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RESEARCH ARTICLE

BIOFILM & MOTILITY STUDIES ON FOOD BORNE PATHOGENS UNDER THE TREATMNET OF SILVER NANOPARTICLES

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ABSTRACT

Foodborne illness (sometimes called "foodborne disease," "foodborne infection," or "food poisoning) is a common, costly—yet preventable—public health problem. Many different disease-causing microbes, or pathogens, can contaminate foods, so there are many different foodborne infections. In addition, poisonous chemicals, or other harmful substances can cause foodborne diseases if they are present in food. The present study was focussed to check the effect of silver nano particles on the food borne pathogens. The study was aimed to screen the activity of the nanoparticles on the bacterila growth and its biofilm forming ability. Motility ability of the species was also assayed by capillary method. Percent inhibition of nanopartciles on the food borne pathogens was dose dependent. There was no much difference between the species. But with increasing concentrations of the nanoparticles the effect was found to be significant. The effect was found to be more at 500µg/ml. Biofilm inhibition assay was done under the treatment of nanopartciles on the food borne pathogens. This assay was also found to be dose dependent. There was no much difference between the species. But with increasing concentrations of the nanoparticles the effect was found to be significant. The effect was found to be more at 500µg/ml. The effect was significant and dose significant. There was no much difference between the species. But with increasing concentrations of the nanoparticles the effect was found to be significant. The effect was found to be more at 500µg/ml. The motility behaviour of the isolates was confirmed and was found to more with increasing time. The number of colonies was found to be more with the increase in time.

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INTRODUCTION

More than 250 different foodborne diseases have been described. Most of these diseases are infections, caused by a variety of bacteria, viruses, and parasites that can be foodborne. Other diseases are poisonings, caused by harmful toxins or chemicals that have contaminated the food, for example, poisonous mushrooms. The spectrum of foodborne pathogens includes a variety of enteric bacteria, aerobes and anaerobes, viral pathogens, and parasites, as well as marine dinoflagellates, bacteria that produce biotoxins in fish and shellfish, and the self-inducing prions of transmissible encephalopathies (Tauxe, 2002).

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Viruses are the most common pathogens transmitted via food, for example they cause 66.6% of food related illnesses in the United States, compared to 9.7% and 14.2% for salmonella and campylobacter, respectively (Mead *et al*., 1999). In the Europe the Norwalk-like caliciviruses and hepatitis A virus are currently recognised as the most important human foodborne pathogens with regard to the number. People commonly get infected by eating products that have been contaminated during processing. Contamination may occur through: (i) Contact with human or animal faeces, or water contaminated with faeces (washing, irrigation, etc.) (ii) Contact with hands, objects soiled with faeces (iii) Contact with vomitus or water contaminated with vomit (iv) Contact with the environment where infected persons were previously present (v) Virus-containing aerosol produced by infected persons (Cliver, 1997a; Koopmans and Duizer, 2004). The current investigation

aims to evaluate the effect of silver nanopartciles on the food pathogens. Food spoilage is the report of bactericidal activity on the food medium causing breakdown of food components. Studies have shown that the various types of food pathogenic bacteria resistant found to be different antibiotics. The bacterial strains such as Salmonella and Vibrio species are the major microorganisms which spoil the food. Contamination of food products by using these bacteria is able to cause health hazards to human beings. Hence we tried to investigate the effect of the nanoparticles on the percent inhibition and the biofilm assays. The study was also aimed to screen the effect of nanopartciles on the motility behaviour.

Nanotechnology in the food manufacturing can get a number of forms. It takes in the use of nanotechnology in food packaging materials. Nanotechnology will increase antimicrobial covering for food products. The nanoparticles are dispersed during the synthesis and are capable to building block CO2, O2, and moisture from reaching fresh fruits, vegetables, meats and other foods. Packaging can give to the control of bacterial growth in food stuffs (J.-W. Rhim, 2007), which can the way to in the container of pathogenic bacteria, illness and disease. A serious problem in food packaging is that of migration and permeability (G. L. Robertson, 2006; B. Finnigan, 2009): no stuff is totally resistant to water vapor, atmosphere level, or moisture controlled within the food being packaged. *Salmonella* are one of the most challenging bacteria for food manufacturers, and are a major cause of gastroenteritis. It is estimated that 93.8 million cases of salmonellosis occur globally each year, with 80.3 million of these being attributed to the consumption of contaminated food products (Majowicz *et al*., 2010). Further- more, an estimated 155,000 deaths are reported annually due to *Salmonella* infection (Majowicz *et al*., 2010). The majority of reported food borne illness outbreaks related to low moisture products occur as a result of *Salmonella* contamination.

It is well known that *Salmonella* can form biofilms under numerous conditions and in response to starvation stress (Solano *et al*., 2002). It is possible therefore, that biofilm formation may play a role in the survival of *Salmonella* in response to desiccation and low-*a*w stress. Production of curli fimbriae, one of the main components of biofilms, and cellulose have both been shown to enhance long term desiccation survival (White *et al*., 2006). However, in a non-dry environment one study demonstrated the presence of curli fimbriae is not important in persistence on convey or belts, instead surface type may be a more influential factor (Stocki *et al*., 2007). No upregulation of the biosynthetic genes involved have been observed in two studies investigating the transcriptome of desiccated *Salmonella* (Li *et al*., 2012). Bacterial biofilms are structured communities of cells enclosed in self produced hydrated polymeric matrix, adherent to an inert or living surface. Biofilms form when bacteria adhere to surfaces in aqueous environments and begin to excrete a slimy, glue-like substance that can anchor them to all kinds of material – such as metals, plastics, soil particles, medical implant materials and, most significantly, human or animal tissue. The challenges to antibacterial discovery have kept the output of novel antibacterial drug classes to extraordinarily low levels over the past 25 years, even though discovery programs have been in place at large and small pharmaceutical companies as well as academic laboratories over this period. While it is easy to find compounds that kill bacteria, it is hard to find novel antibacterial classes worthy of development. The present study is mainly focussed to trace out the effect of silver nanoparticles on the food borne pathogens. Salmonella species and Vibrio species were studied in this context. The activity of the silver nanoparticles on the bacterial growth and biofilm was traced out. The percent of inhibition of the strains was calculated based on the results from tube method. Motility assay was also studied using the capillary method.

MATERIALS AND METHODS

Sample Collection: Kitchen Waste forms a significant part of domestic waste. Food waste is an unwanted raw or cooked food discarded during or after food preparation that is no longer fit for consumption or desirable (N. Jean *et al*, 2009). There is a large variety of micro organisms present in waste such as bacteria, fungi, protozoa. Sample was collected directly from waste receiving dustbin of domestic kitchen in many replicates. Sterilized 10ml vials were used for collection purpose. Following collection, the samples were immediately transferred to the laboratory for examination and subsequent analysis.

Isolation of Bacterial strains: For serial dilution, 1gm of sample was dissolved in 10ml of sterile double distilled water in a 100ml conical flask, and agitated at 120rpm for 30 min at 37°C on an orbital shaker. The sample (aqueous slurry) was further serially diluted up to 10^{-10} dilution using 0.8% saline. 100µl of each dilution was spread on Thiosulfate-citrate-bile salts-sucrose agar (TCBS) for selective isolation of Vibrio species. The plates were incubated at 37°C for 24-72 hours. Out of 20 isolates, about 9 were found prominent with regard to the vibrio species. The TCBS agar is both selective and differential. It is highly selective for Vibrios and differential due to the presence of sucrose and the dyes. Sucrose fermentation produces acid, which converts the colour of bromothymol blue or thymol blue. Two dyes rather than one make the medium produce an array of yellow, green or blue so that differentiating among various *Vibrios* is possible.

Bacterial strains: To confirm the activity on the pure cultures, food inhabiting pathogens like Salmonella were procured from MTCC. *Salmonella typhii* and *Salmonella paratyphii* were also selected for the experimental study. The strains of salmonella were procured from the MTCC repository. Two serovars of Salmonella *Salmonella typii* (MTCC 8767) and *Salmonella paratyphii* (MTCC 735) were used for the study for the comparative analysis of their resistance to the nano particles. All these cultures were maintained on nutrient agar plates at 4^0C.

Biochemical characteristics of *Vibrio* species: The isolates obtained were done with the biochemical characteristics like oxidase test, Nitrate Reduction test, Myoinositol fermentation, L-Arginine dihydrolase and Lysine Decarboxylase Test (LDC).

Nanoparticle suspension: Silver nanoparticles of size 10nm were purchased from Himedia laboratories.

Antibacterial Assay: Antimicrobial activity of the silver nanoparticles (10nm) was tested against the two species. Overnight cultures were prepared in Nutrient broth (NB) media by inoculation with a single colony from agar plates and incubated at 37^0C for 12 hrs. Overnight cultures were diluted with fresh NB media to approximately 10^4 colonies forming units (CFU) and used for further assays.

Antibacterial activity using the agar cup plate method: Both the cultures of Salmonella were pour plated onto nutrient agar plates and about 20µl of nanoparticle suspension was added into each well. The antibacterial activity was confirmed first on the plate and later assayed using tube method.

Antibacterial activity using the tube method: Silver nano particles of different concentrations 50µg/ml, 100µg/ml, 200µg/ml, 300µg/ml, 400µg/ml and 500µg/ml were prepared and used for the antibacterial assay. Eight test tubes filled with 10ml of NB media were used in the study. The first one being negative control without inoculum, and second being the control without treatment. The tubes labelled from 3-8 were used for the treatments. About 10µl of the culture suspension was added to all the tubes labelled from 2-8. *Salmonella typhii*; *Salmonella paratyphii*; *Vibrio cholera*e; *Vibrio parahemolyticus*; *Vibrio harveyi* are used as inoculums. The tubes labelled for treatments were added with 20µl of the nanopartcile suspension of varying concentrations (50µg/ml, 100µg/ml, 200µg/ml, 300µg/ml, 400µg/ml and 500µg/ml).The experiment was repeated twice for the confirmation. The percentage inhibition was calculated by using the formula: Percentage Inhibition (%) = ((dc - dt)/dc) x 100, where dc and dt represent OD600 of control and treated sample strains respectively.

Cultivation of biofilms: Although many antibiotics can freely penetrate the EPS, cells within the biofilm are often still protected. Biofilm bacteria show much greater resistance to antibiotics than their free-living counterparts. The creation of starved, stationary phase dormant zones in biofilms seems to be a significant factor in the resistance of biofilm populations to antimicrobials (Anderl *et al.*, 2003; Walters *et al.*, 2003), particularly against antibiotics such as β-lactams, which are effective against rapidly dividing gram positive bacteria by interruption of cell wall synthesis. Briefly overnight cultures grown in Luria Broth was taken and diluted to a ratio of 1: 200 using Luria Broth+Glucose solution. *Salmonella typhii*; *Salmonella paratyphii*; *Vibrio cholera*e; *Vibrio parahemolyticus*; *Vibrio harveyi* are used as inoculums and done separately in wells. The wells from 2-7 were added with 20µl of the nanoparticle suspension of varying concentrations as done in the antibacterial assay. The 1st well being the control (without treatment).The plate was incubated for overnight at 370C. After incubation the wells were washed with 200µl of PBS pH7.4 three times. The plates were then air dried and stained with 2% crystal violet for 15 minutes. The plates were then rinsed under running tap water, air dried and then crystal violet was solubilised in 200µl of ethanol: acetone 80:20. Absorbance was recorded at 590nm.

Primary adherence assay: The process of surface adhesion and biofilm development is a survival strategy employed by virtually all bacteria and refined over millions of years. This process is designed to anchor microorganisms in a nutritionally advantageous environment and to permit their escape to greener pastures when essential growth factors have been exhausted (W. Michael Dunne, Jr. 2002).

Primary adherence assay: The cell suspension of the cultures was inoculated into respective flasks with Nutrient broth containing 0.5% glucose. *Salmonella typhii*; *Salmonella paratyphii*; *Vibrio cholera*e; *Vibrio parahemolyticus*; *Vibrio harveyi* are used as inoculums. One of the flask containing nutrient broth with glucose is labelled as control. The control is used separately for each species. Flasks labelled with 1-7 were treatments with varying concentrations of nanoparticles. The flasks were then incubated at 37□C overnight. Following incubation, 200µl of the broth with the culture was diluted to an absorbance of 0.1 at 578 nanometres with nutrient Broth containing 0.5% glucose. 10µl of the suspension was added to slides and incubated for two hours at 37□C.After incubation the slides was washed three times with PBS (pH 7.4). The cells were then fixed with glycerine solution and then carried with the gram staining process. Adherent bacterial cells were observed under 40X and mean count was taken for 5 microscopic fields.

Isolation of genomic DNA: Briefly 2ml O/N culture of the five samples *Salmonella typhii*; *Salmonella paratyphii*; *Vibrio cholera*e; *Vibrio parahemolyticus*; *Vibrio harveyi* was taken in a centrifuge tube and centrifuged at 5000rpm for 10 minutes. The pellet obtained was resuspended in 300µl of lysis buffer (1M Tris HCl, 0.5M EDTA, 1% SDS) and added with 500µl of TE buffer (1M Tris HCl, 0.5M EDTA) and 500µl of chloroform. The contents are centrifuged at 9000rpm for 10 minutes and the upper aqueous layer is collected in a fresh eppendorf tube. To the aqueous layer $1/10^{th}$ volume of 3M sodium acetate is added and the DNA was precipitated with 2 volumes of chilled ethanol. The precipitated DNA is resuspended in 30-50µl of TE buffer and further stored at -20^0C.

Bacterial homotyping assay: Homtyping assay was done to confirm the similarity of the strains isolated. Restriction enzymes (HindIII) are used for the assay. The DNA isolated from all the three *Vibrio* isolates were screened for their homotyping. Briefly to 30µl of nuclease free water 4µl of assay buffer (HIND III) were added to each tube. To the respective tube 8µl of DNA was added. 1µl of enzyme (HINDIII) were added and the tubes were incubated for 1-2 hours at 37ºC. The digested samples were analysed on 0.8% agarose gel. 1kb ladder is used as molecular marker.

Motility assay by Capillary method: The assay was done according to the protocol mentioned elsewhere (Adler 1969). The effects of the duration (T) of the test and the concentration (Co) of the bacterial suspension on the number (N) of bacteria that entered the capillary were measured. Briefly the isolates were grown on nutrient agar for 24hours and the bacteria were washed from the plates with 0.1 M-phosphate buffer pH *7.0.* All the cultures were transferred to the pretreated buffer 5 to 12 h before use. The concentrations of nanoparticles used were 100µg/ml, 200µg/ml and 400µg/ml. Capillaries with a capacity of 1µl (length 32 mm) were filled with phosphate buffer and

closed at one end. The open end was placed in a bacterial suspension on a glass slide. The number of bacteria that entered the capillary after the prescribed time was determined by diluting its contents with buffer and plating portions on nutrient agar. Colonies were counted after 3 days, and their number was multiplied by the dilution factor.

RESULTS AND DISCUSSION

Sample isolation and subculture: A total of 20 bacterial isolates were isolated from the sample by serial dilution. The isolates were done with gram staining procedure and were found about 12 samples to be gram negative and the remaining gram positive. Only the gram negative strains were used for further studies. They are selected for the culturing on the TCBS agar plates. The TCBS agar plates showed about 5 different colonies which are variable in size and shape (Figure 4). Among them 3 largely different colonies were further screened and selected.

Biochemical characteristics of *Vibrio* species: The biochemical characteristics test done for the 3 screened samples and basing on the morphology studies, the colonies V1, V2, and V3 were identified to be *V.cholerae, V. parahemolyticus, and V. harveyi.*

Antibacterial Assay: Antimicrobial activity of the silver nanoparticles (10nm) was tested against the two species of Salmonella and the Vibrio species isolated. Overnight cultures were prepared in Nutrient broth (NB) media by inoculation with a single colony from agar plates and incubated at 37^0C for 12 hrs. Overnight cultures were diluted with fresh NB media to approximately 10^4 colonies forming units (CFU) and used for further assays.

Antibacterial activity using the agar cup plate method: Both the cultures of Salmonella were pour plated onto nutrient agar plates and about 20µl of nanoparticle suspension was added into each well. The antibacterial activity was confirmed first on the plate and later assayed using tube method. Antimicrobial assay of the silver nanoparticles (10nm) was examined against both the species of Salmonella. A zone of inhibition was found towards both the strains.

Antibacterial activity using the tube method: Silver nano particles of different concentrations 50µg/ml, 100µg/ml, 200µg/ml, 300µg/ml, 400µg/ml and 500µg/ml were prepared and used for the antibacterial assay. Seven test tubes filled with 10ml of NB media were used in the study. The first one being control without inoculums. The tubes labelled from 2-7 were used for the treatments. About 10µl of the culture suspension was added to all the tubes labelled from 1-8. The tubes labelled for treatments were added with 20µl of the nanopartcile suspension of varying concentrations (50µg/ml, 100µg/ml, 200µg/ml, 300µg/ml, 400µg/ml and 500µg/ml).The experiment was repeated twice for the confirmation. The percentage inhibition was calculated by using the formula: Percentage Inhibition (%) = ((dc - dt)/dc) x 100, where dc and dt represent OD600 of control and treated sample strains respectively.

A two way ANOVA between the species and treatment was conducted to compare the percent of inhibition of bacterial cells at different concentrations of nanopartciles. All effects were statistically significant at the 0.05 significance level. There was a significant effect of the nanopartciles on the species remembered at the $p<0.05$ level. The species showed significance to the treatment ($F(5,20) = 149.4254$, $p = 3.97E-15$) and to the adherence of the bacterial cells ($F(4,20)= 4.452008$, $p= 0.009797$). Percent inhibition of nanopartciles on the food borne pathogens was dose dependent. There was no much difference between the species. But with increasing concentrations of the nanoparticles the effect was found to be significant. The effect was found to be more at 500µg/ml.

Cultivation of Biofilms: Biofilm inhibition assay was done under the treatment of nanoparticles on the food borne pathogens. This assay was also found to be dose dependent. There was no much difference between the species. But with increasing concentrations of the nanoparticles the effect was found to be significant. The effect was found to be more at 500µg/ml.

A two way ANOVA between the species and treatment was conducted to compare the biofilm inhibition of bacterial cells at different concentrations of nanoparticles. All effects were statistically significant at the 0.05 significance level. There was a significant effect of the nanoparticles on the species remembered at the $p<0.05$ level. The species showed significance to the treatment ($F(5,20) = 37.9087$, $p = 1.55E-09$) and to the adherence of the bacterial cells ($F(4,20)= 3.202157$, $p= 0.034752$).

Primary adherence assay: Primary adherence assay of the bacterial samples was tested under the treatment of nanoparticles. The effect was significant and dose significant. There was no much difference between the species. But with increasing concentrations of the nanoparticles the effect was found to be significant. The effect was found to be more at 500µg/ml. A two way ANOVA between the species and treatment was conducted to compare the primary adherence of bacterial cells at different concentrations of nanoparticles. All effects were statistically significant at the 0.05 significance level. There was a significant effect of the nanoparticles on the species remembered at the $p<0.05$ level. The species showed significance to the treatment ($F(5,20) = 125.7148$, $p = 6.35E-17$) and to the adherence of the bacterial cells ($F(4,20)= 89.68814$, $p= 4.52E-14$).

Bacterial homotyping assay: Homtyping assay was done to confirm the similarity of the strains isolated. Restriction enzyme HindIII are used for the assay. The cultures were then analysed by restriction digestion. The three vibrio isolates had dissimilar banding patterns by electrophoresis using *Hind*III restriction enzyme digestion. The banding pattern showed dissimilarity confirming of the different homotypes. This confirms of their dissimilarity among the species.

Table 1. The biochemical characteristics specific for the vibrio species

Characteristics	V1	V2	V3
TCBS agar	Green	Green	Green
Gram staining	-	-	-
Lysine decarboxylase	+	+	+
Oxidase test	+	+	+
Nitrate Reduction test	+	+	+
Myo inositol fermentation	-	-	-
L-Arginine dihydrolase	-	-	-

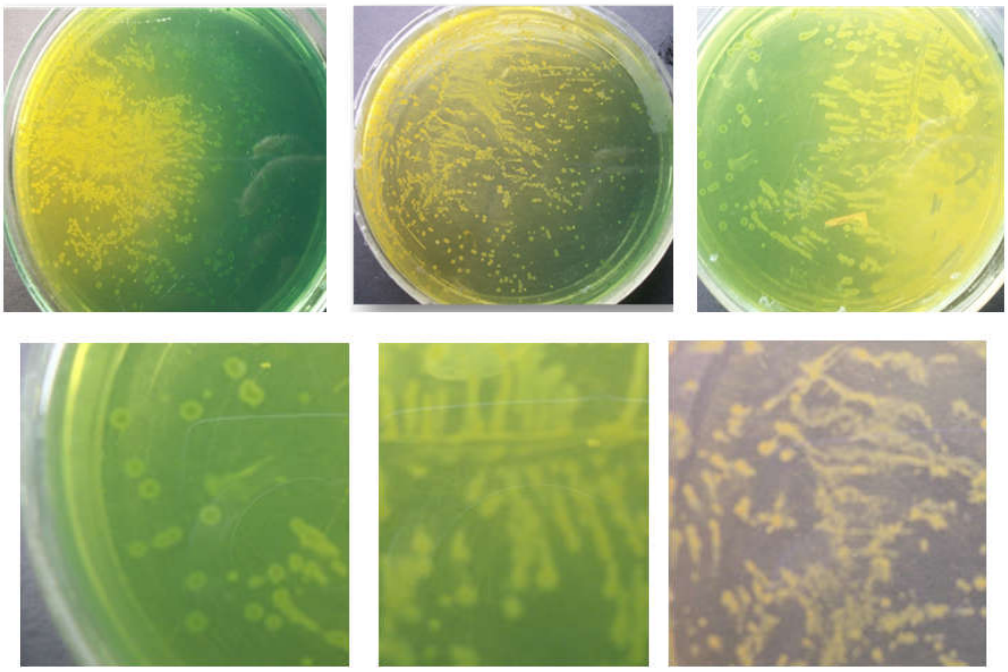

Fig. 1. Above: Vibrio colony isolates on TCBS agar plates. Below: Colony morphology of the isolates

Fig. 2. Figure showing the plates with zone of inhibition. Left: *Salomella typhii*, Right: *Salmonella paratyphii*

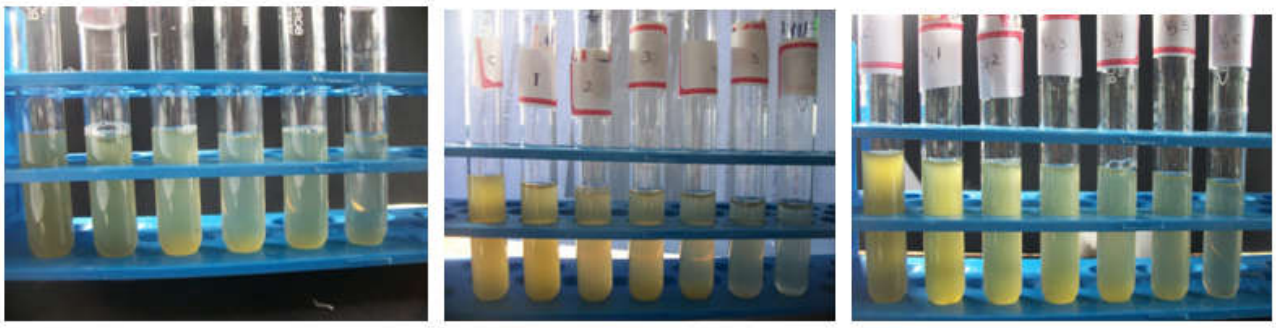

Fig. 3. Antibacterial activity uisng tube method. Left: Culture V1; Middle: Culture V2; Right: Culture V3. Tubes labelled 1 to 6 were 50µg/ml, 100µg/ml, 200µg/ml, 300µg/ml, 400µg/ml and 500µg/ml. All the experiments were done twice. The first tube C being the control without the treatment

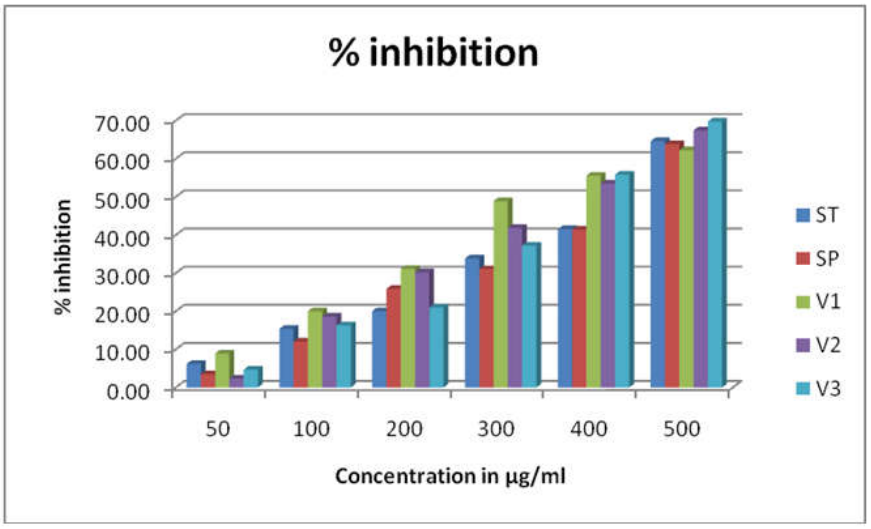

Fig. 4. Graph showing the percent inhibition of nano particle son the food borne pathogens. The concentration of the nanoparticles were expressed in µg/ml. all the values are average of triplicates. ST: *Salmonella typhii*; SP: *Salmonella paratyphii*; V1: *Vibrio cholera*; V2: *Vibrio parahemolyticus*; V3: *Vibrio harveyi*

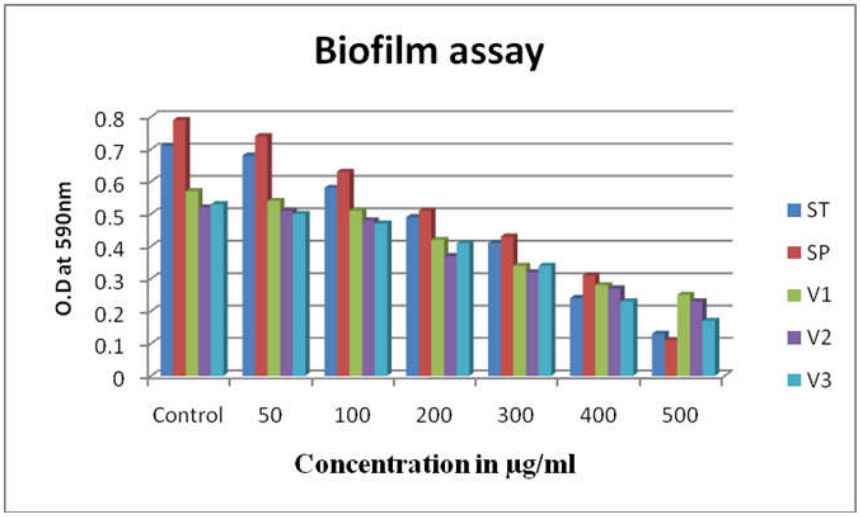

Fig 5. Figure showing the biofilm formation under the treatment of nanoparticles of 10nm size on the food borne pathogens. The concentration of the nanoparticles were expressed in µg/ml. all the values are average of triplicates. ST: *Salmonella typhii*; SP: *Salmonella paratyphii*; V1: *Vibrio cholera*; V2: *Vibrio parahemolyticus*; V3: *Vibrio harveyi*

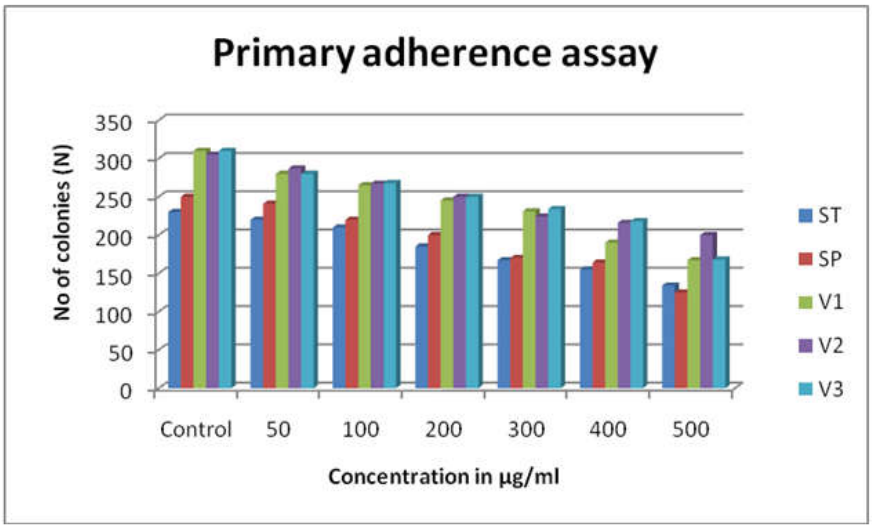

Fig. 6. Figure showing the primary adherence assay under the treatment of nanoparticles of 10nm size on the food borne pathogens. The concentration of the nanoparticles were expressed in µg/ml. All the values are average of triplicates. ST: *Salmonella typhii*; SP: *Salmonella paratyphii*; V1: *Vibrio cholera*; V2: *Vibrio parahemolyticus*; V3: *Vibrio harveyi*

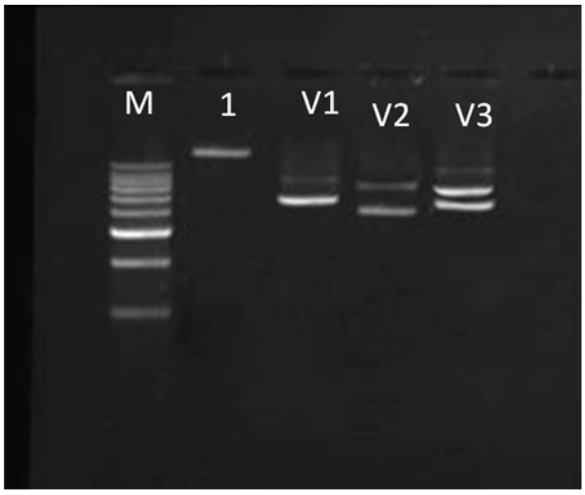

Fig. 7. 0.8% agaorse gel showing the restriction digestion. Lane M is 10kb marker, Lane 1: DNA (control); Lane V1: *Vibrio cholerae*; Lane V2: *Vibrio parahemolyticus*; Lane V3: *Vibrio harveyi*

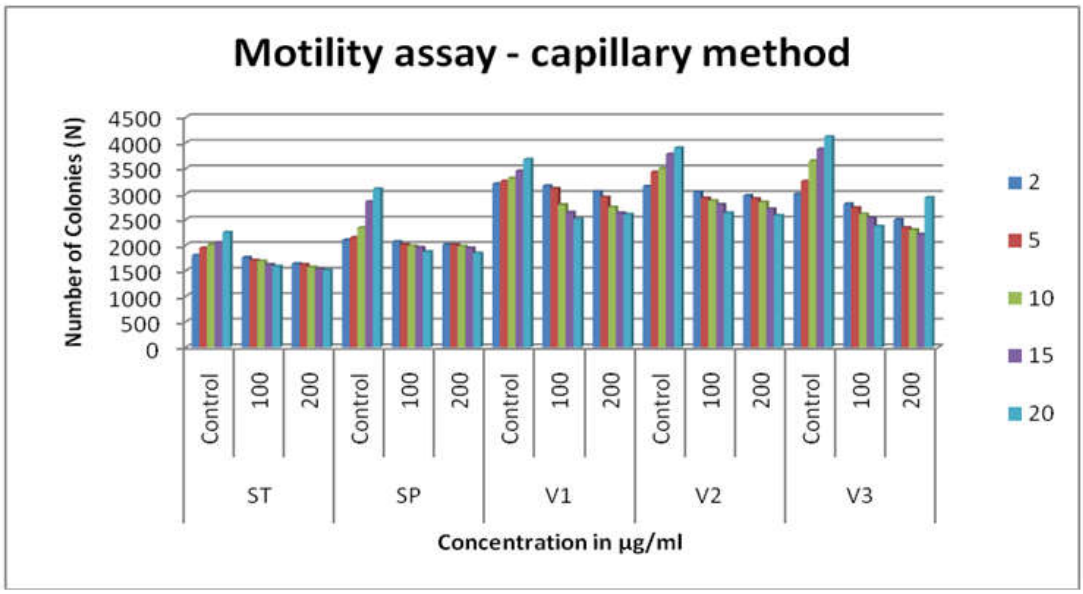

Fig 8. Graph showing the effect of duration on the number of bacteria (N). The effect was screened under the treatment of nanoparticles at a concentration of 100µg/ml and 200µg/ml. Time is expressed in minutes. ST: *Salmonella typhii*; SP: *Salmonella paratyphii*; V1: *Vibrio cholera*; V2: *Vibrio parahemolyticus*; V3: *Vibrio harveyi*

Motility assay by Capillary method: The assay was done according to the protocol mentioned elsewhere (Adler 1969). The effects of the duration (T) of the test of the bacterial suspension on the number (N) of bacteria that entered the capillary were measured.

Our results suggest that the effect of nanoparticles on the motility of the bacterial species was found to be significant. The motility behaviour of the isolates was confirmed and was found to more with increasing time. The number of colonies was found to be more with the increase in time. The effect of the treatment was checked at two concentrations of 100µg/ml and 200µg/ml. with the increase in concentration of the nanoparticles the motility behaviour of the bacterial samples were found to be decreased. The same effect was found in all the species tested. But the loss in motility behaviour was found to be in the order of *Salmonella paratyphii* > *Salmonella typhii* > *Vibrio harveyi* > *Vibrio cholera* > *Vibrio parahemolyticus*.

Conclusion

A total of 20 bacterial isolates were isolated from the sample by serial dilution. The isolates were done with gram staining procedure and were found about 12 samples to be gram negative and the remaining gram positive. Only the gram negative strains were used for further studies. They are selected for the culturing on the TCBS agar plates. Percent inhibition of nanoparticles on the food borne pathogens was dose dependent. There was no much difference between the species. But with increasing concentrations of the nanoparticles the effect was found to be significant. The effect was found to be more at 500µg/ml.

Biofilm inhibition assay was done under the treatment of nanoparticles on the food borne pathogens. This assay was also found to be dose dependent. There was no much difference between the species. But with increasing concentrations of the nanoparticles the effect was found to be significant. The effect was found to be more at 500µg/ml. Primary adherence assay of the bacterial samples was tested under the treatment of nanoparticles. The effect was significant and dose significant. There was no much difference between the species. But with increasing concentrations of the nanoparticles the effect was found to be significant. The effect was found to be more at 500µg/ml. The motility behaviour of the isolates was confirmed and was found to more with increasing time. The number of colonies was found to be more with the increase in time. The effect of the treatment was checked at two concentrations of 100µg/ml and 200µg/ml. with the increase in concentration of the nanopartciles the motility behaviour of the bacterial samples were found to be decreased. The same effect was found in all the species tested. But the loss in motility behaviour was found to be in the order of *Salmonella paratyphii > Salmonella typhii > Vibrio harveyi > Vibrio cholera > Vibrio parahemolyticus*.

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