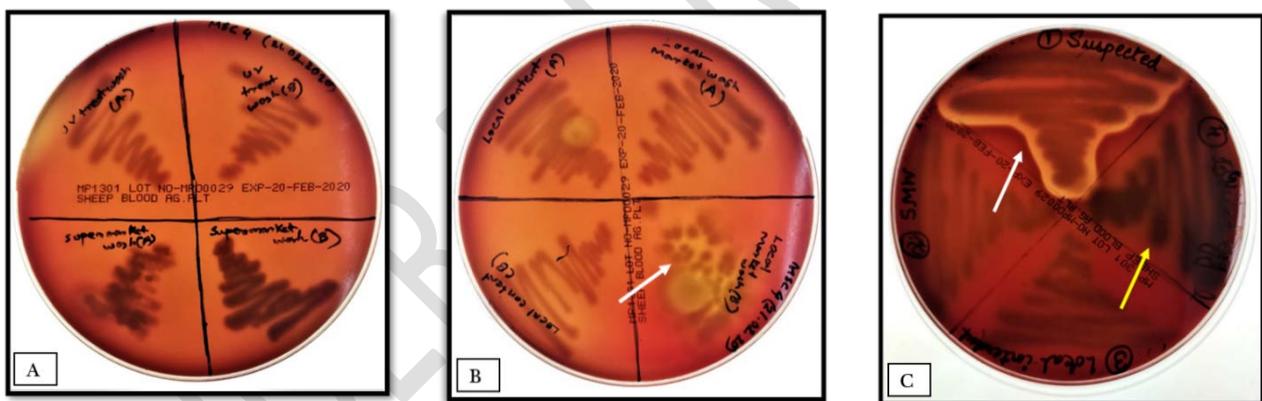


Figure 4: Blood Agar plates streaked with suspected *Salmonella* culture. White Arrows indicate hemolysis after streaking with suspected pathogenic culture of *Salmonella* and yellow arrow indicate no hemolysis after streaking with isolated cultures from boiled egg (control).

3.4. Biofilm formation

The isolated suspected colonies showed significant biofilm formation. More than 50% of the cultures exhibited biofilm formation which is a clear indication of the potential risk that it may pose after egg consumption. The ability to form a biofilm represents a significant risk not only to contamination of food items prepared with eggs but also in the kitchen environment. The results presented here are consistent with the findings of a previous study that examined the biofilm formation capacity of 78 different isolates of eight *Salmonella* serovars (Schonewille et al. 2012). *Salmonella* isolates are able to attach and form biofilm on eggshells and genes associated with curli, fimbriae and cellulose production might be responsible for biofilm formation, which may be further studied as a future prospect. Further, biofilm formation on the eggshell observed in this study could be a food safety and public health concern, as the persistence of such biofilm producing *Salmonella* strains would make their eradication more difficult, and enable them to develop resistance to antimicrobials and common disinfectants.

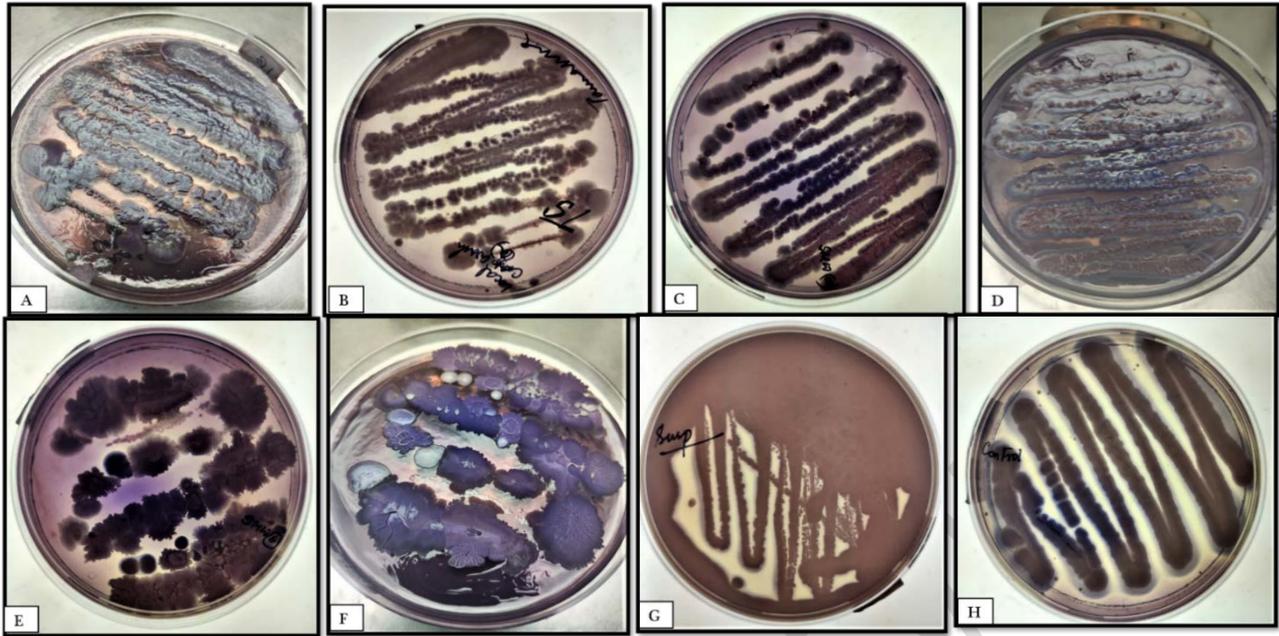


Figure 5: LB plates (without NaCl and with Congo Red and Coomassie Brilliant Blue) (A-H) after incubation with the isolated suspected organisms were checked for biofilm formation. Key: A-B: Local egg wash for colony a, C-D: Supermarket egg wash for colony a, E-F: Supermarket egg wash for colony b, G: Suspected local egg wash, H- Control organism showing no biofilm formation. The control organism used was *Bacillus subtilis*.

3.5. Total protein isolation and analysis by SDS-PAGE

The total protein was isolated and was analyzed by SDS PAGE and the bands were studied. Apart from the metabolic proteins that were observed, two bands (33 and 36kDa) unique to the toxin found in *Salmonella* cultures. Though the band corresponding to the 33kDa has been shown previously by several experiments to belong to a toxin, however, the 36kDa protein needs to be further investigated to explore its characteristics and avenues in biological application (Figure 6). In a [reviously conducted study, Ngwai et al. (2005) reported analysis of the whole cell proteins of *S. Typhimurium* strains by SDS-PAGE and detected 36.5 and 65 kDa proteins while the protein profiles of all strains were similar. Hassanain (2008) noted that protein profiles of *Salmonella* strains show many bands between 77.5 and 11.4 kDa and protein bands of 77.5, 55.2, 33.1 and 16.2 kDa are common. In our study, unique 34 and 43 kDa protein bands were observed. However, further definitive characterization and differentiation of *Salmonella* serovars are required, using alternative molecular techniques such as plasmid analysis, PFGE or RFLP.

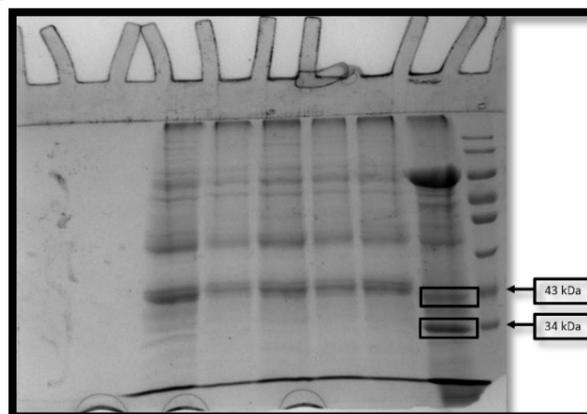


Figure 6: Gel picture after SDS gel electrophoresis showing the bands corresponding to the toxin isolated (arrows indicating the bands corresponding to the ladder sizes 34 and 43 kDa and the bands in the box indicates toxin bands of sizes 36 kDa (upper band) and 33 kDa (lower band).

3.6. Effect of toxic protein on human squamous epithelial cheek cells

The human cheek cells were smeared onto the slides and incubated with the toxin isolated from the total protein pool and were observed microscopically (Figure 7) to observe the effect of the bacterial culture on the morphology of the cells. It was clearly observed that the toxin induced morphological changes and cell distortions in the squamous cheek cells. We can infer that consumption of contaminated egg may cause damage to the oral cavity and affect the oral microflora as well. Subsequently, the toxic substances or proteins from pathogenic *Salmonella* might also enter the gastrointestinal tract and may elevate the risk of developing food-borne infections. This preliminary observation of toxin effect further needs to be substantiated with testing on cell lines.

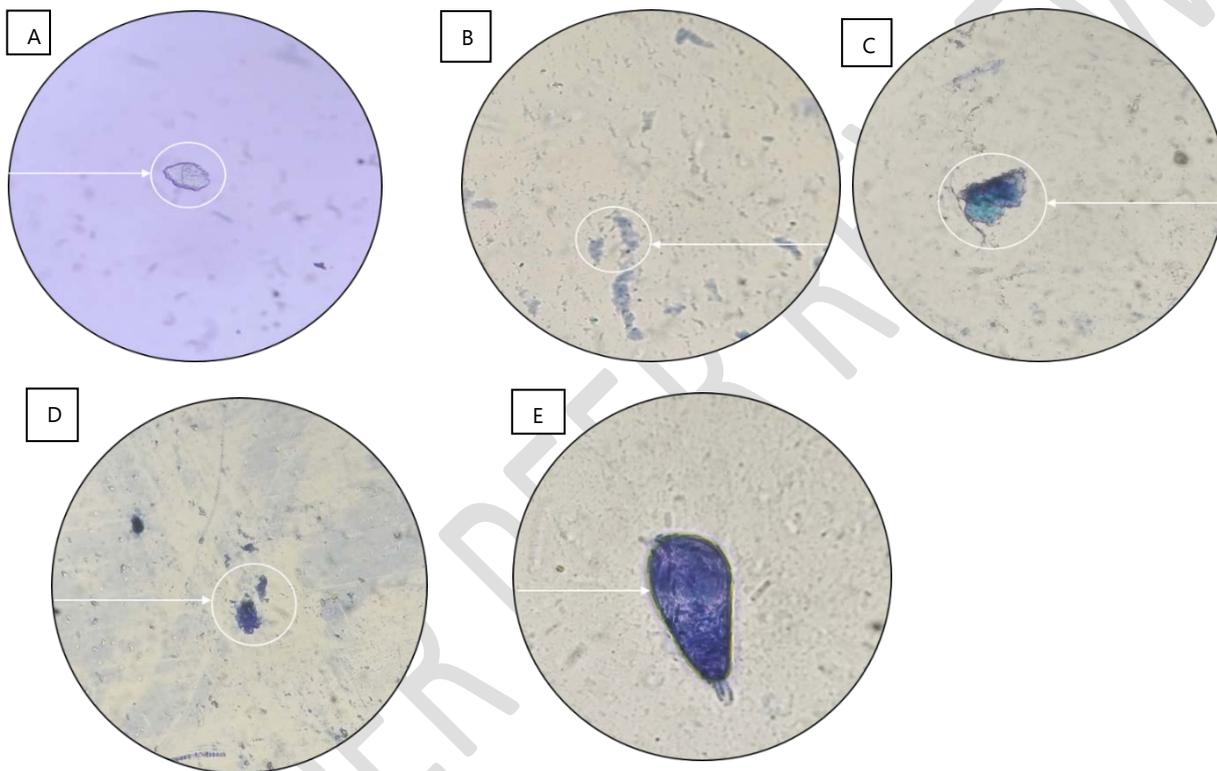


Figure 7: Toxicity Assay performed on Human Cheek Cells (HCCs) shows normal squamous cell (A) in the absence of toxin isolated; deformity in structure was observed in presence of toxin (B-E) [400x magnification].

4. CONCLUSION

The *Salmonella* species isolated were checked for pathogenicity by studying antibiotic sensitivity, pellicle formation, biofilm formation and production of any potential toxin. Microscopic and biochemical observations revealed the presence of *Salmonella* spp. in 68% egg samples. About 22% of the identified *Salmonella* spp. cultures were resistant to the antibiotics used in the study. The isolated suspected *Salmonella* culture was observed to be resistant towards all the six different antibiotics - Cefotaxime (30 mcg), Levofloxacin (5 mcg), Aztreonam (30 mcg), Imipenem (10 mcg), Amikacin (30 mcg) and Ceftazidime (30 mcg). The culture was also observed to form pellicle as well as biofilm, both of which indicate pathogenicity of the isolated bacteria. More than 50% of the cultures exhibited biofilm formation which is a clear indication of the potential risk that it may pose after egg consumption. The total protein was isolated and was checked for the presence of any potential toxin of size 33 kDa and 36 kDa. The bacteria produced toxin of size 36 kDa which was purified and subsequently its affects were studied on Human Cheek Cells (HCCs). 15% of the isolates were

seen to affect the normal morphology of the Squamous epithelial cells. Thus, we can conclude that the microorganism isolated from egg samples is a potentially pathogenic culture of *Salmonella* and thus necessary measures are needed to be taken to ensure proper microbial safety of the eggs before human consumption. We further aim to characterise the specific species of the *Salmonella* cultures obtained. The investigation and its associated study provide interesting information regarding the current prevalence of *Salmonella* in different types of eggs from various procurement sources but raises many more questions regarding the present surveillance and monitoring of the safety according to the nationally approved guidelines. The toxin isolation clearly is in accordance with previous studies and consequently caters to the initiative of designing novel vaccination strategies for ensuring food safety.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

REFERENCES

1. Gomez TM, Motarjemi Y, Miyagawa S, Käferstein FK & Stöhr K. Foodborne salmonellosis. World Health Statistics Quarterly 1997; 50(1-2):81-89.
2. Tessema K, Bedu H, Ejo M, Hiko A. Prevalence and Antibiotic Resistance of *Salmonella* Species Isolated from Chicken Eggs by Standard Bacteriological Method. Journal of Veterinary Science Technology 2017; 8: 421. doi: 10.4172/2157-7579.1000421.
3. Shahunja KM, Leung DT, Ahmed T, et al. Factors Associated with Non-typhoidal *Salmonella* Bacteremia versus Typhoidal *Salmonella* Bacteremia in Patients Presenting for Care in an Urban Diarrheal Disease Hospital in Bangladesh. PLoS Neglected Tropical Diseases 2015; 9 (9): e0004066. doi: 10.1371/journal.pntd.0004066.
4. Van, T. T. H., Moutafis, G., Istivan, T., Tran, L. T., & Coloe, P. J. Detection of *Salmonella* spp. in Retail Raw Food Samples from Vietnam and Characterization of Their Antibiotic Resistance. Applied and Environmental Microbiology 2007, 73(21), 6885–6890. doi:10.1128/aem.00972-07.
5. Mead, P. S., Slutsker, L., Dietz, V., McCaig, L. F., Bresee, J. S., Shapiro, C. & Tauxe, R. V. Food-Related Illness and Death in the United States. Emerging Infectious Diseases 1999; 5(5), 607–625. doi:10.3201/eid0505.990502.
6. Ahmed, M. M., Rahman, M. M., Mahbub, K. R., & Wahiduzzaman, M. Characterization of Antibiotic Resistant *Salmonella* spp. Isolated from Chicken Eggs of Dhaka City. Journal of Scientific Research 2010; 3(1). doi:10.3329/jsr.v3i1.6109.
7. Gantois I, Ducatelle R, Pasmans F, et al. Mechanisms of egg contamination by *Salmonella enteritidis*. FEMS Microbiology Reviews 2009; 33(4):718-738. doi:10.1111/j.1574-6976.2008.00161.x.
8. World Health Organization (WHO) Risk assessment of *Salmonella* in eggs and broiler chickens. World Health Organization; Geneva, Switzerland: 2002.
9. Zubair, Al., Berfkani, M. and Issa, A. Prevalence of *Salmonella* species from Poultry eggs of local stores of Duhok, Int J Res Med Sci. 2017 Jun;5(6):2468-2471.
10. Vassiliadis, P., Mavrommati, V., Kalapothaki, G, et al. *Salmonella* isolation with Rappaport-Vassiliadis enrichment medium seeded with different sized inocula of pre-enrichment cultures of meat products and sewage polluted water. Journal of Hygiene 1985, 95(1): 139-147.
11. Bauer, Kirby, Sherris & Turck. American Journal of Clinical Pathology 1966, 45: 493
12. Orf, K. and Cunningham, A. Infection-related hemolysis and susceptibility to Gram-negative Bacterial co-infection, Frontiers in Microbiology 2015, 6: 666.
14. Pande, V., McWhorter, AR. And Chousalkar KK. *Salmonella enterica* isolates from layer farm environments are able to form biofilm on eggshell surfaces, Biofouling 2016, 32 (7): 699-710.
15. Aksakal, A., Analysis of whole cell protein profiles of *Salmonella serovars* isolated from chicken, turkey and sheep faeces by SDS-PAGE, Veterinarni Medicina 2010, 55 (6): 259–263.

16. Hu, X., Laguerre, V., Packert, D., Nkasone, A. nadMoscinski, L. A simple and efficient method for preparing cell slides and staining without using cytocentrifuge and cytoclips, International Journal of Cell Biology 2015, Article ID 813216, 4 pages. <https://doi.org/10.1155/2015/813216>.
17. Im, MC., Jeong, SJ., Kwon, YK., Jeong, OM., Kang, MS. and Lee YJ. Prevalence and characteristics of *Salmonella* spp. isolated from commercial layer farms in Korean Poultry Science 2015;94:1691–8.
18. Performance standards of Antimicrobial Disc Susceptibility Tests, CLSI Vol. 32 No.3, Jan 2012.

UNDER PEER REVIEW