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Research Article

Oligo-Probe Conjugated Gold Nanoparticles for Rapid Detection of Unamplified Bluetongue Viral RNA

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Abstract

Bluetongue (BT) is an insect born, non-fatal disease of small ruminants, particularly sheep; characterized by inflammation of buccal mucosa and coronary bands, nasal discharge, facial edema and cyanosis of the tongue, hence the name Bluetongue. The etiological agent being BT virus belonging to the *Reoviridae* family is a ds-RNA virus with a segmented genome. Though the disease is non-fatal, causes significant losses to wool and meat industry due to decreased wool production and weight losses in animals. Gold nanoparticles (GNPs) is regarded as one of the most stable nanoparticles and also because of their surface charge, they are able to be activated with majority of bio-molecules. Hence in this study we have developed a novel GNPs-based nucleic probe for detection of BT virus with sensitivity of 14.4pg/ul. The GNPs based robust technique can be implemented for diagnosis of other Arboviruses as a potential point of care assay. There is no earlier report on designing of nucleic acid nanoparticles-based assay for detection of Bluetongue virus in India and abroad to the best of our knowledge.

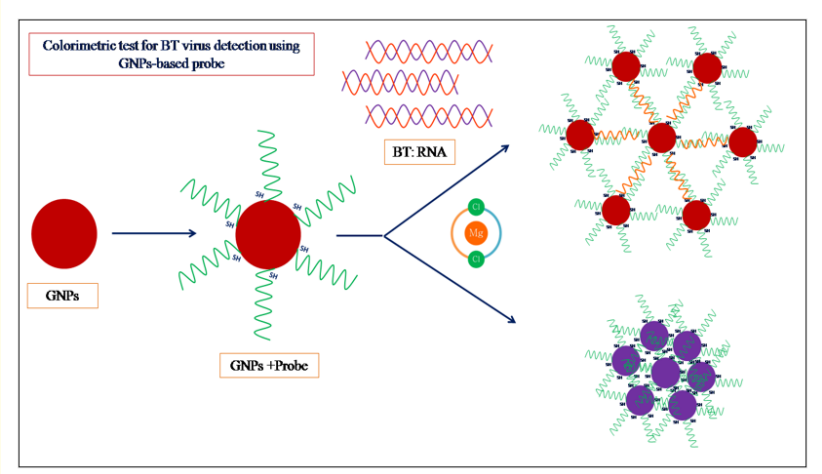

Figure a: Graphical Abstract: Colorimetric test for detection using GNPs-based probes.

Keywords: Nanoparticles; Unamplified; Bluetongue; Viral RNA

Introduction

Bluetongue (BT) is a non-contagious, vector born, viral disease of small and wild ruminants and occurs in several parts of the world. The aetiological factor is BT virus belonging to the family Reoviridae and genus Orbivirus. The vectors responsible for the transmission of BT virus are species of flies belonging to the genus Culicoides [1]. Though the mortality of BT is low, the higher percentages of morbidity causes significant losses by causing still births, foetal abnormality, abortion, weight loss, wool break, reduced meat and milk yield causing a significant cumulative economic losses to the poor farmers, livestock, wool, leather and meat industries [2]. The clinical form of BT is characterized by pyrexia, cyanosis of tongue, swelling of lips and muzzle and coronitis. In sub-clinical form of infection BT leads to reduced milk yield, body condition loss and abortion leading to infertility in animals [3]. Therefore, due to severe economic losses, BT has been included in OIE list of diseases (OIE) Surveillance and trade barriers have been imposed on movement of ruminant animals, their products and germplasm from BT endemic to BT areas [4].

BTV is icosahedral virus having ten segmented linear double-stranded RNA (dsRNA) genome. The BT genome segments encode 7 structural (VP1 to VP7) and 4 non-structural proteins (NS1, NS2, NS3/NS3a and NS4). The structural proteins play essential role in viral nucleic acid replication and viral capsid assembly. However, non-structural proteins have role in egression of viral particle from infected cell [5]. NS1 is proven to be one of the most conserved segments [6]. For diagnosis of BT, various kind of techniques have been designed including serological assays such as ELISA, AGID, CFT, HA, HI; molecular tools using RNA-PAGE, RT-PCR, q-PCR as well as whole genome sequencing. Though these assays are highly sensitive and exhibit precise results, but they require long hours to perform, sophisticated laboratories, expertise and are quite expensive.

Mankind has been fascinated by gold nano-particles since centuries and the Lycurgus cups are a prime example of their unique optical properties [7]. In the 21st century, research involving gold nanoparticles has witnessed significant growth with applications in drug delivery [8] and biosensors [9]. Along with being the most stable metallic nanoparticles, gold nanoparticles flaunt several outstanding features, including facile reactivity with bio-molecules, high surface area to volume ratios, and environment dependent optical properties, which make them the ideal candidate for use in colorimetric biosensors [10]. Since last decade GNPs-based nucleic acid detection methods have started getting popular, because of rapid and sensitive results. Pathogens-including bacteria, viruses, fungi, and protozoa-are a leading cause for loss of lives in the developing world, as well as rural areas of developed countries, due to lack of infrastructure and resources [11]. The most effective method for preventing the spread of infectious diseases is early diagnosis, which is challenging using conventional methods because of expensive equipment, specialized sample preparation, and slow data output [11]. Modern biosensors have overcome these obstacles by miniaturizing devices and providing simple rapid output that can be analyzed at the point-of-care without specialized training [12,13].

Gold nanoparticles have been implemented for the detection of pathogens, which contaminate food, water, and hospital surfaces [11,14]. A major focus of research is to improve conventional genomic analysis methods using gold nanoparticles such that the assays have lower detection limits and faster response times. Unamplified nucleic acids can be detected by functionalizing gold nanoparticles with specific thiolated probes. As compared to amplification-based methods, these assays are simpler and faster. In this study we have developed a GNPs based oligonucleotide probe for the detection of BT virus [10].

Material and Methods

Bluetongue virus isolation, cultivation

Cell culture adapted Indian isolate of BTV was used for the experimentation. The TN09CIBT isolate was grown and used. Baby hamster kidney-21 (BHK-21) cell line (National Facility on Animal Tissue Culture Collection, Pune, India) was grown and maintained by routine subculture in autoclavable minimum essential medium (MEM, Sigma) with Earle salts, with 0.03% L-glutamine (GIBCO-BRL), 10% fetal calf serum (Life Tech), Antibiotic (100 IU penicillin and 100 µg streptomycin per 100 ml), 1 M HEPES and 7.5% sodium bicarbonate.

BHK-21 cell cultures were infected with virus when cell culture attained 70-80% confluency. The cultures were inoculated with 1 ml of virus suspension for (Greiner Biocon) 0.5 ml for 25-cm^2 tissue culture flask after decanting the growth medium. Flasks were incubated at 37°C until appearance of characteristic cytopathic ef-

fect (CPE). Control cell culture flasks were processed similarly except that these were inoculated only with maintenance medium.

Viral RNA extraction

The virus was harvested by three cycles of freezing (-20˚C) and thawing (37˚C) for further inoculation in cell culture. For viral genomic RNA extraction, the cell sheet was detached and along with growth medium and centrifuged in 15 ml plastic tube at 1500 rpm (MPW-350R and REMI model C-24 BL centrifuge) for 20 min. The pellet was re-suspended in 0.5 ml of supernatant and used for RNA extraction. Remaining virus was stored at 4oC or in liquid nitrogen. RNA was isolated by TRIZOL method and the isolated RNA pellet was air dried and then dissolved in 20 µl RNase free water. The segmented dsRNA genome of the virus was analyzed by RNA-PAGE.

Synthesis of gold nanoparticles

Aquaregia was prepared by mixing three parts hydrochloric acid (HCl) to one-part nitric acid (HNO3) by volume in a beaker. The aquaregia was prepared in a well-ventilated fume hood with protective clothing and gloves. The glassware and the magnetic bead were washed with aquaregia prior to nanoparticles synthesis.

The synthesis of citrate-stabilized gold nanoparticles was based on the Turkevich method (1951) [15]. Fifty milliliters of 0.1% HAuCl4 was transferred to a 250 mL conical flask and boiled on a magnetic hot plate. Two mL of 1% trisodium citrate was added to the boiling solution and constantly stirred using a magnetic stirrer until a ruby-red color appeared. The mixture was cooled to room temperature and stored at 4°C.

Designing of probe

The probes were designed by using various bioinformatics tools. The NS1 gene belonging to segment 5 of BT genome was selected for the purpose of probe designing because of its conserved nature. Region between 447 to 895nt was considered. By using IDT PrimerQuest tool, two potential regions were identified. By using MEGA-7 and Clustal-W multiple sequence alignment was carried out. Using BioEdit tool certain changes were made in the sequences. At last at the 5' end of both the sequence a Thiol group (-SH) along with a ten-mer linker (C10) was added. The designed probes were ordered from Integrated DNA Technologies.

Functionalization of GNPs

The GNPs were functionalized by protocol mentioned by [16] by applying minor modifications. In summary, 990 µl of GNPs were transferred in a sterile 2ml centrifuge tube and was added with 10 µl of 100 µM thiol modified probe and mixed thoroughly. The pH of the solution was adjusted to 3 by adding 1M HCl and vortexed. This mixture was allowed to incubate at 4ºC for 2 hours followed by centrifugation at 8000 RPM for 15 minutes. The supernatant was discarded and then re-suspended in 1ml of 10mM phosphate buffer (PB) pH 7.2 The centrifugation step was repeated and final functionalized GNPs were dispersed in 150 µl of 10mM PB pH 7.2.

Characterization of GNPs

- **UV-V is spectrometry:** GNPs were characterized by UV-V is spectrometry before and functionalization and the difference in the absorption spectrum was measured. Different concentrations of salt NaCl was added in GNPs till they get aggregated which was followed by UV-V is spectrometry for characterization of GNPs.
- **TEM:** Transmission electron microscopy was performed by outsourcing (Jeol/JEM 2100, LaB6, Japan) the GNPs and GNPs-based probe samples to the Sophisticated Test and Instrumentation Centre (STIC), Cochin University of science and technology, Cochin, Kerala.

Optimization of detection

To know the appropriate conditions for the assay, optimization was done by using varying volumes and concentrations of RNA and GNPs-based probes as well as hybridization temperature and hybridization time for the assay and volume of $MgCl_2$ salt solution. The UV-V is spectrum was measured after each test by using Nano Drop spectrophotometer. The optimum condition was determined by getting highest absorbance and shortest lambda max in UV-V is spectrum.

Colorimetric test development

The method for colorimetric detection of RNA using GNPs-based probe was adopted from methods described previously [17] with some necessary modifications. In brief, 2 µl of extracted RNA was used as a template, which was diluted in 8 µl TE buffer pH 8. The diluted RNA was allowed to get denatured at 95º C for 5 minutes and snap chilled on ice in a 200 µl PCR tube. This was followed by addition of 5 µl of GNPs-based probe and mixed by pipetting. This was kept for incubation at 62ºC for 15 minutes. In the end, 3 µl of 0.5 M $MgCl_2$ was added and mixed by pipetting. Blank and negative control was prepared by using TE buffer and PCR purified DNA from other organisms respectively. No color change attributes

to positive result and a color change from magenta red to purple or blue attributes to negative result. Sensitivity of the assay was determined by performing tenfold serial dilutions of sample RNA and followed by same protocol.

C-DNA synthesis and PCR

The viral nucleic acid was used for cDNA preparation using Moloney Murine Leukemia virus reverse transcriptase (Mo- MuLV-RT) enzyme (Sibzyme, Russia) and random decamer primer (Ambion, USA) in thermal cycler (Bio-Rad iCycler, USA) as per manufacturer's protocol. The cDNA was allowed for group specific ns1 gene based PCR to confirm the samples as BTV. The group specific PCR was performed using ns1 gene specific primers pair forward and reverse which produced an amplicon of 123 bp size in agarose gel electrophoresis [18]. The cDNA was subjected to PCR in a 20 µl reaction mixture having 20 µM of NS1 specific primers [19], 2 µl cDNA, 3% DMSO, 0.4 µl of 10mM dNTPs mix (Finnzyme, Finland), 2 µl 10X Taq buffer in thermal cycler (Bio-Rad I Cycler, USA). The PCR amplification cycle was set as initial denaturation at 95°C for 3 minute, followed by 30 cycles of denaturation at 94°C for 30 second, primer extension at 72°C for 30 second and annealing for 30 second at 60°C. The final PCR extension was allowed at 72°C for 8 minute. The PCR products were visualized using gel documentation system (Biovis, USA) in 2% agarose gel (Lonza, Seakem LE Agarose, USA) electrophoresis. Tenfold serial dilutions were made using cDNA and then subjected to PCR to evaluate and compare the sensitivity of the PCR with the GNPs-based probes.

Results

Virus propagation and confirmation

In the present study, BHK-21 cell line was used for propagation of TN09-CIBT Indian isolate of BTV. The TN09-CIBT isolate adapted to BHK-21 cell line produced BTV specific CPE such as vacuolation in cells, aggregation and rounding of cells, floating of dead cells in medium within 36 hours (Figure 1B). The viral nucleic acid was extracted using Trizol method from pelleted cell culture materials and screened by RNA-PAGE followed by silver staining. The RNA-PAGE analysis showed characteristics BTV specific migration pattern (3:3:3:1) of viral ds-RNA (Figure 2B). The viral nucleic acid was subjected to group specific ns1 gene-based RT-PCR. Theamplicon of 123 bp confirmed the samples as BTV on agarose gel electrophoresis (Figure 2A). The characteristic CPE in BHK-21 cell culture, specific migration pattern of viral nucleic acid (3:3:3:1) in RNA-PAGE and RT-PCR confirmed the presence of BT virus. The sensitivity of RT-PCR was analyzed by performing tenfold serial dilutions of cDNA. The sensitivity of assay was observed till the dilution of 1:100000.

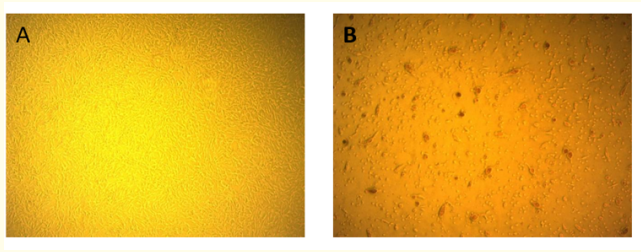

Figure 1: Microphotograph showing (A) confluent monolayer of uninfected BHK-21 cell line (X100);
(B) cytopathic effects produced by BTV suspected new isolates (X10).

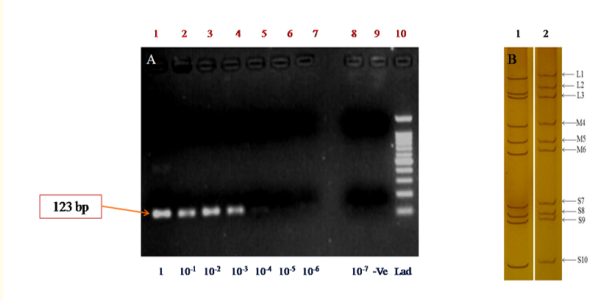

Figure 2: A: PCR of NS1 gene, Lane 1-8: tenfold serial dilutions of c-DNA to know the sensitivity of RT-PCR; Lane 9: negative control; Lane10: 100bp Ladder; **B:** Electrophoretic migration pattern (3:3:3:1) of genomic ds-RNA segments of isolate of BTV in PAGE gel.

Characterization of GNPs and GNPs-based probes

The colloidal GNPs were synthesized using citrate reduction method producing negatively charged nanoparticles and having wine red color. The negative charge due to citrate stabilizes and prevents aggregation of GNPs and red color appears. After func-

tionalization the color becomes more intense. Gradual addition of NaCl in the GNPs causes aggregation of GNPs turning the color purple from red. The GNPs were characterized by adding gradual increased volumes of NaCl solution in the GNPs solution, followed by measuring absorbance in UV-V is spectrophotometer. A pattern of gradual decrease in the peak was observed as the volume of NaCl solution was increased (Figure 3). The decreasing pattern of absorbance peak indicates homogenous nature of GNPs. In the TEM imaging, it is evidently seen sphere shaped gold nanoparticles of uniform size less than 20nm (Figure 4) the size of GNPs was determined to be 18nm.UV-V is spectrometry was performed to check the absorbance of the GNPs and GNPs-based probs. The absorbance peak for GNPs was observed at 520 nm for GNPs and after functionalization, the peak was lowered and absorbance was shifted on higher wavelength (Figure 5).

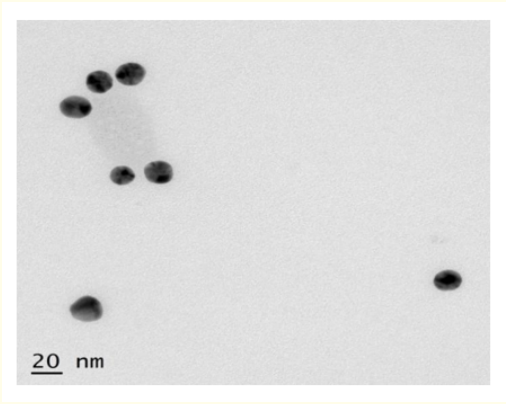

Figure 4: Transmission electron microscopy of GNPs showing presence of spherical shaped GNPs with uniform size 18nm.

Colorimetric DNA detection

The ability of the GNPs-based probe to detect BT-RNA was tested using RNA extracted from BT virus infected BHK-21 cell culture. The detection was performed by hybridizing of the GNPs based probe to the BT-RNA. The hybridized DNA fragment to the GNPs-based probe acts as a stabilizing network acting antagonistically for aggregation. The GNPs-based probe hybridized to the positive control resisted color change even after adding 3 µl of 0.5M MgCl2. On the other hand the negative control and TE buffer (blank control) both showed no hybridization to the probe, thus no stability

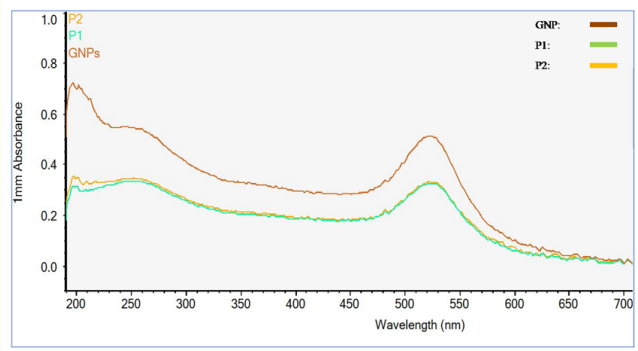

Figure 5: UV-V is spectrum of GNPs showing absorbance peak at 520 nm and absorbance of GNPs-based probes indicating peak lowered and shifted to higher wavelength.

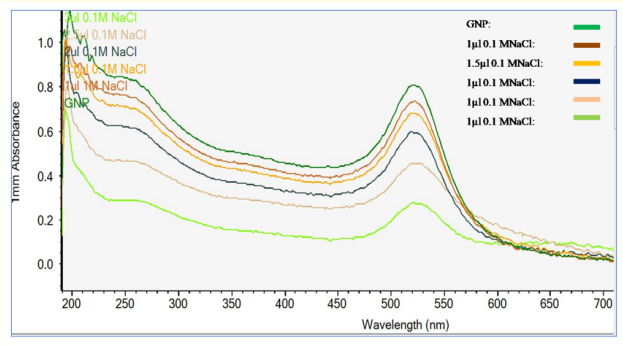

Figure 3: Characterization of GNPs with UV-V is spectrometry by adding increasing volumes of NaCl solution. The decreasing pattern of absorbance peak indicates homogenous nature of GNPs.

leading to the aggregation of GNPs followed by addition of MgCl2. This resulted in generation of purple or black color in the tube after MgCl2 addition (Figure 6). The difference in the UV-V is spectrum absorbance was also observed (Figure 7). To determine the sensitivity of the GNPs-based probes, the RNA was serially diluted; the maximum detection limit was observed till 1:1000th dilution (Figure 8). The initial concentration of BT-RNA was determined as 14.4 ng/µl. Therefore the sensitivity of the GNPs-based probe was estimated to be 14.4 pg/µl.

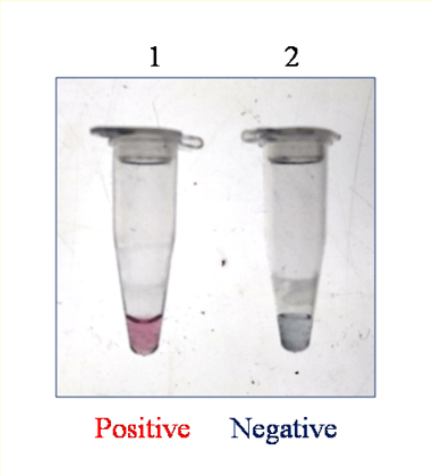

Figure 6: Colorimetric test for detection BT viral RNA using GNPs-based probe. Tube1: Positive test indicated by red color of GNPs; Tube2: The purple/black color indicating negative test result.

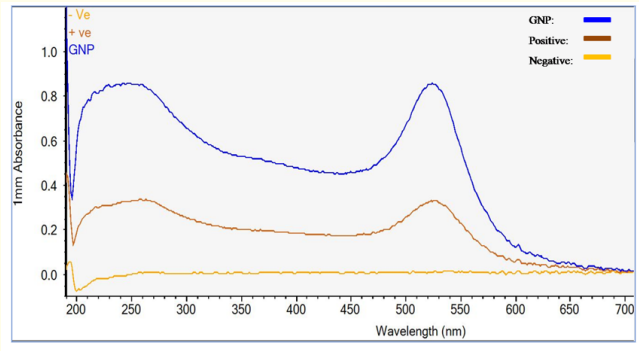

Figure 7: UV-V is spectrometry depicting the absorbance of GNPs, positive test and negative test.

Discussion

Bluetongue is a very infectious, vector born disease of wild and small ruminants caused by a virus Bluetongue virus. The transmission occurs through *Culicoides* species of flies. The genome of the virus is RNA, thus making it more prone to mutations (More than 29 serotypes have been reported) and spread throughout the world. The economic impact of BT is severe on the livestock industry and wool industry. Hence BT has been included in OIE list of diseases. The primary strategy to control vector born disease is the diagnosis of the disease before spread throughout the population. The current diagnostic tests available are very specific and sensitive but they consume a lot of time, require expertise to perform and very expensive. Therefore in this study we have developed a novel GNPs-based oligonucleotide probes for detection of Bluetongue virus RNA.

The electrophoretic migration pattern of genomic RNA segments of BTV isolates in RNA-PAGE observed was 3:3:3:1 which is characteristic to BTV as reported by earlier workers [20]. RNA-PAGE has been used not only to identify and detect BTV in samples but also to analyze genomic diversity within serotypes and re-assortments in mixed infection [21]. In the present study, the isolate TN01-CIBT also showed the same electrophoretic migration pattern 3:3:3:1. With this it was confirmed that the viral genome was segmented having same migration pattern as observed in the BTV.

Since NS1 gene of BTV being most conserved gene, exhibits the least sequence variation among different serotypes as compared to other segments [22]. The viral genome segment 5 encoding nonstructural protein (NS1) has 97-100% nucleic acid sequence homology with other serotypes [23,24]. Therefore, it was targeted for detection of BTV in PCR amplification. Earlier workers have reported the use of serogroup and serotype specific primers based on NS1 and vp2 gene sequence variation, for the detection of BTV and its serotypes respectively [19]. In the present study, we used already published BTV specific primers for detection of BTV with the amplicon size of 274 bp in the samples. cDNA was synthesized from Trizol extracted viral RNA using random primer which were shown to have better results as compared to the reverse transcription by the BTV specific PCR primers [25]. The cell culture adapted virus isolate yielded an expected product of 123bp after RT-PCR amplification using NS1 gene based serogroup specific primer pair. A similar report of BTV group specific amplification using same primer pair was reported by earlier workers [19].

The ability of the GNPs-based probe to detect BT-RNA was weathered using BT-RNA extracted from BT virus grown in BHK-21 cell line. The detection was performed by hybridizing of the GNPs based probe to the BT-RNA. RNA diluted in TE buffer 10µl was denatured at 95ºC for 5 minutes followed by snap chilling and later

the GNPs-based probe were added (5 µl) and allowed to hybridize at 62 ºC. The GNPs-based probe hybridized with RNA segment forms stabilizing network, which intern acts antagonistically for aggregation in presence of salts [10,26,27]. The GNPs-based probe hybridized to the positive control resisted color change even after adding 3 µl of 0.5M MgCl2. On the other hand the negative control and TE buffer (blank control) both showed no hybridization to the probe, thus no stability leading to the aggregation of GNPs followed by addition of MgCl2. This resulted in generation of purple or black color in the tube after MgCl2 addition. The difference in the UV-V is spectrum absorbance was also observed. To determine the sensitivity of the GNPs-based probes, the RNA was serially diluted; the maximum detection limit was observed till 1:1000th dilution. The initial concentration of BT-RNA was determined as 14.4 ng/µl. Therefore the sensitivity of the GNPs-based probe was estimated to be 14.4 pg/µl. similar results were obtained by Liandris and coworkers [28] for DNA detection of Mycobacterium spp. Similarly in another study by Ganeral and coworkers, Mycobacterium paratuberculosis DNA was detected using GNPs-based probes [16]. In another study carried out by Bakthvathsalam and coworkers, [17] the detection of E. coli DNA was performed by targeting *malB* gene. Similarly, in another study, Leishmania DNA was targeted with GNPs-based probes producing similar results [29]. Many other studies have been done for DNA detection, but our study is novel because we have devised it to hybridize with RNA target.

Conclusion and Summary

In the present study, GNPs-based probes for the colorimetric detection of BT-RNA have been developed. The GNPs-based probe was functionalized by reacting thiol-modified single stranded oligonucleotide DNA probe with GNPs. UV-V is spectrometry was used to characterize the functionalized GNPs. Colorimetric detection of BT-RNA was done by hybridization of denatured RNA with GNPs-based probes and color reaction was observed following addition of salt solution. The optimum conditions for the RNA detection were optimized by using checkerboard method. The conditions were, 10 µl of TE diluted RNA, with 5 µl of GNPs-based probes at 62ºC for 15 minutes and followed by addition of 3 µl 0.5M MgCl2. The blank and negative reactions were also shown to be negative. In the summary, we have developed GNPs-based probe for detection of BT viral RNA making it novel in comparison with other literature. The functionalization methods can be improved in the future to make the assay more sensitive. The colorimetric assay can be utilized for the detection of BT viral RNA for rapid diagnosis of BT.

Bibliography

1. Ranjan K., *et al*. "Molecular Typing of Bluetongue Virus 16 From Karnataka State of India". *JMRR* 2.1 (2016): 43-49.

2. Prasad G., *et al*. "Genomic diversity in Indian isolates of bluetongue virus serotype 1". *Indian Journal of Microbiology* 38 (1998): 161-163.

3. Osburn BI. "Bluetongue virus". *Veterinary Clinics of North America: Food Animal Practice* 10 (1994): 547-560.

4. Minakshi P., *et al*. "Virus-host interactions: new insights and advances in drug development against viral pathogens". *Current Drug Metabolism* 18 (2017): 942-970.

5. Ratinier M., *et al*. "Identification and Characterization of a Novel Non-Structural Proteinof Bluetongue Virus". *PLOS Pathogens* 7.12 (2011): 1-14.

6. Prasad G and Minakshi P. "Comparative evaluation of sensitivity of RNA-polyacrylamide gel electrophoresis and dot immunobinding assay for detection of bluetongue virus in cell culture". *Indian Journal of Experimental Biology* 37 (1999): 157-160.

7. Kumar N., *et al*. "Green Synthesis of Silver Nanoparticles and its Applications A Review". *Nano Trends: A Journal of Nanotechnology and Its Applications* 19.3 (2017): 0973-1418.

8. Prasad M., *et al*. "Nanotherapeutics: An insight into healthcare and multi-dimensional applications in medical sector of the modern world". *Biomedicine and Pharmacotherapy* 97 (2018): 1521-1537.

9. Mayer KM and Hafner JH. "Localized surface plasmon resonance sensors". *Chemical Reviews* 111 (2011): 3828-3857.

10. Lambe U., *et al*. "Nanodiagnostics: a new frontier for veterinary and medical sciences". *JEBAS* 4.3S (2016): 307-320.

11. Tallury P., *et al*. "Nanobioimaging and sensing of infectious diseases". *Advanced Drug Delivery Reviews* 62 (2010): 424-437.

12. Mao X., *et al*. "Disposable nucleic acid biosensors based on gold nanoparticle probes and lateral flow strip". *Analytical Chemistry* 81 (2009): 1660-1668.

13. Skottrup PD., *et al*. "Towards on-site pathogen detection using antibody-based sensors". *Biosensors and Bioelectronics* 24 (2008): 339-348.

14. Upadhyayula VK. "Functionalized gold nanoparticle supported sensory mechanisms applied in detection of chemical and biological threat agents: a review". *Analytica Chimica Acta* 715 (2012): 1-18.

15. Turkevich J., *et al*. "A study of the nucleation and growth processes in the synthesis of colloidal gold, Discuss". *Faraday Society* 11 (1951): 55-75.

16. Ganareal TACS., *et al*. "Gold nanoparticle-based probes for the colorimetric detection of Mycobacterium avium subspecies paratuberculosis DNA". *Biochemical and Biophysical Research Communications* 496.3 (2018): 988-997.

17. Bakthavathsalam P., *et al*. "A direct detection of Escherichia coli genomic DNA using gold nanoparticles". *Journal of Nanobiotechnology* 6.10 (2012): 8-10.

18. Prasad G., *et al*. "Genomic diversity in Indian isolates of bluetongue virus serotype 1". *Indian Journal of Microbiology* 38 (1998): 161-163.

19. Vishwaradhya TM., *et al*. "Sensitive detection of novel of Indian isolate of BTV21 using ns1 gene based real-time PCR assay". *Veterinary World* 6.8 (2013): 554-557.

20. Squire KRE., *et al*. "Rapid methods for comparing the double stranded RNA genome profiles of bluetongue virus". *Veterinary Microbiology* 8 (1983): 543-553.

21. Ramesha. "VP7 gene based molecular characterization of Indian isolates of bluetongue virus and evaluation of nucleic acid-based serogroup specific diagnostics. M.V.Sc. thesis submitted to CCS Haryana Agricultural University, Hisar, India (2003).

22. Roy P. "Bluetongue virus genetics and genome structures. Review article". *Virus Research* 13 (1989): 179-206.

23. Prasad G., *et al*. "RT-PCR and its detection limit for cell culture grown bluetongue virus I using NSI gene specific primers". *Indian Journal of Experimental Biology* 37 (1999): 1255-1258.

24. Malik Y., *et al*. "Comparison of cultural characteristics and genomic profiles of two strains of bluetongue virus 1 of Indian origin". *The Indian Journal of Agricultural Sciences* 70 (2000): 3-7.

25. Malik S. "Genetic diversity and evolutionary relationship of Indian isolates of bluetongue virus serotype 1 based on variable regions of VP2 gene. Ph.D. thesis submitted to CCS Haryana Agricultural University, Hisar, India (2004).

26. Dahiya S., *et al*. "Silver Nanoparticles Embedded Photosensitive Silicon as Catheter Materials". *Advances in Animal and Veterinary Sciences* 3.1s (2015): 10-15.

27. Malik YS., *et al*. "Nanotechnology: applications in animal disease diagnosis. In a multi volume set book on Recent Developments in Biotechnology". Publisher Studium Press LLC, USA (2013).

28. Liandris E., *et al*. "Direct detectionof unamplified DNA from pathogenic mycobacteria using DNA-derivatized goldnanoparticles". *Journal of Microbiological Methods*78 (2009): 260-264.

29. Andreadou M., *et al*. "A novel non-amplification assay for the detection of Leishmania spp. in clinical samples using gold nanoparticles". *Journal of Microbiological Methods* 96 (2014): 56-61.