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SYNTHESIS AND ANTIMICROBIAL ACTIVITIES OF GOLD NANOPARTICLES AGAINST *SALMONELLA* SER. *TYPHI*

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Abstract

Salmonella ser. Typhi is common pathogenic bacteria found in the environmental reservoirs, responsible for major disease outbreaks. Prevalence of drug-resistant strains of *Salmonella* ser. *Typhi* in environment is alarming for human health. The present study aims to generate drug-resistance profiling of *Salmonella* ser. *Typhi*, isolated from potable water of Gwalior and develop an alternative antimicrobial agent. Gold nanoparticles (AuNPs) were synthesized by chemical approach and characterised by UV-visible spectroscopy and Electron Microscopy. The morphology of nanoparticles was found to be quite spherical within the size range of 20-30 nm. Synthesized AuNPs were used as antimicrobial agent against drug-resistant *Salmonella* ser. *Typhi*. Study revealed that the synthesized AuNPs were effective against *Salmonella* ser. *Typhi*. The study can further be used in development of a potential antimicrobial agent against drug-resistant pathogenic bacteria.

Key words : *Salmonella* ser. Typhi, Gold nanoparticles, Antimicrobial resistance and Minimum Inhibitory Concentration.

Introduction

Contamination of safe water is a serious public-health problem in rural-urban interface. Deterioration in the microbiological quality of water often leads to water-borne disease outbreaks, which adversely effects human health. Water is said to be regenerative, as it can absorb pollution loads up to the certain levels without affecting its quality, beyond which water is harmful to both human and non-humans (Reynolds *et al.,* 2008, Ercumen *et al.,* 2014, Dickin *et al.,* 2016).

Salmonella ser. Typhi, causative agent of typhoid fever, is one of the prevalent pathogens in potable water. Typhoid results nearly 22 million cases and 216,500 deaths per year in Asia, especially countries like India exhibit high load for typhoid fever (Date *et al.,* 2014). Typhoid is a communicable disease and spreads in India by different ways. The bacterium can spread through typhoid patients, carried in large quantity by stool and vomit. It further travels to water bodies by different means. After consumption of contaminated water, the human population gets exposed to the bacterium and results in typhoid fever. In countries of South Asia, particularly Southeast Asia, typhoid fever is very much prevalent. India falls in the category of zone of typhoid by *Salmonella* ser. Typhi (CDC, 2017). Outbreaks of *Salmonellae* infections have been reported due to consumption of contaminated foods including vegetables, egg etc. According to CDC Report of 2014 ciprofloxacin resistance is developing in *Salmonella* ser. Typhi strains.

Classical methods for the identification of pathogens include pre-enrichment and biochemical assays (Law *et al.,* 2015, Biswas *et al.,* 2016). Polymerase Chain Reaction (PCR), being specific and sensitive have been established as the gold-standard for detection of pathogenic bacteria. *Salmonellae* isolate harbouring *invA* gene has been identified in potable water using molecular methods (Jyoti *et al.,* 2010, Agarwal *et al.,* 2017).

The indiscriminate use and easy availability of antibiotics without prescription leads to the dissemination of drug resistance among *Salmonellae* to most of the available antibiotics (Lima *et al.,* 2013). Combination of antibiotics with non-antibiotic drug seems to be a new choice to overcome bacterial resistance (Farha *et al.,* 2013). As a result, the drug-resistant (DR) water-borne

Salmonella ser. Typhi are prevalent in environment and spread worldwide, resulting in high rate of morbidity and mortality. This leads to necessity for the development of potential new alternative materials in order to combat this problem. Novel and effective antibiotic synthesis and implementation has many roadblocks including higher cost, time and shorter life cycle (Ventola, 2015). The development of antibiotics is indeed expensive, time consuming, risky and is also unattractive because of their short life cycle and rapid bacterial resistance. Therefore, the development of a novel antimicrobial agent which can overcome drawbacks of existing antibiotics and can work against *Salmonella* ser. Typhi is an urgent need.

Nanomaterials are materials ranging from 1-100 nm (FDA, 2014). They possess exceptional properties as compared to their corresponding bulk materials and have successful impact on biology and medicine (Azam *et al.,* 2012). Smaller dimension and high surface area are the outstanding properties of metallic nanoparticles (NPs) which allow them to actively interact with various recognition elements in biological system (Spivak *et al.,* 2013, Shrivastava *et al.,* 2019). At nano-scale size, the surface electrons became active which results into unique optical and mechanical properties (Guo *et al.,* 2014). Nanoparticles are frequently used in different bio-applications such as therapeutics and also as antimicrobial agents (Ranghar *et al.,* 2014) due to their high bio-availability. The interaction of Silver nanoparticles and their effectiveness are based on the different types of capping agents used (Ganandhas *et al.,* 2013). Several metallic nanoparticles, including Silver and Gold are able to inactivate bacterial enzymes. Therefore, nanoparticles in synergy with antibiotics could be more effective as antimicrobial agent (Hari *et al.,* 2014). The additive effect can be seen in antibiotics against several bacterial strains (Bhardwaj *et al.,* 2016).

Materials and Methods

Bacterial Culture

Potable water samples were collected from pre-identified sites of Gwalior. Each sample was filtered using membrane filtration technique. An aliquot of 500 ml sample from each site was concentrated and re-concentrated and brought up to 500 µL using repeated centrifugation at 18000 x g for 10 min at 4°C. This was followed by spreading the concentrated sample on Hi-Chrome Improved *Salmonella* Agar (Hi-media, Mumbai) and incubated for 18 h. Colonies showing pink colour on Hi-Chrome media were preliminary confirmed as *Salmonella* spp. and were preserved -70°C, as glycerol stock (15% v/v) for further characterization. *Salmonella* ser. Typhi MTCC 733, procured from Microbial Type Culture Collection (IMTech, Chandigarh) was used as positive control

Isolation of Genomic DNA

Genomic DNA was isolated from the sample by boil prep method. A 500 µL of concentrated water (after repetitive centrifugation) was boiled in a water bath at about 90°C. The debris of lysed cells was removed by centrifugation at 7000 x g for 4 min at 4°C. DNA was precipitated using boil prep method (Jyoti *et al.,* 2010). The extracted DNA was in kept 100 µL TE (pH 8.0) for further use.

Identification of *Salmonella* ser. Typhi

Salmonellae isolated from different sites of Gwalior city were further revived for the molecular characterization. Specific signature genes were selected for the molecular characterization of *Salmonellae.* The target virulent gene *invA* was found to be highly conserved among wide *Salmonella* species and almost in all serovar.

Primers

To detect *Salmonellae* harbouring *invA* gene in drinking water samples, primers (Table 1) were adopted from Jyoti *et al.,* 2010.

Detection of *Salmonella*e using PCR

Genomic DNA from pure cultures and environmental isolates were prepared and purified. In order to detect the signature gene *invA* in isolated strains, Polymerase Chain Reaction was performed using specific primers. The PCR assay was performed in 50 µL reaction mixture using 2x Hi-media PCR kit, containing dNTPs (200 µM), Taq polymerase, 10 X reaction buffer, $MgCl_2$, primer pairs (0.4 µM each) and DNA (5 µl). The optimised temperature conditions were as: initial denaturation for 3 min at 95°C and then 30 cycles at 95°C for 30s, 54°C for 45s, and 72°C for 40s. All the assays were done in triplicate. Purified multigenomic DNA (5 µL) from environmental samples were also used as template. Post PCR the amplicons were run on agarose gel containing ethidium bromide. *Salmonella* ser. Typhi MTCC 733 was used as the positive control for *invA* gene.

Molecular Characterization for identification of *Salmonella* ser. Typhi

Primers

In order to detect *Salmonella* ser. Typhi, in drinking water samples (samples were first confirmed for *Salmonellae* and were then check for *Salmonella* ser. Typhi), PCR reactions were performed harbouring signature genes-*ViaB, FliC-D* and *Prt.* Primer pair for

all the genes were adopted from Kumar *et al.*, 2006.

Detection of *Salmonella* ser. Typhi Using PCR

Genomic DNA isolated from water samples after biochemical characterization was further identified using PCR. Specific primers of virulent signature gene *Via B*, *Fli-D* & *Prt* genes of *Salmonella* ser. Typhi were used for the identification of bacteria and were followed by Kumar *et al.*, 2006 with slight modifications. PCR was performed in 50 µL reaction volume containing 0.2 µM of each primer pair (separate for each reaction), 200 µM of each dNTPs, 1 unit of Taq DNA polymerase, 2.5 mM $MgCl_2$, 1X PCR reaction buffer and 5 µl of template DNA. PCR was run using optimised temperature conditions as: denaturation at 94°C for 30s, annealing temperature was varied in all the cases, (*ViaB* gene: 56°C for 45 s, *Flic-D* gene 60°C for 45 s and *Prt* gene 62°C for 45 s and extension at 72°C for 30 s. PCR was performed through 35 cycles in Gradient Thermo Cycler (Bio-Rad Laboratories, Hercules, CA, USA). Amplicon were analysed on 1.8% agarose gel and observations recorded. *Salmonella* Typhi MTCC 733 was used as positive control for *Via B, Fli-D* and *Prt* gene.

Drug resistance profiling for *Salmonella* ser. Typhi

Drug resistance profiling was done at phenotypic level using disc diffusion method. After confirmation, they were further checked for drug resistance at genotypic level, using antibiotic resistance genes. Antibiotic resistance gene was first checked on NCBI Blast and then they were checked for specificity and selectivity.

Phenotypic Characterization

Drug-resistance profiling was done for the identified isolates of *Salmonella* Typhi against several antibiotics of major classes as per Clinical and Laboratory Standards Institute (CLSI) guidelines. These are Clindamycine (10 µg/disc), Cefoxitin (30 µg/disc), Ciprofloxacin (5 mcg/disk), Chloremphenicol (30 mcg/disk), Fosfomycine (200 mcg/disk), Aztreonain (30 mcg/disk), Ampicillin/sulbactem (10 mcg/disk), Ertapenem (10 mcg/disk), Meropenem (10 mcg/disk), Amikacin (30 mcg/disk), Doripenem (10 mcg/disk), Amoxyclav (30 mcg/disk), Trimethoprim (5 mcg/disk), Ceftazidime (10 mcg/disk), Cefuroxime (30 mcg/disk), Cefaxolin (30 mcg/disk). Each test was performed in triplicate. Data for drug-resistance were recorded as resistant (R), intermediate (I) or sensitive (S), based on CLSI guidelines. Overnight culture was spread onto Mueller Hinton (MH) agar medium plate and antibiotics were placed on the MH agar plates followed by overnight incubation at 37 °C.

Synthesis of Gold Nanoparticles

Synthesis of Gold nanoparticles was achieved by reducing auric chloride ($HAuCl_4$) with sodium borohydride ($NaBH_4$). In an Erlenmeyer flask, add 18.5 ml of de-ionized water and kept the flask on magnetic stirrer, then add 0.5 ml of 0.01 M $HAuCl_4$, stir it properly so as to maintain homogeneous mixing. After proper mixing add 0.5 ml of 0.01M Sodium citrate (addition of sodium citrate should be very precise, a small drop rate should be maintained throughout the addition) and then keep it for constant stirring for 30 min at 15°C. During the Au NPs synthetic process, the solution colour changed from yellow to pink then to red wine in colour, indicating the formation of AuNPs. AuNPs were kept for overnight in dark at room temperature, so as to settle the nanoparticles and then collecting then by centrifugation at 15000 rpm for 15 min. A repetitive wash was done with de-ionized water and pellet was collected and particles were dried in oven at 55°C.

Characterisation of Nanoparticles

Synthesised nanoparticles were characterised using various biophysical techniques. Size, concentration, shape and crucial factors for optical properties of gold nanoparticles. UV-Visible spectroscopy was used to observe the spectra of synthesised gold nanoparticles.

Morphology and size of the NPs was determined by Electron Microscope at the 120 KV (JEOL 2000). Samples after preparation and preliminary characterization were sent to sophisticated test and instrumentation centre (STIC), Cochin, Kerala. The size and particle distribution of nanoparticles was confirmed using transmission electron microscopy (TEM). Briefly, the sample was prepared by placing a drop of collected nanoparticles on a carbon coated copper grid and subsequently drying the sample in an oven at 60°C before transferring it into microscope and the size and morphology was characterized by TEM (JEOL 2000, STIC Cochin, Kerala).

***In-vitro* experiment for antimicrobial activity of nanoparticles against *Salmonella* ser. Typhi**

Salmonella ser. Typhi MTCC 733 was grown at 37°C in Luria Bertani broth. Various concentrations of gold nanoparticles (0, 10, 20, 30, 40, 50 and 100 µg/mL) were added to the grown bacterial culture (~1 x 10^5 CFU), followed by incubation at 37°C for 16-20 h in shaking incubator at 120 rpm. Bacterial cultures showing poor growth were spread (100 µL aliquots) onto the control MH agar plates (without any antibiotics) to further examine the culturability of culture and bactericidal effect of nanoparticles.

Estimation of Minimum inhibitory concentration for *Salmonella* ser. Typhi using Agar dilution method

MIC and MBC were evaluated by agar dilution

method. Agar dilution was followed using Wiegand et al 2008. Briefly, the Muller Hinton Agar (MHA) was prepared and sterilized. After autoclaving, the AuNPs in different concentrations were added before the agar solidified. This was followed by mixing the bacterial suspension (~1 x 10^5 CFU/ mL). The surface of the agar along with bacterial culture was dried and incubated at 37°C for 20 h. Grown colonies after incubation were counted next day.

Results and Discussion

Isolation of *Salmonellae*

Environmental water samples were analysed for the presence of *Salmonellae*. *Salmonellae* were confirmed primarily on the basis of colour of each individual colony on Hi-Chrome *Salmonella* Agar.

Molecular Characterisation of *Salmonellae*

Positive samples were further analysed using molecular method for the presence of *Salmonellae*. The 147 bp amplicon was observed on gel electrophoresis (Fig. 1). Results revealed that *Salmonellae* strains isolated from environmental sample exhibited virulent gene *invA*. The present study suggests the prevalence of *Salmonellae* in environmental potable water samples. Similar observations were also reported in previous studies (Jyoti *et al.,* 2010 and Agarwal *et al.,* 2015).

Molecular characterisation for identification of *Salmonella* ser. Typhi

Table 1: Nucleotide sequence of candidate oligomers of *invA* gene of *Salmonellae.*

Gene	Primer Sequence (5-3)	Tm(°C)	Amplicon
inv A	Forward: 5'-CGCACCGTCAAAGGAACC-3'	56.8	147 bp
	Reverse:5'-GCCCGATTTTCTCTGGATGG-3'	56	

Table 2: Nucleotide sequence of candidate oligomers of *via B, FliC-D* and *Prt* genes of *Salmonella* ser. Typhi.

Gene	Primer Sequence (5-3)	Tm(°C)	Amplicon
Via B	5'CACGCACCATCATTTCACCG3'	57	738 bp
	5'AACAGGCTGTAGCGATTTAGG3'	57.5	
FliC-D	5'GCTTAATGTCCAAGATGCCTAC3'	59	587 bp
	5'GAGCAACGCCAGTACCATCTG3'	58.6	
Prt	5'CGTTTGGGTTCCTTGGATCACG3'	60.5	369 bp
	5'CTATAATGGCGGCGGCGAGTTC3'	61	

Table 3: Nucleotide sequence of candidate oligomers of *via B, FliC-D and Prt gene of Salmonella* ser. Typhi.

Gene	Amplicon	Result
Via B	738bp	+
FliC-D	587bp	+
Prt	369bp	+

Different sized PCR products corresponding to each gene were observed in gel electrophoresis (Table 3). Our interpretation on virulence markers indicate that the potable water is contaminated by *Salmonella* ser. Typhi isolates exhibiting *Via B, Fli-D* & *Prt* virulent genes. Prevalence and survival of *Salmonella* ser. Typhi in water has also been reported previously and. Various regulatory genes are expressed while facilitating the survival of pathogen in water. Kingsley et al have reported the role of gene regulatory adaptation in *S.* Typhi which helps survival in water (Kingsley *et al.,* 2018). Liu et al have discussed the prevalence of S. Typhi in irrigation water and suggested the entry of pathogen in viable but non-culturable (VBNC) state of *S.* Typhi (Liu *et al.,* 2018).

Phenotypic Characterisation of drug resistance profiling for *Salmonella* ser. Typhi

Drug resistance profiling was performed using disc diffusion method at phenotypic level. Selected strains showed high resistance against Clindamycin, Aztreonam, Trimethoprim, Ceftazidime, Cefuroxime, Cefaxolin, Oxacilline, Erythromycin, Tetracycline, Co-trimoxazole (Table 4). Similar observations were reported in *Salmonella* spp., isolated form potable water (Agarwal *et al.,* (2015). However, intermediate to Amikacin Amoxyclav, Tetracycline and Streptomycin were observed among some isolates. Some of the strains were found to be sensitive against Chloremphenicol, Fosfomycine, Ampicillin/Sulbactam, Ertapenem, Meropenem, Doripenem. Nair *et al*., have discussed the prevalence of various antibiotic-resistant Salmonella serotypes in food animals and food supply (Nair *et al.,* 2018). Aregbo et al have reported the presence of drug-resistant bacteria in water reservoir (Aregbo *et al.,* 2018). Prevalence of antibiotic-resistant bacteria in water is alarming and crucial for public health.

Synthesis and Characterisation of Gold Nanoparticles

Highly stable Au nanoparticles were synthesized using Sodium citrate as capping agent. Sodium borohydrate was used as reducing agent. Finally, synthesized product was characterized using standard characterization techniques, to check the shape size and morphology of the particle.

Characterization of nanoparticles was done using different methods:

(I) UV-Visible Spectroscopy

Table 4: Antimicrobial susceptibility pattern of *Salmonella* ser. Typhi isolates was determined by disk diffusion assay.

S. No.	Antibiotics	Zone of Inhibition (mm)	Results
1.	Clindamycine(CD ^{10})	13	Resistance
2.	Cefoxitin (CX ^{30})	20	Intermediate
3.	Ciprofloxacin (CIP ^{5})	10	Intermediate
4	Chloremphenicol (C^{30})	30	Sensitive
5.	Fosfomycine (FO ^{200})	33	Sensitive
6.	Aztreonain (AT ^{30})	11	Resistance
7.	Ampicillin/sulbactem (A/S 10/10)	28	Sensitive
8.	Ertapenem (ETP ^{10})	30	Sensitive
9.	Meropenem (MRP ^{10})	33	Sensitive
10.	Amikacin (AK ^{30})	14	Intermediate
11.	Doripenem(DOR ^{10})	32	Sensitive
12.	Amoxyclav (AMC ^{30})	18	Intermediate
13.	Trimethoprim (TR ^{5})	NIL	Resistance
14.	Ceftazidime (CAZ ^{10})	12mm	Resistance
15.	Cefuroxime (CXM ^{30})	NIL	Resistance
16.	Cefaxolin (CZ ^{30})	NIL	Resistance
17.	Oxacilline (Ox^{1})	NIL	Resistance
18.	Erythromycin (E^{15})	NIL	Resistance
19.	Tetracycline (TE^{30})	13	Intermediate
20.	Streptomycin (STR^{10})	17	Intermediate
21.	Co-trimoxazol (Cot^{25})	12	Resistance
22.	Norfloxin (NOR^{10})	21	Sensitive

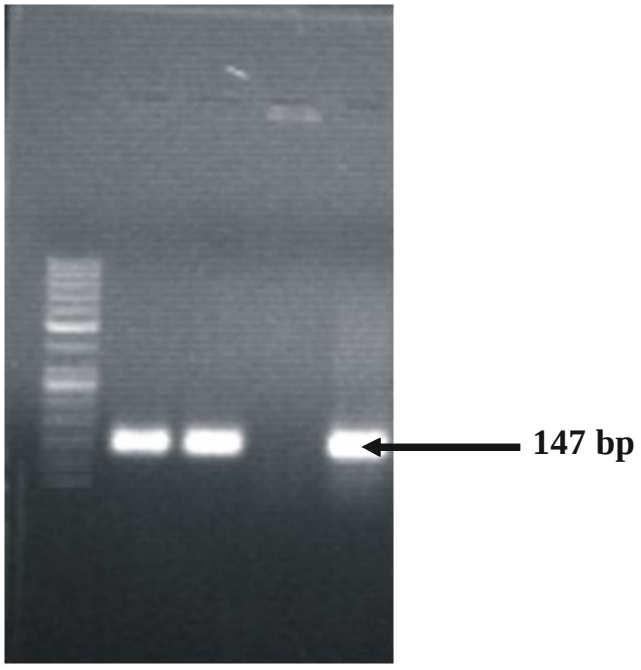

Fig. 1: PCR amplification of *invA* gene in *Salmonellae* isolates Lane 1: 50 bp DNA ladder; Lane 2 and 3: Positive isolates; Lane 4: Negative control and Lane 5: Positive control.

UV-Visible spectrum was taken in an optical quality quartz cuvette with a 1 cm path length. A visible colour changes from yellow to pink due to Surface Plasmon Resonance (SPR) vibration was observed indicating the formation of nanoparticles. Spectra were seen at room temperature, while double distilled water was used as a blank. The absorption spectrum was recorded from of 200 to 700 nm. The Gold nanoparticles synthesized by chemical method exhibit Surface plasmon resonance spectra at 515 nm.

(II) Transmission Electron Microscopy

Size of Gold NPs was in the range of 20 to 30 nm as evident from Transmission Electron Microscopy. Nanoparticles were prominently spherical in shape (Fig. 2). The selected area diffraction pattern of Au nanoparticles evidenced the crystalline planes of the face-centred-cubic structured gold (Fig. 3), which suggested the crystalline nature of synthesised gold nanoparticles.

***In-vitro* experiment for antimicrobial activity of gold nanoparticles against *Salmonella* ser. Typhi and evaluation of MIC**

Growth inhibition of *Salmonella* ser. Typhi was examined in broth containing varying concentrations (0, 10, 20, 30, 40, 50 and 100 µg/mL) of AuNPs. Gold nanoparticles were highly effective at concentration $\geq$ 50 µg/ml. At concentration of 40 µg/ml, the gold nanoparticles began to show modest antimicrobial effect on *Salmonella* Typhi and fewer viable cells were observed as compared to control samples. Further, it was observed that at concentrations $\geq$ 40 µg/

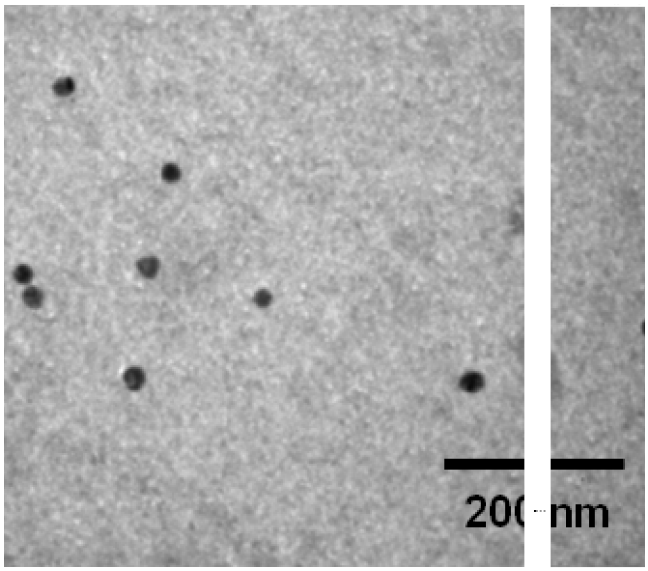

Fig. 2: TEM micrograph of synthesised Gold nanoparticles.

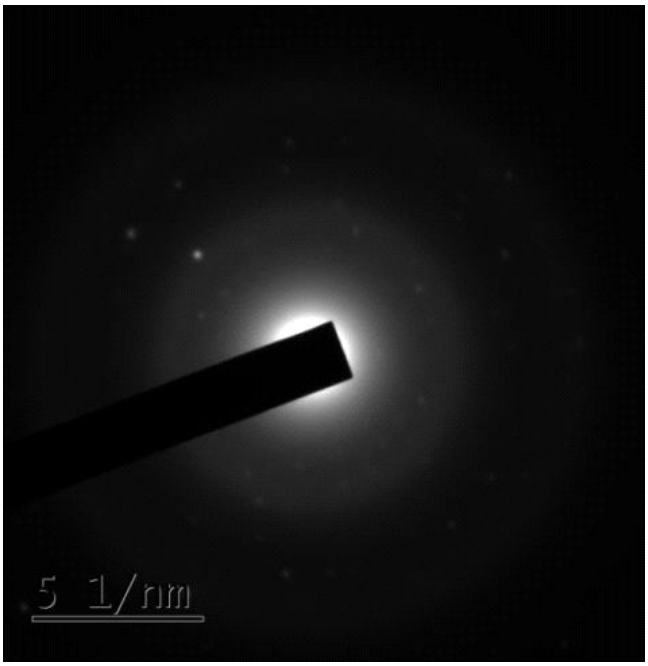

Fig. 3: SAED Pattern of synthesised Gold nanoparticles.

ml of the nanoparticles the *Salmonella* ser. Typhi culture lost its culturability. The MIC of gold nanoparticles was evaluated to be 50 µg/mL. Previous reports have demonstrated that NPs of size less than 5 nm can enter human tissues easily and may disrupt the cell normal biochemical environment (Bahadar *et al.*, 2016, Vishwakarma *et al.*, 2010).

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