PAKISTAN JOURNAL OF HEALTH SCIENCES
https://thejas.com.pk/index.php/pjhs
Volume 3, Issue 7 (December 2022)

Original Article

Real Time Paper Based Detection of *Streptococcus bovis* using Chromogenic Substrate in Resource Constrained Environments

Amna Mahmood[1] and Amtul Jamil Sami[1*]

[1]School of Biochemistry and Biotechnology, University of the Punjab, Pakistan

ARTICLE INFO

Key Words:
Streptococcus bovis, Paper Based Detection, Bacterial Identification, Diagnosis

How to Cite:
Mahmood, A. ., & Jamil Sami, A. (2022). Real Time Paper Based Detection of Streptococcus bovis using Chromogenic Substrate in Resource Constrained Environments: Real Time Paper Based Detection of Streptococcus bovis. Pakistan Journal of Health Sciences, 3(07). https://doi.org/10.54393/pjhs.v3i07.344

***Corresponding Author:**
Amtul Jamil Sami
School of Biochemistry and Biotechnology, University of the Punjab, Pakistan
2amtuljamilsami@gmail.com

Received Date: 10th December, 2022
Acceptance Date: 27th December, 2022
Published Date: 31st December, 2022

ABSTRACT

Healthcare management faces a variety of difficulties in Circumstances with limited resources. Accurate diagnosis is the first step in disease prevention and therapy that works However, the diagnostic tools that are available in the economically advanced world are frequently of little utility in underdeveloped nations including Pakistan. The gram-positive bacterium *Streptococcus bovis* is an opportunistic pathogen that can cause various infections from superficial skin infections to severe and potentially fatal invasive diseases. **Objective:** To design a platform in environments with limited resources for the quick detection of *Streptococcus bovis*. **Methods:** A paper based analytical device (PAD) has been created with enrichment in sterile PYP broth for 1.5 hours and used for the detection of alkaline phosphatase activity using the chromogenic substrate Para-Nitrophenyl Phosphate (PNPP), *the specie* could be found in clinical samples. A coagulase test and sample Gram staining was conducted with the test. Qualitative detection was evaluated by visual detection while quantitative analysis is carried out using Image J software. **Results**: Alkaline Phosphatase (*S. bovis*) reacts with the PNPP substrate (5.7 mM) in the presence of PAD. Concentrations below 4.5x104 cfu mL resulted in the detection of a color change. The micro PADs were incubated at 37 °C for 3-4 hours before reaction. Colored product (yellow) indicated the presence of *S.bovis*. **Conclusion:** Within 2 hours including enrichment time the test may identify *Streptococcus bovis* up to 104 CFUmL-1.

INTRODUCTION

Over the years, the molecular diagnosis of infectious diseases has become the standard of care for the identification and diagnosis of viral, bacterial infections and several bacterial infections. Although molecular testing has a number of benefits for precise diagnosis and the best therapeutic care of patients infected with microorganisms, many test methods are not practical in underdeveloped or resource-constrained environments. From ethical, financial, and scientific aspects, the disparity in testing methodologies is debatable [1]. Because skilled employees are frequently lacking. The World Health Organization states that affordable, sensitive, specific, user-friendly, rapid and robust diagnostic gadgets which is equipment free and deliverable to the required person for poor nations. This will be named as ASSURED. Microfluidic paper-based analytical devices (PADs), a new platform created for ASSURED diagnostic assays which are composed of patterned papers [2]. *S. bovis* is a facultative anaerobe that is non-motile, non-sporulating, and both oxidase and catalase negative [3]. The standard procedure for identifying *Streptococcus bovis* entails direct Gram staining at first, then isolation and biochemical testing. These conventional techniques are time-consuming and laborious. Numerous fast methods for identifying *Streptococcus bovis* directly from cultures have been reported, including nucleic acid-based and immunological methods. However, the majority of these methods can be prohibitively expensive or challenging to use in environments with limited resources [4]. *Staphylococci Micrococci*, *Streptococci*, and *Enterococci* are gram-

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DOI: https://doi.org/10.54393/pjhs.v3i07.344

positive cocci that can be discovered in dietary or clinical samples. When cultivated in the presence of 0.3% Pi, *micrococci* do not produce phosphatase, but *enterococci* and *streptococci* may produce phosphatase but do not produce coagulase. By doing a catalase test, *Streptococci* and *Staphylococci* can be distinguished therefore, a probable identification of *Streptococcus bovis* can be made using the current test on paper, Gram staining, and Coagulase test. Only circumstances where *Staphylococcus* or *Streptococcus* is anticipated to be the sample's etiological agent require the use of the catalase test [5]. In all dimensions of human existence, recent advancements in nanoscience and nanotechnology have profoundly changed how we recognize, treat, and prevent a wide range of diseases. Silver nanoparticles (AgNPs) are one of the most significant and intriguing nanomaterials among the various metallic nanoparticles employed in biomedical applications. AgNPs in particular are essential for nanomedicine and other nanoscience and nanotechnology disciplines [6]. Silver nanoparticles (AgNPs) are being used more and more in a range of industries, including medicine, food, health care, consumer goods, and industrial applications, as a result of their unique physical and chemical properties. High electrical conductivity, optical, electrical, thermal, and biological properties are some of these [7]. Precise particle characterization is required following synthesis since a particle's physicochemical characteristics may have a big impact on those particles' biological characteristics [8]. Recent studies have demonstrated that biologically-mediated nanoparticle synthesis is an easy, affordable, dependable, and ecologically friendly method [9]. In several investigations, AgNPs were synthesized without the use of harmful chemicals by biological processes that were both affordable and environmentally friendly [10].

METHODS

A technique created for Laboratory development of paper-based electronics [11] For this, 11 cm 9 cm Whatman filter paper No. 3 (GE Healthcare Life Sciences) was utilized. A simple well-plate design using a 9-mm diameter spot array served as the basis for the PAD's design. *Streptococcus bovis* American type culture collection (ATCC 33317) from a microbial type culture that is known to produce ALP For the creation of the standard graph was utilized. Additionally, hospital isolates of *S. bovis* were used to assess alkaline phosphatase (ALP) activity. ATCC *25922 Escherichia coli*, *Pseudomonas aeruginosa* ATCC 27853 (Laboratory maintained) were used as known negative cultures. The PYP medium, which contained 20 g of peptone, 10 g of yeast extract, 1 g of glucose, and 3.0 g of mixture of mono- and dibasic phosphates, 15 g of agar, 1 liter of water, and NaH2PO4 to raise the medium's pH to 7. Thermo Fischer GeneJet Genomic DNA purification kit was used to extract the genomic DNA from this culture in accordance with the manufacturer's instructions. At -20 degrees purified DNA was stored. Primers for gene amplification were designed against *streptococcus bovis* Strain sequence mentioned in NCBI databank (table 1).

Name of primers	Sequence of primers
(F-*tuf*)	5-ATGGTCCAATGCCACAAACACGTGAAC-3
(R-*tuf*)	5-CTACAGTACCACGACCAGTGATTG-3

Table 1: Primers sequence

F-*tuf* and R-*tuf* Primers were used for the amplification of the *tuf* gene. Total 25 µl reaction was made. 1 min Initial denaturation was done at 94, annealing was done at 63 for 30 seconds, extension at 72 for 90 seconds and final extension was done at 72 for 17 min followed by a hold at 4°C. After PCR the amplified product was run on 1% agarose gel. Once Electrophoresis was done the amplified product evaluated under UV light for the confirmation of size of the product. Advance Bio-sciences carried out the sequencing. Sanger Dideoxy Chain Termination was used for the ABI-310 sequencer to perform the sequencing. Using BLASTn, the similarity of each gene to known sequences acquired from the gene bank was calculated. Different Bioinformatics tools were used for the current study. The primer binding site on the *tuf* gene was discovered using the bioinformatics tool EMBOSS-needle. The amino acid sequence was converted from the nucleotide sequence using the ExPASy translate programme. The protein 3-D structure of the EF-Tu derived from SWISS-MODEL. To find the site of interaction with the primer, the protein sequence encoded by the targeted *tuf* gene was discovered on the protein 3-D structure. P-Nitrophenylphosphate (PNPP) solution was prepared as per manufacturer's instruction by adding 1 mg/ 1 mL of Glycine Buffer at pH 8.8. Para-Nitophenylphosphate (PNPP) was used as substrate and prepared according to manufactures instruction. the aforementioned paper sheets were used for conducting the test protocol. The paper wells had PNPP poured into them (5 µL). The paper was given 30 minutes to dry. It was followed by the addition of Silver Nanoparticles (5 µL) allowing the paper to dry to proceed next step. Afterwards culture broth or enzymes (5 µL) were added. Devices were kept alive in a foil-covered petri dish at 37 ° C throughout the incubation phase. Alkaline Phosphatase causes the reagent to change color from without color or ranging from extremely light to dark yellow. On a smart phone, a picture of the color development was taken. A qualitative test was conducted to determine the presence *Streptococcus bovis* in samples collected from different Hospitals. Different healthy individuals and clinical samples were also used.

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DOI: https://doi.org/10.54393/pjhs.v3i07.344

Streptococcus is routinely detected in clinical samples. Blood, Nasal swabs, Pus, Abscess, oral swabs were among the samples received from a pathology lab with a good reputation and examined. As previously noted, samples were prepared and processed for quantitative analysis. Enriched Medium (broth) was utilized for both the Coagulase test and the paper test. Every sample was simultaneously stained and put on a plate. NaCl broth test was used for *Streptococcus bovis* presence confirmation. Results from clinical samples were compared to the pathological laboratory report. Specificity Test was performed to assess the viability of the paper based sensor designed for *S. bovis.* Known Positive and known Negative controls were used. Positive Control (*S. bovis* ATCC),Test samples *S. bovis* isolated from (Clinical, Dental and Blood) and Negative Controls were (*B. subtilis*, *E.coli* and *Pseudomonas*) collected from different Hospitals of Lahore. After identification based on Gram staining, Biochemical analysis pure cultures were used for the experiment

RESULTS

Agarose Gel Electrophoresis was applied to samples of DNA that had been extracted and for Gene amplification as well. 1kb ladder was utilized, and 1% Gel was operated at 90 volts. Sequences of S.bovis that were collected from clinical samples were compared to sequences that had already been published in the gene data bank. For a better knowledge of pathogenicity and management of the diseases brought on by the specie, this homology analysis aids in the identification of mutations as well as homologous sequence areas. The comparison revealed sequence similarities between the isolated sequences of the two S.bovis strains. The isolated species identity as S.bovis was established by this comparison. S. bovis clinically isolated strain sequence product was aligned with reported gene sequence of Streptococcus bovis elongation factor Tu (tuf) gene with sequence ID AF276258. Overall similarity was 95%.S. bovis clinically isolated strain sequence product was aligned with reported gene sequence of Streptococcus bovis elongation factor Tu (tuf) gene with sequence CP046919. Overall similarity was 95%.

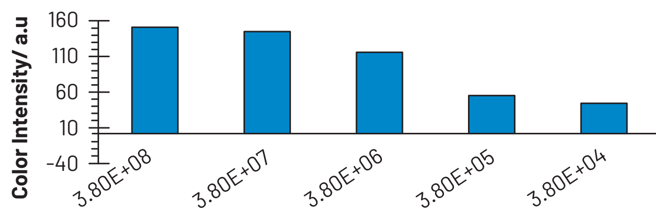

Figure 1: Lowest number of cells exhibiting an observable positive reaction on paper was 3.8×104 CFU mL-1

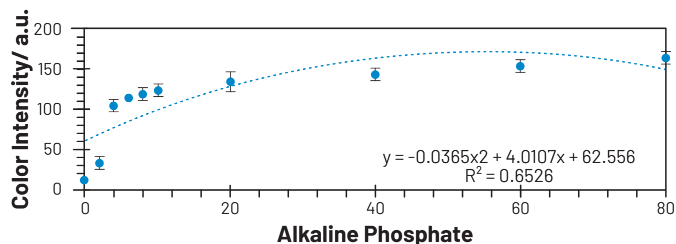

Figure 2: Estimation of cell number in Clinical Samples (Experiments were done in triplicates, (n=3)

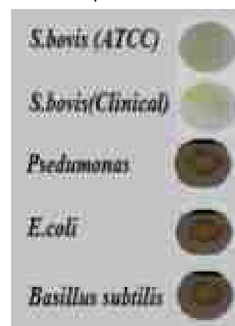

Figure 3: Specificity Test. Positive Control(S.bovis ATCC),Test samples S.bovis isolated from (Clinical) Negative Controls (B.subtilis, E.coli and Pseudomonas)

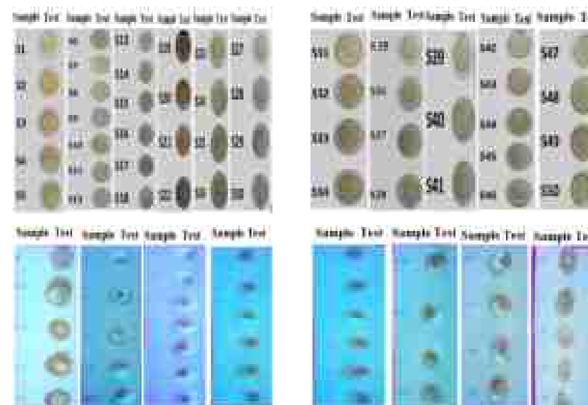

Figure 4: (A) Stripes comprises of filter paper based Enzyme-Substrate interaction in Clinical isolates (S.bovis) form of a color product (yellow) (B) Stripes comprises of filter paper analyzed under UV-Vis Spectrophotometer for the Fluorescence and measurement of the fluorescence intensities evaluated by image J software

DISCUSSION

Facilities in underdeveloped nations generally do not have access to the tools and cutting-edge technologies needed to produce test findings that are quick, accurate, and repeatable. As a result, the kinds of molecular assays that can be used as standards of care for patients in resource-poor nations are constrained. Unfortunately, infectious diseases predominate in these low-resource environments and serve as the foundation for prospective global hazards [12]. These difficulties have pushed lab professionals to develop effective molecular laboratory testing for infectious diseases that satisfies the needs of

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DOI: https://doi.org/10.54393/pjhs.v3i07.344

all nations in terms of resources, the environment, and health care [13, 14]. This study addresses the issues encountered by *Streptococcus* species. Samples were Collected from different hospitals of Lahore and characterized by performing different Biochemical Tests and Plate Methods. Because PYP medium have a high salt concentration that encourages the development of gram-positive bacteria, they were specifically utilized for the secondary culture of *S. bovis*. Phosphatase and coagulase tests were used to do qualitative analysis on *S. bovis*. a biofilm-forming organism, has high levels of alkaline phosphatase, which it uses to hydrolyze phosphate from various sources in the media. PNPP was employed in this work as a chromogenic reagent to carry out the test. The enzyme found in the inoculation media hydrolyzed the phosphate group from the reagent, and the phenol group appeared as yellow rings on the filter paper [15]. A typical *Streptococcus bovis* graph preparation demonstrates a linear relationship between the quantity of *Streptococcus bovis* cells and the average values for fluorescence intensity 3.0 × 104 CFU mL-1 was found to be the limit of detection. Out of the five hundred (500) samples analyzed for qualitative tests, twenty clinical samples that produced false positive results may have contained flora other than *Streptococcus bovis* and were capable of producing alkaline phosphatase. Gram staining and coagulase tests was performed for the confirmation of the strains isolated from clinical samples [5, 16]. The designed procedure produces data in 3–4 hours, enrichment time included, and requires no expensive analysis tools. Both qualitative and quantitative testing can be done with the designed platform. Although it offers a very accurate estimate precision of quantitative analysis still needs improvement. This technique can find up to 104. CFU mL-1 and can be used by the food industry, to find out the microbial contaminants in water and in medical field as a screening test. Previously this methodology was used for identification of *Staphylococcus aureus* [17]. They have used BCIP as chromogenic substrate and we have used Paranitrophenylphosphate. We have also implied AgNPs to add up uniqueness of the test. A group of study proposed by Galia and co-workers for the identification of methicillin resistant *Staphylococcus aureus* [18]. Previously another group has proposed a study for the identification of bacteria by using antibody coated microspheres [19]. A Paper based Analytical device was designed and fabricated by a group of researchers for the identification of *E.coli* in real urine samples [20]. Our results were in line with the previous results reported as given above. Given that the Limit of Detection (LOD) of this method is 104 CFU mL-1 and counts above 104 CFU mL-1 yield results in less than 30 minutes, this test could provide speedy results for certain applications. Medical professionals in distant areas can utilize the test to make a preliminary *Streptococcus* species diagnosis in conjunction with Gram staining, coagulase and catalase as well as a clinical picture. In addition to being quick, the test is also reasonably economical. The test does not require any complicated equipment or skilled workers because the number of reagents needed is measured in microliters. Visual color detection is the foundation of the qualitative test. Only a smartphone is needed for quantitative analysis in order to record and process the image. The paper can be incinerated securely for disposal. Thus, a quick test for *Streptococcus bovis* identification that may be utilized in areas with low resources is standardized.

CONCLUSION

The procedure produces result swiftly within 2 hours including enrichment time and the best part it requires no expensive analysis tools. Both qualitative and quantitative testing can be done with the designed assay. Precision of quantitative analysis is excellent, although it offers a very accurate estimate but still needs improvement to switch the whole platform to electrochemical chips. The food industry can utilize this technique as a screening test because it can identify up to 104 CFU mL-1. The limit of detection of this method is 104 CFU mL-1 and counts above 104 CFU mL-1 yield results in less than 30 minutes, this test could provide speedy results for certain applications. Medical professionals in remote areas can utilize the test to make a preliminary Streptococcus diagnosis in continuation with Gram staining, Coagulase and catalase as a clinical picture.

Conflicts of Interest

The authors declare no conflict of interest

Source of Funding

The author(s) received no financial support for the research, authorship and/or publication of this article

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