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RESEARCH ARTICLE

RAPID DETECTION OF *STAPHYLOCOCCUS AUREUS* USING PAPER BASED MICROFLUIDIC DEVICES FOR RESOURCE LIMITED SETTINGS.

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Abstract

A microfluidic paper-based analytical device (**µPAD**) has been developed for rapid detection of *Staphylococcus aureus* in resource limited settings. *Staphylococcus aureus* can be detected from food or clinical samples with enrichment in Sterile PYP broth for 1.5 h and used for detection of Alkaline Phosphatase activity using chromogenic substrate Blue Phos. The test is accompanied by Gram staining of the sample and coagulase test. Qualitative detection is based on visual detection while quantitative analysis is done using ImageJ^{TM} software. The test can detect up to 10^4 CFUmL^{-1} of *Staphylococcus aureus* within 3-4 h. This test is cost effective, does not need any trained personnel or sophisticated instruments for analysis. Analysis can be done on a smart phone. The test is ideal for presumptive diagnosis of *Staphylococcus aureus* for clinicians operating in remote locations and can be used as a screening test by food industries.

Introduction:-

There are a number of challenges faced by healthcare management in resource limited settings. The first step in proper prevention and treatment of disease is accurate diagnosis. But diagnostic technologies that are available in the economically developed world often are of limited use in developing countries. People who live in these countries frequently cannot afford even modestly expensive tests, and basic infrastructures i.e. reliable power, refrigeration, and trained personnel are often not available (Rodrigues, Desai, & Fernandes, 2016). According to the World Health Organization, diagnostic devices for developing countries should be ASSURED: affordable, sensitive, specific, user-friendly, rapid and robust, equipment free and deliverable to end-users. Diagnostic devices made of patterned papers which are called as microfluidic paper-based analytical devices (**µPADs**) are a new platform designed for ASSURED diagnostic assays (Martinez, Phillips, Whitesides and Carrilho, 2010).

The current work focuses on the use of such an ASSURED µPAD to be used as a rapid test for presumptive diagnosis of *Staphylococcus aureus* in resource limited settings.

Staphylococcus aureus is a Gram positive, non spore forming, facultative coccus. It is an opportunistic pathogen that can cause various infections from superficial skin infections to severe and potentially fatal, invasive disease. (Minnesota Department of Health, 2016). It is also one of the common food poisoning organisms.

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Routine identification of *Staphylococcus aureus* by conventional method includes initial direct Gram staining, followed by isolation and biochemical testing. These traditional methods are cumbersome and time consuming. (El-Hadedy and Abu El-Nour 2012). A number of rapid tests for identification of *Staphylococcus aureus* directly from cultures have been reported which include nucleic acid-based, biosensor-based and immunological-based methods but most of these tests can be cost-prohibitive or difficult to carry out in resource-limited settings.

Amongst staphylococci, major differentiation is based on coagulase test. Staphylococci are divided into coagulase-positive *Staphylococcus aureus* and Coagulase-negative *Staphylococcus* (CoNS). (Hébert, Crowder, Hancock, Jarvis, & Thornsberry, 1988) When grown on media supplemented with 0.3% Pi, only *S.aureus, S. epidermidis* and *S. xylosus* are phosphatase positive. *Staphylococcus aureus* is coagulase positive while the other two are coagulase negative. Hence, when samples enriched in medium with 0.3% Pi are used to perform phosphatase and coagulase test, samples giving both tests positives will be presumptively having *S. aureus* (Soro, Grazi, Varaldo, & Satta,1990). At present there are no commercially available stand alone tests for detection of phosphatase activity for staphylococci, although several commercial kit systems include phosphatase test in the biochemical test battery. All of them need 18-24 hours of incubation (Winn & Koneman, 2006).

Other gram positive cocci that can be found in food or clinical samples are micrococci, streptococci and enterococci. Micrococci do not produce phosphatase when grown in presence of 0.3 % Pi (Satta, D'andrea, Grazi, Soro, & Varaldo, 1993) while enterococci and streptococci may produce phosphatase but are coagulase negative. Staphylococci and streptococci can be differentiated by Catalase test. Hence, the current test on µ PAD along with Gram staining and Coagulase test can give presumptive identification of *Staphylococcus aureus* within 3-4 hours. Catalase test needs to be used only in cases where in the sample is expected to have either *Staphylococcus* or *Streptococcus* as an etiological agent. Therefore this test can be of great importance to the clinicians working in remote locations for presumptive diagnosis of *Staphylococcus*, as well as for food industry as a primary screening test to avoid SFP.

Besides detection of *Staphylococcus*, this test can be used for for detection of Alkaline Phosphatase (ALP) for other industrial applications.

Various Substrates are available for phosphatase test. BluePhos® is a commonly used chromogenic substrate for ELISA and can detect less than 0.5 pg of phosphatase. It is a soluble, proprietary form of 5-bromo-4-chloro-3-indolyl phosphate (BCIP). BluePhos® in addition to BCIP, also contains a different tetrazolium salt. It develops an intense blue color and provides good contrast between control and test and is also easier for visual detection for qualitative tests. Hence this was the substrate of choice for this work.

Material & Methods:-

Paper Based Device Fabrication:-

Laboratory prototyping of paper based devices was made using 'FLASH' (Fast Lithographic Activation of Sheets), a method developed by Andres W. Martinez and coworkers (Martinez, Phillips, Wiley, Gupta, & Whitesides, 2008). Whatman® filter paper no. 3 (GE Healthcare Life Sciences) of dimension 11 cm × 9 cm was used for this purpose. The design of the µPAD consisted of a 9-mm diameter spot array based on a simple well-plate design done using CorelDRAW.

Test Protocol:-

Substrate used was BluePhos® reagent (KPL Life Sciences) and was prepared as per manufacturer's instructions. All the paper wells were loaded with BluePhos® reagent (6 µL). Paper was allowed to dry for 30 minutes. It was followed by addition of enzyme or culture broth (6 µL). Devices were incubated in a petri dish covered with foil at 37 °C for the duration of incubation period. In presence of Alkaline Phosphatase, the reagent changes colour from colourless or very light blue to dark blue or bluish violet. Image of the colour developed was captured on a smart phone. The resultant images (JPG format) were analyzed for measurement of color intensity using ImageJ™ software (National Institutes of Health, Bethesda, Maryland, USA). Invert intensity was measured and readings for control were subtracted from the readings of Test. Proof of concept of feasibility of this assay on paper was done using commercially available pure ALP (Sigma Aldrich, St. Louis, MO).

Statistical Analysis:-
Statistical analysis was done on Stata/IC 14.1. A box plot was drawn of average grey intensities for each ALP concentration or for various log cell numbers of *Staphylococcus aureus* as applicable. This was done to identify outliers and compare distribution of the data. Outliers were removed based on the readings of the box plot.

A *t*-test was conducted to test whether the grey intensity for a particular value of concentration of alkaline phosphatase or log cell number was significantly different from that of the control. If the value for a particular reading was not significantly different at 5% level of significance from the control mean, it was excluded from the analysis.

Cultures Used:-
Known positive ALP producing *Staphylococcus aureus* ATCC 25923 obtained from Microbial Type Culture Collection & Gene Bank, Microbial Technology, Chandigarh, India, was used for preparation of standard graph as well as for proof of concept studies with spiked samples. Hospital isolate *Staphylococcus aureus* K1 was also used to compare the detection of ALP production with *Staphylococcus aureus* ATCC 25923. *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa*, *Streptococcus pyogenes* (Laboratory maintained) were used as known negative cultures.

Media:-
Medium used for enrichment was PYP medium - 20 g of peptone,10 g of yeast extract, 1 g of glucose, 3.0 g of mixture of mono- and dibasic phosphates,15 g of agar,1 liter of water, and NaH_2PO_4, to bring the medium to pH 7 (Soro, Grazi, Varaldo, & Satta,1990)

Quantitative Assay:-

Standard Graph Preparation Using *Staphylococcus aureus*:- Dense overnight culture of *Staphylococcus aureus* was serially diluted 10 fold up to 10^{-6} dilution and each dilution was inoculated at 10% inoculum level in two, 1 mL sterile PYP broth tubes for 1.5 h of enrichment. One tube was used to perform viable count, while broth from the other tube was used directly to perform the test. A pilot assay was carried out where assay was conducted in triplicates and was followed by actual assay wherein tests were done in eight replicates. The same set was used for determination of Limit of Detection (LOD) and for determination of optimum incubation time. Determination of optimum incubation time was done earlier with pure enzyme but was repeated with *Staphylococcus aureus* as crude enzyme from the culture may give different results. Pure ALP was incubated only up to 60 min while here three incubation times namely 30, 60 and 120 minutes were tested to determine the optimum time of incubation for the test.

Specificity test:- Overnight slant cultures of *Staphylococcus aureus*, *Staphylococcus aureus* K1, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa*, *Streptococcus pyogenes* were enriched in St. PYP broth for 1.5 h and the broth was used for the test as per the protocol above.

Proof of Concept with Spiked Samples:- Samples commonly found to be involved in SFD like cake, milk, vegetables like Coriander, Spinach were selected for this study. 10 g of chopped vegetable or food sample was added to a flask containing 40 mL Ringer's Solution, mixed in a blender for 2 minutes. Added additional 50 mL of Ringer's solution. For preparation of spiked food samples known amount of *S. aureus* was added to 10 mL of such food or vegetable preparation. 1.0 mL PYP broth tube was inoculated with 10% inoculum of such spiked sample. Enriched for 1.5 hours and the broth was used for the test on the paper wells. A pilot assay in triplicate was done earlier with same set of experimental conditions.

Actual assay was carried out with eight replicates. A viable count of each sample was performed simultaneously.

Qualitative Assay:- Qualitative test was carried out to show presence or absence of *Staphylococcus aureus* using various food as well as clinical samples. Clinical samples commonly known to show presence of *Staphylococcus* namely sputum, Broncho Alveolar Lavage (BAL), Urine, Pus, Abscess, CSF, Pleural and Ascitic fluid were obtained from a reputed pathological laboratory. Milk, Pedha (dry sweet made from milk), coriander, spinach, chutney (raw vegetable preparation) were used as spiked samples while fruit juice, pastry, lettuce were used as it is without spiking. Samples were prepared and processed as mentioned earlier for quantitative analysis. Enriched broth was used for the test on paper and Coagulase test. All samples were simultaneously gram stained and plated on Baird Parker Agar for confirmation of presence of *Staphylococcus aureus*. Viable count was performed only for all spiked and actual food and vegetable samples (Miles and Misra method). Results obtained for clinical samples were compared with report given by pathological laboratory.

Results:-

Quantitative Assay:-

Standard Graph Preparation using *Staphylococcus aureus*:-

Optimization of time of incubation:-

A linear correlation was observed between log cell number and average grey intensity at all the times of incubation that were tested. Thirty minutes of incubation was found to be satisfactory as the R^2 value for the graph is highest (0.8764) at 30 minutes. Values of average grey intensity at 60 minutes and 120 minutes is higher than that obtained at 30 minutes but the R^2 values are lower. **(Figure 1)**

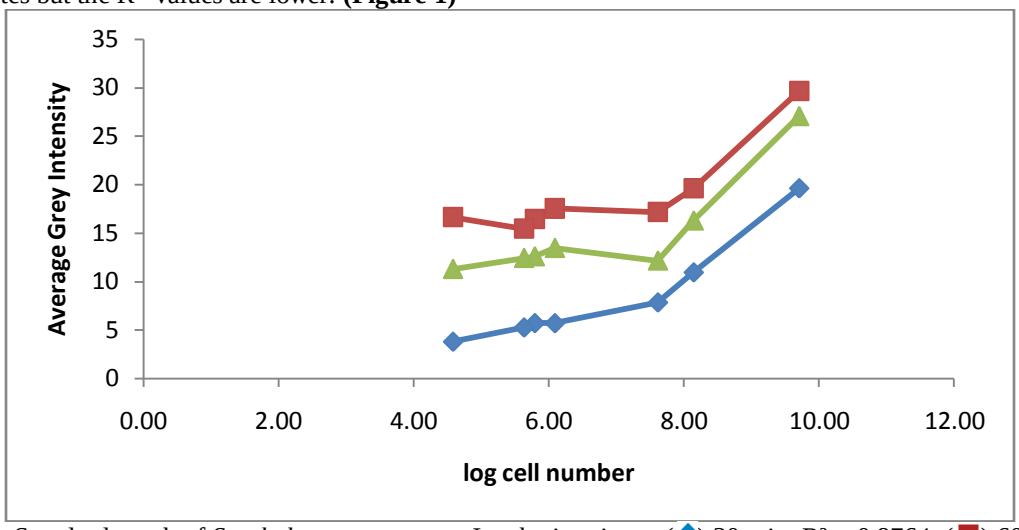

Figure 1:- Standard graph of *Staphylococcus aureus*. Incubation times. (◆) 30 min, $R^2 = 0.8764$; (■) 60 min, $R^2 = 0.7044$; (▲) 120 min, $R^2 = 0.7044$.

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Limit of Detection:-

The LOD was the same for all three times of incubation. According to this set of experiments lowest number of cells giving detectable positive reaction was 3.81×10^4 CFU mL^{-1} i.e. 1.9×10^2 cells on paper well.

Specificity Test:-

Test was found to be specific for Staphylococcus aureus. *Pseudomonas* showed weak positive reaction. **(Figure 2)**

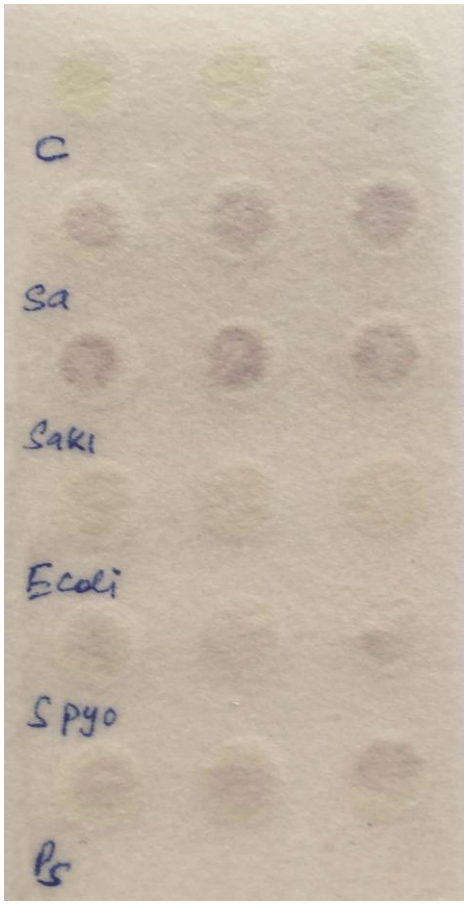

Figure 2:- Specificity Test

Proof of Concept for estimation of cell number using spiked samples:-

Spiked samples were tested along with standard *Staphylococcus aureus* . A linear correlation was observed between log cell number and average grey intensity. The LOD for *Staphylococcus aureus* was 3.0×10^4 CFU mL^{-1}. Results with lower cell densities were not significant according to '*t*- test' . Average grey intensity values of all samples were used to obtain the cell number using equation obtained for the standard graph of *Staphylococcus aureus*. Three samples namely coriander, milk and cake show around 10% percentage difference with the count obtained by viable count. Spinach showed much higher counts than the actual counts. **(Figure 3, Table 1)**

Table 1:- Results of estimated cell number from *Staphylococcus aureus* Standard Graph.

Food/Vegetable Sample	Estimated Log Cell Number based on Linear Regression	Actual Log Cell Number as per Viable Count	% Difference
Coriander	5.90	6.60	10.62
Milk	5.99	6.56	8.65
Spinach	8.07	5.60	-44.11
Cake	4.23	4.48	5.34

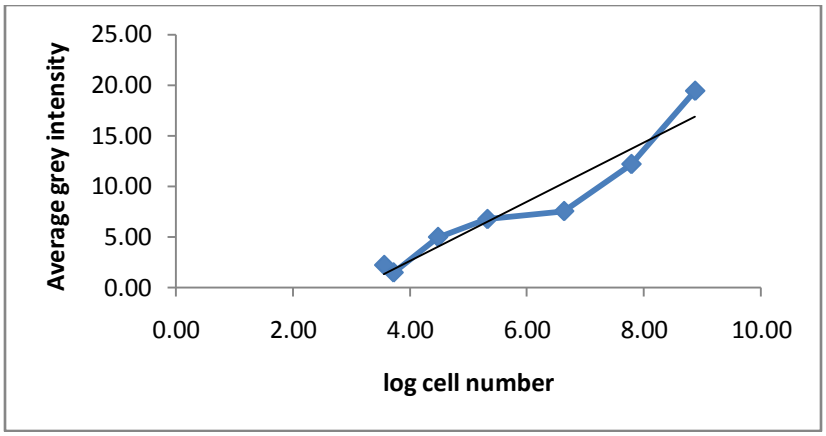

Figure 3:- Estimation of cell number of spiked food and vegetable samples

Qualitative Assay:-

Out of 20 samples tested qualitatively, two clinical samples showed false positive results. All other samples showed results comparable to that obtained by pathological laboratory **(Figure 4, Table 2)**. Both these samples, Ascitic fluid and urine showed a high count of mixed culture. Gram stain revealed mixed morphology. All positive samples gave results within 15-30 minutes while all negative samples were negative even after 20 hours of incubation. All spiked food and vegetable samples showed positive test and had count above 10^4 CFU mL^{-1}while all actual samples showed negative results. Viable count of these samples showed absence of *Staphylococcus aureus* in all of these samples.

Table 2:- Qualitative Analysis.

Sample No.	Samples	Results on Paper Device	Growth of *Staphylococcus aureus* on plate	Coagulase
S1	M342,Ascitic fluid	+	-	-
S2	U1023, Urine	+	-	-
S3	2465, Pleural fluid	-	-	-
S4	476, CSF	-	-	-
S5	1665, Endo	-	-	-
S6	M211, Abscess	+	+	+
S7	M216, Pus	+	+	+
S8	M257, Pus	+	+	+
S9	M203, Ascitic fluid	-	-	-
S10	U712, Urine	-	-	-
S11	R83, BAL*	+(Weak)	+	+(Weak)
S12	Coriander, spiked	+	+	+
S13	Milk, spiked	+	+	+
S14	Pedha, spiked	+	+	+
S15	Chutney, spiked	+	+	+
S16	Cake, spiked	+	+	+
S17	Barfi	-	-	-
S18	Lettuce	-	-	-
S19	Pastry	-	-	-
S20	Fresh fruit juice	-	-	-

* BAL = Bronchoalveolar lavage

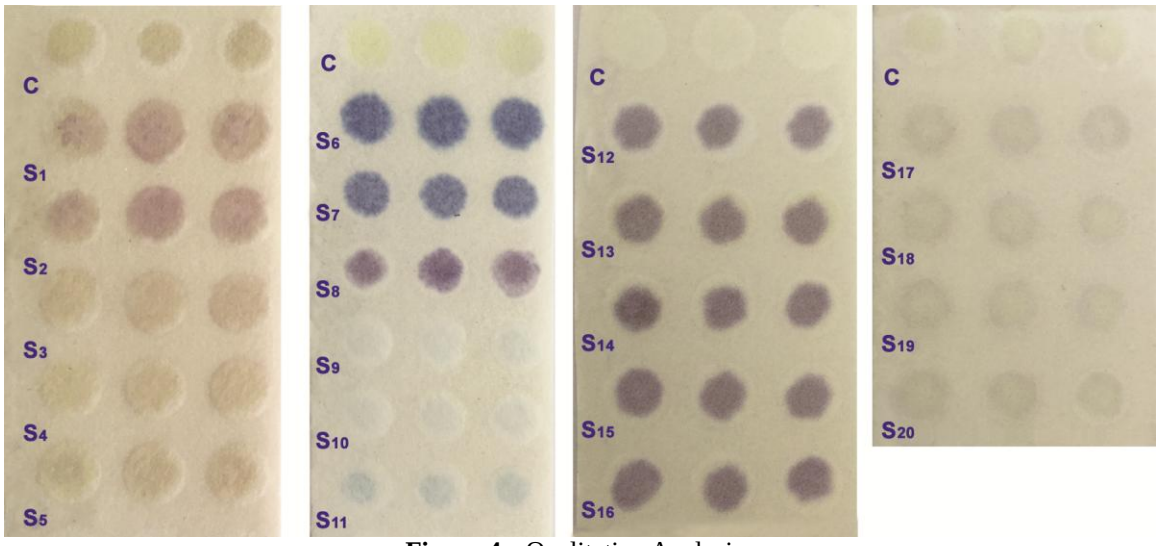

Figure 4:- Qualitative Analysis.

Discussion:-

Standard graph preparation of *Staphylococcus aureus* shows a linear correlation between number of *Staphylococcus aureus* cells and the average grey intensity values. The limit of detection was observed to be 3.0×10^4 CFU mL^{-1}. Densities lower than this were below the detectable range of this method. Using average grey intensity values of the samples, log cell number was calculated using the equation of the trend line obtained with standard graph. Out of four spiked food and vegetable samples, estimated cell number of the three samples namely coriander, milk and cake was within 10% variation of the actual cell number as done by Viable count method. Spinach showed much higher count than the actual cell number. This can be attributed to the fact that Spinach leaves show phosphatase activity of their own. These high levels of ALP are beyond the detectable limits of this method. Therefore this method will give false higher counts or false positive test with spinach or other vegetable samples known to contain high amounts of phosphatases.

This test is specific to Alkaline phosphatase producers. Known positive cultures gave positive results while known negative cultures gave negative results except for *Pseudomonas aeruginosa*. It showed weak positive reaction. It could be due to the bluish green pigment produced by *Pseudomonas*, which interferes with the results. Hence samples having *Pseudomonas* can give false positive test for presence of *Staphylococcus* in those samples. Hence the test should always be accompanied by Gram staining.

In case of qualitative tests, out of twenty samples tested, two clinical samples which gave false positive results could have flora other than *Staphylococcus aureus* and can produce alkaline phosphatase. Hence the test should be accompanied with Gram staining and coagulase. Sample Urine R 83 gave a weak positive reaction on paper as well as weak coagulase. It is possible that the count of *Staphylococcus aureus* is just at the borderline of detectable limit of this method.

The method gives results in 3-4 hours including enrichment time and does not need any sophisticated instruments for analysis. The device can be used for qualitative as well as quantitative testing. Accuracy of quantitative testing needs to be improved, but it gives fairly good estimate within ± 10% variation. This method can detect up to 10^4 CFU mL^{-1} and can be used as a screening test by food industries. The conclusive diagnostic criteria of SFD are based upon the detection of staphylococcal enterotoxins in food or recovery of at least 10^5 *S. aureus* g^{-1} from food remnants. This test can give quick results for such applications as LOD of this method is 10^4 CFU mL^{-1} and counts above 10^4 CFU mL^{-1} give results within 30 minutes. The test can be used by medical personnel in remote locations for presumptive diagnosis of *Staphylococcus* along with Gram staining, coagulase and catalase along with clinical picture.

Besides being rapid, the test is also cost effective. The amount of reagents required is in micro litres, it does not need any sophisticated instruments or trained personnel for the test. Qualitative test is based on visual colour detection. Quantitative analysis requires just a smartphone to capture and analyze the image. The paper can be safely disposed of by incineration. Thus a rapid test for detection of *Staphylococcus aureus* to be used in resource limited settings is standardized.

Conflict of Interest:-

There is no conflict of interest.

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