Original Research Article

A Comparative Study on the Pathogenesis of Egg contamination by different strains of *Salmonella*

ABSTRACT

Aim: Food-borne diseases are an ever-expanding health problem on a global scale. Among the pathogenic bacteria, typhoidal serotype and non-typhoidal *Salmonella enterica* serotype are the root cause of hospitalization and deaths worldwide. *Salmonella enterica* serotype enteritidis is the predominant serotype associated with egg-borne salmonellosis in humans. The present investigation aims to construct a comparative analysis on the pathogenesis of egg contamination caused by *Salmonella*.
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, poultry (chicken) eggs from local and super markets 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 strains 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 pathogenic strains in few samples. In the present study, the total and the outer membrane proteins of specific isolated Salmonella strains were isolated, few of which exhibit antibiotic resistance. A 33 kDa and a 36 kDa protein was isolated 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).
Conclusion: The results hold a promising prospect to develop potential therapeutics and vaccine candidates against selected Salmonella strains to reduce the incidence of food-borne infections in humans.

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

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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 enterica* 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) strains have emerged due to the considerable use of antibiotics in humans [4][5]. The casualty rate in individuals infected with resistant *Salmonella* is 21 times greater than that infected with susceptible strains [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, it was found that the outer microflora is resistant to many of the commonly known antibiotics. In the study concerned, the number of bacteria on the shells and inside the eggs were characterized. The microorganisms were checked for its 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 egg samples.

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), K_2HPO_4 (3.5 g), KH_2PO_4 (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), KH_2PO_4 (0.6 g), K_2HPO_4 (0.4 g), $MgCl_2$. $6H_20$ (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 strains were grown and maintained on enrichment media Brilliant Green Agar (BGA).

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* strain being pathogenic and resistant to antibiotics.

2.4. Antibiotic sensitivity test

Antibiotic sensitivity test was performed on eight different strains isolated, following the Kirby-Bauer Method [9]. 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 strain 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.

2.6. Biofilm assay

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The colony morphology was determined as described by [10] 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 [11]). One colony was transferred from MacConkey agar plate to100 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 95ºC for 10 minutes to bring out the extract. The samples were then centrifuged at 10000 rpm for 2 minutes at 4ºC 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). 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 [12] 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, Na_2HPO_4 – 1.44 g/L and KH_2PO_4 – 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 strains 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.

3. RESULTS AND DISCUSSION

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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 strains 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* strains 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* strain 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* strain being pathogenic and resistant to antibiotics (Figure 2-b).

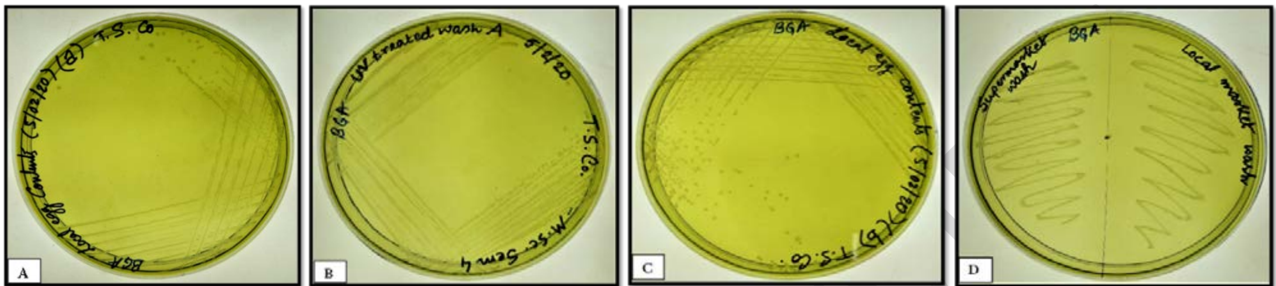


Figure 1: Brilliant green Agar (BGA) plates (**A-D**) showing the growth of suspected *Salmonella* colonies (a and b) from local and supermarket eggs after incubation for 24 hours at 37°C; Key: (**A**- Local egg contents suspected colony b, **B**- UV treated local egg wash suspected colony a, **C**-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

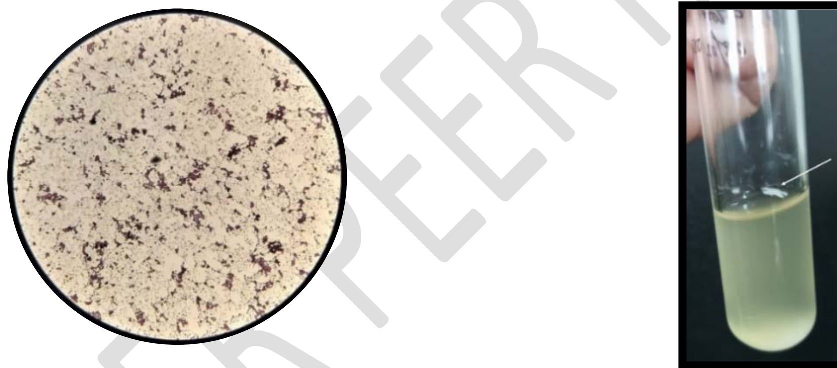
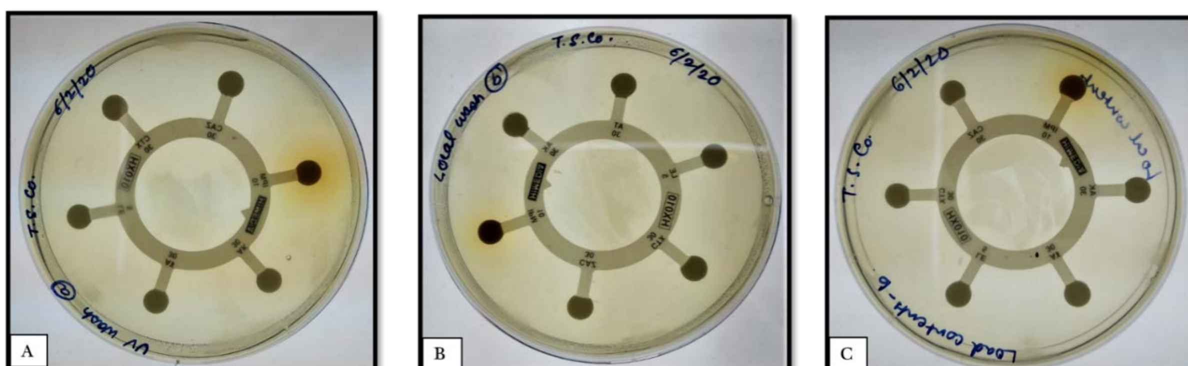


Figure 2: Figure 2a- Gram staining showing the presence of Gram-negative rod-shaped cells of the isolated *Salmonella* strain (400X magnification); **Figure 2b**- **Arrow indicating the pellicle formation** in LB shown by the suspected *Salmonella* species.

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) [13]. 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 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). Thus, it can be inferred that this strain can pose serious health implications by consuming eggs that are not properly cooked or boiled.



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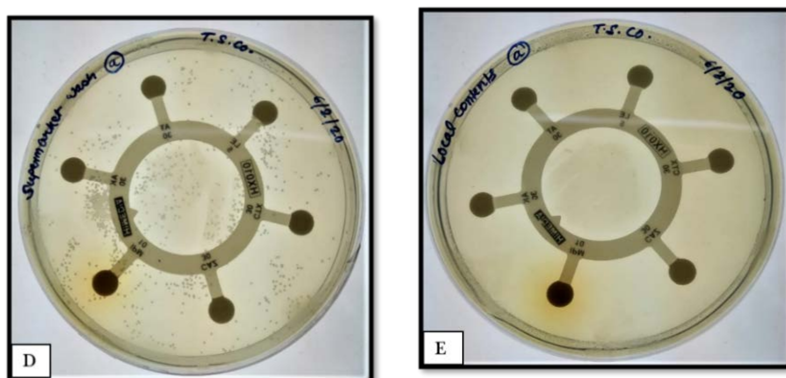

Figure 3: Antibiotic Sensitivity Assay plates (**A-E**) showing sensitivity of the egg microflora (local and supermarket egg shell surface and contents). Key: (**A**- UV treated Local egg wash suspected colony b- sensitive, **B**- Local egg contents suspected colony b- sensitive, **C**- Local egg contents 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±0.50	17±0.50	19±0.80	19±0.35	15±0.75	21±0.20
B	22±0.15	23±0.40	26±0.75	23±0.45	24±0.30	25±0.50
C	23±0.75	21±0.50	22±0.35	20±0.20	21±0.15	23±0.10
D	18±0.30	16±0.45	17±0.10	15±0.15	12±0.20	17±0.75
E	0	0	0	0	0	0

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The Blood agar plates streaked with suspected *Salmonella* strain (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 strains isolated from boiled egg were streaked as negative control (Figure 4).

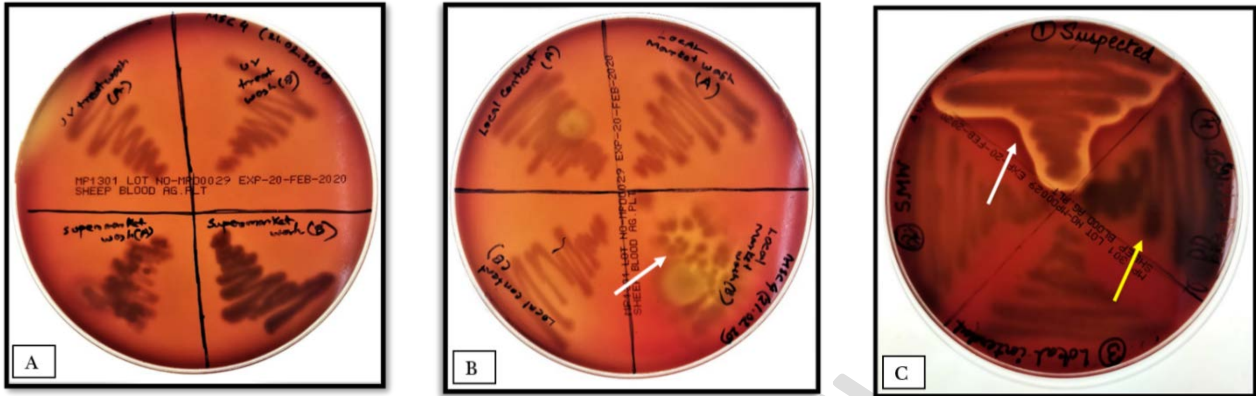

Figure 4: Blood Agar plates streaked with suspected *Salmonella* strain. White Arrows indicate hemolysis after streaking with suspected pathogenic strain of *Salmonella* and yellow arrow indicate no hemolysis after streaking with isolated strains from boiled egg (control).

The isolated suspected colonies showed significant biofilm formation.

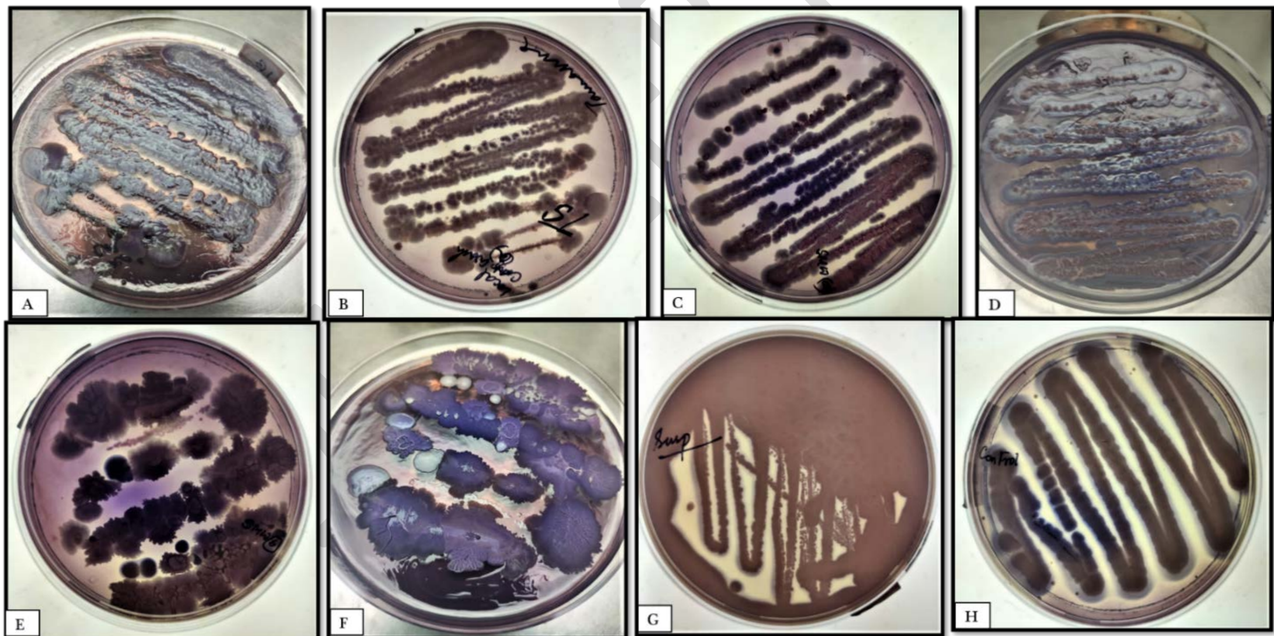

Figure 5: LB plates (without NaCl and with Congo Red and Coomassie Brilliant Blue) (**A-H**) after incubation with the isolated suspected organisms were checked for biofilm formation. Key: **A-B**: Local egg wash for colony a, **C-D**: Supermarket egg wash for colony a, **E-F**: Supermarket egg wash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

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* strains. 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).

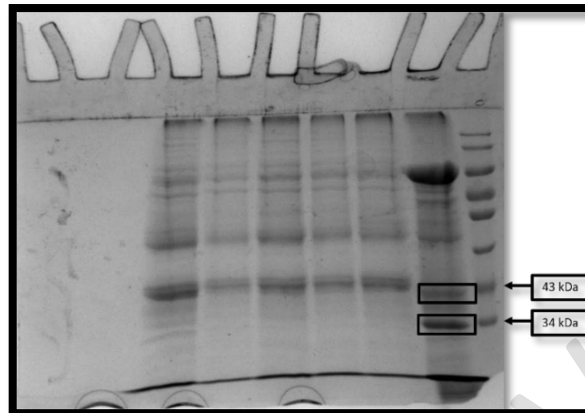

Figure 6: Gel picture after SDS gel electrophoresis showing the bands corresponding to the toxin isolated (arrows indicating the bands corresponding to the ladder sizes 34 and 43 kDa and the bands in the box indicates toxin bands of sizes 36 kDa (upper band) and 33 kDa (lower band). The human cheek cells 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 strain on the morphology of the cells. It was clearly observed that the toxin induced morphological changes and cell distortions in the squamous cheek cells.

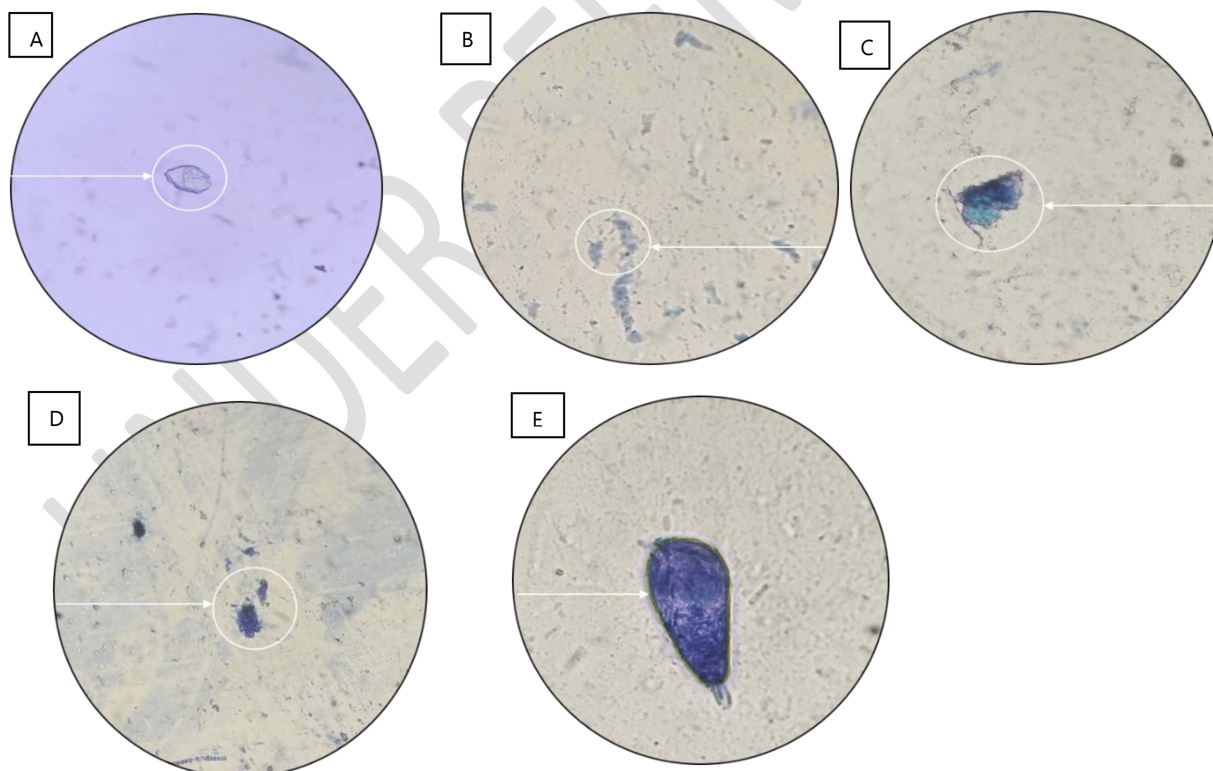

Figure 7: Toxicity Assay performed on Human Cheek Cells (HCCs) shows normal squamous cell (A) in the absence of toxin isolated; deformity in structure was observed in presence of toxin (B-E) [400x magnification].

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4. CONCLUSION

The *Salmonella* species isolated were checked for pathogenicity by studying antibiotic sensitivity, pellicle formation, biofilm formation and production of any potential toxin. The isolated suspected *Salmonella* strain 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 strain was also observed to form pellicle as well as biofilm, both of which indicate pathogenicity of the isolated bacteria. 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). It was 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 strain 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* strain 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.

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