Detection of *Staphylococcus aureus* using Lab-On-Chip Devices

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Abstract

Microfluidic chip (Lab-On-Chip)-device based tests are becoming popular as they use extremely small reagent volumes, have low power consumption and are portable. Thus, they are economical and are advantageous in point of control or rural settings. This work describes the detection of *Staphylococcus aureus* using Alkaline Phosphatase on such a Lab-On-Chip(LOC)device. The test gives results within 15 minutes and can be used for detection of *S.aureus* from food samples as well as clinical samples. This device, coupled with a Gram stain and Coagulase test, can be used to for presumptive diagnosis of *S. aureus* in point of control, resource limited settings. Proofs of concept studies were done using spiked food samples. Actual food and clinical samples were also tested for the presence of *S. aureus*.

KEYWORDS–*Staphylococcus aureus*, Alkaline Phosphatase, Lab-On-Chip, Point-of-Control

Introduction

A Lab on Chip (LOC) device, also known as a microfluidic device is a device that uses extremely small fluid volumes in the order of microliters to picoliters. In these devices, small volumes of fluids are manipulated and transported through micron-sized channels, and the various processes that occur on the device are monitored using various detection systems (Assadollahi, Reininger, Palkovits, Pointl & Schalkhammer, 2009). Miniaturized versions of bioassays provide a variety of advantages, such as small requirements for solvents, reagents, and cells (which is very critical for valuable samples and for high-throughput screening), short reaction times, portability, relatively lower costs, lower power consumption, design versatility and potential for simultaneous operations and for integration with other miniaturized devices (Sia&Whitesides, 2003).

Staphylococcus aureus(*S. aureus*)is an opportunistic pathogen that can cause various infections from superficial skin infections to severe and potentially fatal, invasive diseases. It is one of the main causes of skin and soft tissue infections like abscesses, furuncles and cellulites. ("Causes and Symptoms of *Staphylococcus aureus* - Minnesota Dept. of Health", 2016). It is also one of the common food poisoning organisms. *S. aureus* can cause contamination of food products during food preparation and processing. Foods that are commonly contaminated with staphylococci are meat and meat products, poultry and egg products, milk and dairy products, salads, bakery products, especially cream-filled pastries and cakes and sandwich fillings (JhalkaKadariya, 2014). It is also a common causative agent of bovine mastitis. This can lead to the contamination of milk by *S. aureus* via direct excretion of the organisms in the milk.

The permissive temperature for the growth and toxin production by *S. aureus* is between 6°C and 46°C. Thus, the ideal cooking and refrigeration temperature should be above 60°C and below 5°C, respectively. Maintaining the cold chain is essential for preventing the growth of *S. aureus* in food products. This is a matter of concern in developing or

underdeveloped countries. Therefore *S. aureus* becomes one of the most common food poisoning organisms in places where there is inadequate refrigeration facility.

Routine identification of *S. aureus* by conventional method includes initial direct Gram staining from clinical or food samples, followed by isolation on selective media such as Baird-Parker, Sheep blood agar, Columbia colistin- nalidixic agar (CNA) or Mannitol-salt agar etc. followed by identification of suspicious colonies by biochemical reactions. These traditional methods are cumbersome and time consuming. (El-Hadedy and Abu El-Nour,2012). A number of rapid tests for identification of *S. aureus* directly from cultures have been reported which include nucleic acid-based, biosensor-based and immunological-based methods e.g. RAPIDEC *Staphylococcus* test, latex slide agglutination tests (Skulnick et al., 1994) etc., but most of these tests can be cost-prohibitive or difficult to carry out in resource-limited settings. To overcome these limitations, enzymatic methods of detection have been developed. These assays are specific, sensitive and rapid. Recently, media containing chromogenic substrates have become available for isolation and presumptive identification of *S. aureus*; e.g. CHROMagar (Microbiology, Paris, France), *S. aureus* ID agar (BioMerieux, La Balme Les Grottes, France) etc. All these methods are based on detection of various enzymes of *Staphylococcus* sp.

Staphylococci are divided into coagulase-positive *S. aureus* and Coagulase-negative *Staphylococcus* (CoNS) (Hébert, Crowder, Hancock, Jarvis, &Thornsberry, 1988). When samples enriched in medium with 0.3% Pi are used to perform phosphatase and coagulase test, samples giving both tests positive will be presumptively having *S. aureus* (Soro, Grazi, Varaldo, &Satta, 1990). 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. Therefore Phosphatase test becomes a key test in presumptive identification of *S. aureus*. Hence the current work involves standardisation of Alkaline Phosphatase detection on microfluidic devices for presumptive identification of *S. aureus*.

At present there are no commercially available standalone tests for detection of phosphatase activity for staphylococci, although several commercial kit systems (e.g. API *Staphylococcus*-IDENT, API *Staphylococcus*, ID32 *Staphylococcus* and RapiDEC*Staphylococcus*) include phosphatase test in the biochemical test battery. All of them need 18-24 hours of incubation (Winn &Koneman, 2006).Hence, the current test using microfluidic devices along with Gram staining and Coagulase test can give presumptive identification of *S. aureus* within 3-4 hours. 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 SFD.

Various Substrates are available for phosphatase test. BluePhos® is a commonly used chromogenic substrate for ELISA. BluePhos® offers more sensitivity than commonly used substrate para-Nitrophenylphosphate (pNPP), and can detect less than 0.5 pg of phosphatase. It produces a linear reaction rate for a longer period of time than pNPP with lower background. It is a soluble form of 5-bromo-4-chloro-3-indolyl phosphate (BCIP). It develops an intense blue color. BluePhos®, in addition to BCIP, also contains a different tetrazolium salt, the nature of which is considered proprietary. The reagent is stable beyond 13 months at 4°C and storage at room temperature for 13 months causes an approximately 20% loss of activity (*Phosphatase Substrate System for ELISA*, 2016).

Both pNPP and BluePhos® can be used for detection of alkaline phosphatase (ALP) with all the the microfluidic devices tested in this study. The blue coloured product produced by enzymatic reaction of alkaline phosphatase with BluePhos® provides a good contrast between the control and the test and is therefore more preferred substrate than pNPP.

The current work demonstrates use of this device for presumptive identification of *S.aureus*.Proof of concept studies were done using spiked food samples. Actual food and clinical samples were tested on this device and the results obtained were compared with traditional methods.

Materials and Methods:

Device Fabrication

PDMS-based Lab-on-chip device was fabricated as described byLake et al., 2015(Lake et al., 2015)using locally available SU8 equivalent.Design with three parallel lines was used for this work. (Figure 1)

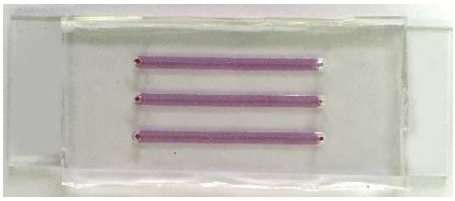

Figure 1: Prepared chip (With coloured reagent for demonstration)

Cultures Used

Known positive ALP producing culture,*S. aureus* ATCC 25923 was used for preparing spiked food samples for proof of concept studies. The cultures were maintained on Nutrient Agar (Hi Media).

Media and Reagents Used

Sterile PYP medium suggested by Soro, Grazi, Varaldo, &Satta, (1990) was used for enrichment of the cultures. BluePhos® reagent was procured from KPL and prepared as per manufacturer's instructions.

Food Sample Preparation

10 g of chopped solid food samples such as vegetable, meat or food sample was weighed and added to a flask containing 40 ml Ringer's Solution (Vegetable samples were taken along with the leaves and 2 inch stalks). This was then mixed in a blender for 2 minutes. Remaining 50 ml of Ringer's solution was added. This food sample was used for further enrichment or direct use. For liquid food samples, 10^{-1} dilution in Ringer's Solution was used.

For preparation of spiked food sample, 1mL of known positive control culture (*S. aureus* ATCC 25923, known ALP producer) suspension of variable cell numbers was addedto 10 mL of such food preparation.

Procedure for enrichment and preparation of Culture broth for Alkaline Phosphatase (ALP) Assay

Samples prepared as per the preceding section were inoculated in 1.0 mL PYP broth at 10% inoculum level and incubated at 37°C for 1.5 hours. This enriched broth was used for the assay.

Assay Protocol

Table 1: Assay Protocol

	Substrate (µL)	Enzyme (µL)	Diluent (µL)
Test	5	5	-
Control	5	-	5

The substrate was loaded first, followed by the enzyme with a micropipette. The contents were mixed within the channel with the help of micropipette. The inlet and outlets were sealed with a scotch tape to control evaporation of the reagents. The devices were incubated at 37°C for 15 minutes in a moist chamber. The results were recorded visually or with a smart phone camera or scanner.

The test reagent BluePhos® is photosensitive, hence chambers with these devices were always wrapped in aluminium foil to prevent exposure to light. In presence of Alkaline Phosphatase (ALP), the reagent changes colour from colourless or very light blue to dark blue or bluish violet.

Proof of Concept with Spiked food Samples

The food samples commonly known to be contaminated by Staphylococcus used were procured from the market. Samples used were Milk, pedha (dry sweet made from milk), coriander, spinach and chutney (raw vegetable preparation). These samples were spiked with known cell densities of positive control *S. aureus* ATCC 25923 culture. Samples were prepared and processed as mentioned in the preceding section. Enriched broth was used for the test on all devices and for Coagulase test. Viable count was performed for all samples.

Test with Food and Clinical Samples

Food samples commonly known to be associated with SFD such as Barfi (Milk Sweet), fruit juice, pastry and lettuce were picked up from the market (non- spiked) and were tested on all the devices. In addition to these food 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 and were tested on the device. Total of 11 clinical samples were tested.

All samples were inoculated in 5.0 mL PYP broth at 10% inoculum level in triplicates and incubated at 37°C for 1.5 hours. This enriched broth was used for the assay in triplicates.

All samples were simultaneously gram stained, plated on Baird Parker Agar and tested for coagulase production for confirmation of presence of *S. aureus*. Viable count was performed for all food and vegetable samples. Results for clinical samples were compared to the report from pathological laboratory as an external standard.

Results and Discussion

Proof of Concept with Spiked food Samples

All spiked samples namely coriander (S1), Milk (S2), Pedha (S3), Chutney (S4) and Cake (S5) showed positive results as seen in Fig.2. Coriander sample which had the

highest number of *Staphylococcus* cells showed strong positive test within 10 minutes. Milk, Pedha and Chutney samples had counts around 10^5 $CFUmL^{-1}$and showed positive results within15-20 minutes while Cake sample had lower count around $10^5 CFUmL^{-1}$, which is just at the borderline of the detectable limit of this device, hence showed weak positive reaction as seen inFigure2 and Table 2.

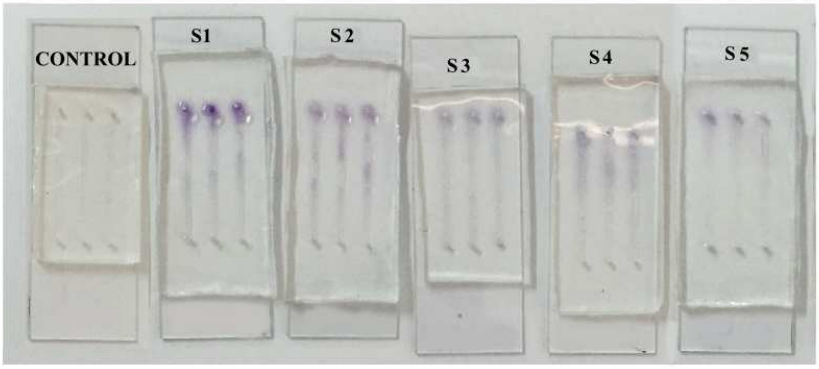

Figure 2: Proof of Concept Test with Spiked Food Samples

Table 2: Results of Proof of Concept Test with Spiked Food Samples

Sample no.	**Samples**	**CFU mL^{-1}**	**Result on LOC Devices**	**Growth of *S. aureus* on Baird-Parker Agar**	**Coagulase**
S1	Coriander	6.1×10^9	+	+	+
S2	Milk	1.0×10^8	+	+	+
S3	Pedha	9.5×10^7	+	+	+
S4	Chutney	2.8×10^8	+	+	+
S5	Cake	4.7×10^5	+	+	+

Test with Food and Clinical Samples

(A) Food Samples

All test food samples obtained from the market (non-spiked) showed negative results. There was no *S. aureus* on Baird Parker agar too. This shows that the results were proper on all the devices. There were no false positive results for any samples.

(B) Clinical Samples

Out of 11 clinical samples tested two clinical samples showed false positive results (Figure 3, Table 3). These were Sample S6 (Ascitic Fluid), and S7

(Urine). Both these samples showed a high count of mixed culture. Gram stain revealed mixed morphology. Coagulase test was negative. Both these samples can have flora other than *S. aureus* and can produce alkaline phosphatase. Hence false positive result can be ruled out when the test is accompanied by Gram staining and coagulase test. Sample Urine R83 gave a weak positive reaction on paper as well as weak coagulase. It is possible that the count of *S. aureus* is just at the borderline of detectable limit of this method. The results were compared to pathologicallab report too and except for the above mentioned two samples, the other sample results matched the pathological laboratory's report.

Figure 3: Test with Clinical Samples

Table 3: Results of Test with Food and Clinical Samples

Sample no.	**Samples**	**Result on LOC Devices**	**Growth of *S. aureus* on Baird-Parker Agar**	**Coagulase**
S6	Ascitic fluid (M342)	+	-	-
S7	Urine (U1023)	+	-	-
S8	Pleural fluid (2465)	-	-	-

S9	CSF (476)	-	-	-
S10	CSF (1665)	-	-	-
S11	Abscess (M211)	+	+	+
S12	Pus (M216)	+	+	+
S13	Pus (M257)	+	+	+
S14	Ascitic fluid (M203)	+	+	+
S15	Urine (U712)	-	-	-
S16	BAL* (R83)	+ (W)	+	+ (W)
* BAL = Bronchoalveolar lavage				

Conclusion

TheLab on Chip device described above for Staphylococcus detection is a rapid, cost effective test for use in the field for point of control settings. Besides being rapid, the test is also cost effective. The amount of reagents required is in microlitres, it does not need any sophisticated instruments or trained personnel for the test. The test is based on visual colour detection. The conclusive diagnostic criteria of staphylococcal food poisoning 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 15 minutes. Hence this test can be used as a screening test by food industries. The test can also be used by medical personnel in remote locations for presumptive diagnosis of Staphylococcus along with Gram staining, coagulase and catalase along with clinical picture.

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