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Research Article

Develop of Lateral Flow Immunochromatographic Test and PCR for Detection of *Salmonella* Enteritidis in Poultry Farm

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ABSTRACT

Salmonellae are responsible for considerable losses in the poultry industry through the death of birds and loss in production especially *Salmonella* Enteritidis (SE). Two diagnostic tools were developed and compared to detect SE in poultry farms. Lateral flow immunochromatographic test (LFIT) was considered a field and laboratory test while PCR was absolutely laboratory test. The minimal bacterial count that gave positive LFIT and PCR was 10^2 CFU/0.1 ml and 10 CFU/ 0.1 ml respectively. For LFIT we could increase the sensitivity results to 1CFU/0.1ml by pre incubating the sample in trypticase soya broth at 37˚C for 6 hour. About 100 samples were tested by the two developed methods and the results were compared with bacteriological methods. The sensitivity, specificity and accuracy of LFIT as compared to bacteriological examination were calculated and were found to be 91%, 80% and 90% respectively and for PCR was 98.8%, 82.3% and 96% respectively.

Key words: Lateral flow, Immunochromatographic Test, PCR, *Salmonella* Enteritidis

INTRODUCTION

Salmonella is considered as one of the most important causes of acute gastroenteritis and foodborne infections worldwide (Ranjbar *et al*., 2016). Gastroenteritis and diarrheal diseases remain one of the most important health problems worldwide, especially in developing countries (Kumar and Subita, 2012). More than 2500 serotypes have been described; mostly belonging to species *Salmonella* enterica (Ranjbar *et al*., 2007); some of these serotypes *S*. Enteritidis (SE) and *S*. Typhimurium (ST) can infect humans and poultry. These serotypes can be responsible for disease outbreaks leading to severe economic losses (Calenge *et al*., 2010). *S*. Enteritidis can produce a systemic infection in young chicks that may further lead to the infection of egg contacts (De Buck *et al*., 2004).

Control of *Salmonella* infections in poultry is posing itself as one of the difficult problems not only for those who are concerned with poultry industry but also for public health hazard because most of the serovars of *Salmonella*e which poultry harbor can act as potential pathogens for human (Galis *et al*., 2013). The isolation and culture of conventional detection method of *Salmonella* cannot meet the testing requirements of quick and easy detection at the grassroots level (Wu *et al*., 2015).

In vitro amplification of DNA by the polymerase chain reaction (PCR) method is a powerful tool in microbiological diagnostics. Several genes have been used to detect *Salmonella* in natural environmental samples as well as food and fecal samples. Virulence chromosomal genes, including *invA*, *invE* and *himA*, *phoP*, are target genes for PCR amplification of *Salmonella* species (Karmi *et al*., 2013). The *invA* gene of *Salmonella* contains sequences unique to this genus and has been proved as a suitable PCR target with potential diagnostic applications (Ibrahim *et al*., 2016).

Lateral flow tests, also known as immune-chromatographic strip tests, are point of care tests that have reduced the time spent waiting for test results from hours to minutes. It requires no specialized equipment, less technical training for operators and has reduced the cost of device development as well as simplicity and rapidity when compared to the molecular and the conventional detection methods also it is suitable for qualitative detection, semi quantitative detection, and mass sample screenings. Results are visualized within 5–10 min by the naked eye (Chen *et al*., 2015; Guo *et al*., 2015; Kong *et al*., 2017).

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The present work aimed to develop two detection methods for SE antigen in poultry farms (molecular detection methods and lateral flow immune chromatographic test method) comparing with conventional bacterial isolation method. This work aimed to present a simple rapid test of high sensitivity, specificity, and accuracy that can improve and facilitates rapid field surveillance of *Salmonella* Enteritidis in the poultry farm.

MATERIALS AND METHODS

Biochemical identification for SE strain

The SE bacterial strain No K482/9A was obtained from the strain bank department at the central laboratory for evaluation of veterinary biologics (CLEVB). The biochemical identification was carried out using analytical profile index API 20 E (Biomerieux –france #20-100).

Preparation of whole SE cells (Soliman et al., 2015)

S. Enteritidis was grown separately on salmonella - shigella ager for 24 hrs at 37°C. Selected separate colonies were inoculated in10 ml of tryptose soya broth and incubated for 24 hrs at 37°C. The culture was inoculated in100 ml of tryptose soya broth and incubated for 24 hr at 37°C, then were inoculated in 250 ml of tryptose soya broth and inoculated for 24 hrs at 37°C. The 250 ml were inoculated in one liter of tryptose soya broth and incubated for 24hr at 37°C. Then the bacterial suspension was centrifuged at 5000 rpm at 4°C for 30 min. to pellet the bacterial strain. A separate final suspension was prepared and the count was adjusted to 10^{10} CFU/0.5 ml using colony count technique.

Preparation of SE somatic (O) antigen (Ibrahim et al., 2017)

SE culture in slope agar was inoculated in 5 ml sterile glucose broth. This suspension was inoculated to 45 ml of sterile glucose broth in gently mixed and incubated at 37°C for 48 h. Two ml of glucose broth culture was inoculated in Roux containing thiosulphate glycerin agar and incubated at 37°C for 48 h. The bacterial growth in Roux flasks was harvested using sterile buffered formal saline and sterile glass beads. The bacterial suspension was filtered by sterile gauze to remove glass beads. The filtrate was tested for purity and morphology by staining film with Gram-stain. Seven hundred ml of absolute alcohol were added to 100 ml of bacterial suspension and left undisturbed for about 36 h until precipitation was completed. The cell suspension was centrifuged for 1 h at 3000 rpm. The antigen was washed 3 times by using normal saline then adjusted to 5 mg/ml by spectrophotometry.

Preparation of Polyclonal antibodies (PAb) of whole SE antigens in Guinea Pig (Gulbenkian et al., 1987)

Suspension from SE strain was mixed with equal volume of complete Freund's adjuvant (10^9CFT/ dose). The emulsion was originally injected subcutaneously. Booster doses containing antigen at a dose of 100µg /dose mixed with incomplete Freund's adjuvant was injected S/C in the immunized guinea pig at 2nd, 4th, 6th and 8th weeks intervals. After one week of the last injection, the serum containing the antigen-specific polyclonal antibodies was collected. The serum thus containing Guinea Pig polyclonal antibody specific to all SE antigens.

Preparation of Polyclonal antibodies (PAb) for SE somatic (O) antigen in Rabbit: (Elke et al., 2008)

The somatic (O) antigen was mixed with equal volume of complete Freund's adjuvant. The emulsion injected intradermally at a dose of 1 mg/kg into male rabbit. Booster doses containing antigen at a dose of 0.5 mg/kg mixed with oily incomplete Freund's adjuvant were injected S/C in the pre immunized rabbits at 2nd, 4th, 6th and 8th week's intervals. After 10 days of the last injection, the serum containing rabbit polyclonal antibody specific Somatic (O) SE antigen, was harvested.

Purification of IgG from Rabbit and Guinea pigs Polyclonal antibodies using Caprylic acid: (Elke et al., 2008)

Twenty five of each serum was centrifuged at 10000 xg for 20-30 min. and discarded the pellet. twice serum volume of 0.06 M sodium acetate buffer pH 4.6 was added in a beaker and placed on a magnetic stirrer. 2.02 ml of caprylic acid was added slowly drop wise drop while stirring at room temperature for 30 min and then centrifuged at 10000 xg for 20 min. the supernatant was retained and the pellet was discarded. The supernatant was dialyzed against PBS buffer at 4˚C overnight with two or three buffer changes; finally the concentration of purified IgG was measured by spectrophotometer.

Preparation of colloidal gold (CG) nanoparticles (Herizch et al., 2014)

Colloidal gold nanoparticles were adjusted at 40 nm diameter size. Fifty ml of purified water contained 0.1% HauCl4 was boiled with vigorously stirring and one ml of 1% (w/v) sodium citrate was added rapidly. When the color of solution was changed to red (about 2 min.) the solution was boiling for another 10 min. After cooling 0.02% (w/v) of sodium azide was added then the diameter of the papered nanoparticles was checked by scanning range 400-600nm using spectrophotometer.

Conjugation of the purified rabbit IgG against O antigen with colloidal gold (CG) (Kong et al., 2017).

Firstly the CG was adjusted to pH 8.5 using 0.02 M K_2CO3. With gently stirring 0.5 ml of purified rabbit IgG (1mg/ml) was added to 50 ml of adjusted CG then gently mixed for 10 min. PEG (20000 1% m/v final concentration) was added for blocking with gently stirring for another 15 min and centrifuged at 10000g for 30 min. The conjugated CG was suspended in 1 ml conjugated CG diluted buffer (20m M Tris contain 1% (w/v) BSA, 3% (w/v) sucrose and 0.02 %(w/v) sodium azide) and stored at 4˚C.

Preparation of lateral flow immunochromatographic test (LFIT) (Guo et al., 2015)

Sample pad: was glass fiber and pretreated with sample pad treated solution pH 8.5 (purified water contained 3.81% (w/v) Borax, 1%(w/v) PVP, 2% (w/v) titronX100, 0.1% (w/v) casein sodium salt, 0.5% (w/v) sodium cholate,0.15% (w/v) SDS, .02% (w/v) sodium azide) then dried at 37C.

Conjugation pad: was also glass fiber and pretreated with conjugation treated solution pH 7.4 (20mM PBS contained 2% (w/v) BSA, 2.5% (w/v) sucrose, 0.3% (w/v) PVP, 1%(w/v) titron x100 and 0.02% (w/v) sodium azide) then dried at 37C and kept in dry condition. Then saturated with conjugated CG finally dried at 37C for 1hr and kept in dry condition.

Nitrocellulose (NC) membrane: The dispenser **(**Iso flow) was used to dispense two lines on the NC membrane (25 mm × 300 mm).the purified IgG Guinea pig antibodies (1 mg/1 ml)was dispensed around the bottom a the test line(1 µl/1 cm line) while goat antirabbit antibodies (0.5 mg/ml) was dispensed at the upper position as the control line (1 µl/1 cm line).The distance between two lines was 5 mm. the loaded NC membrane was dried at 37C for 2 hrs and kept in dry condition.

The treated sample pad, treated conjugation pad, loaded NC membrane and absorption pad were stick down in the PVC card. After that, the collected PVC card was cut into 3.9 mm width test-strips by using an automated cutter machine.

Principles: if sample contained SE was applied to a sample pad, it rapidly wets through to the conjugate pad and the SE Somatic O antigen was bind with rabbit IgG conjugated GC (conjugation complex) would be solubilized this component begins to move with the sample flow front up the nitrocellulose membrane. The conjugation complex passes over the test line to which the capture purified Guinea pigs antibody is immobilized, the complex binding to the another antigens of SE (all sites beyond the somatic O antigen) forming the test line complex (immobilized Purified IgG guinea pig antibody with antigen- rabbit IgG conjugated GC) was subsequently bound forming a red band in the test line. The conjugation complex was trapped by the control zone containing (goat anti rabbit antibody) forming a control line complex (immobilized goat anti rabbit antibody with antigen- rabbit IgG conjugated GC) was subsequently bound forming a red band in the control line, So the sample give two red (test and control line) is considered positive

If sample didn't contain SE, the conjugation complex and the test line complex weren't be formed but rabbit IgG conjugated GC bind directly to goat anti rabbit antibody at the control line forming complex (immobilized goat anti rabbit antibody with rabbit IgG conjugated GC) So the sample give single control red line is considered negative. If no band developed at both zones, the test is invalid.

Molecular identification of SE using multiplex PCR: (Ibrahim et al., 2016)

DNA extraction was carried out for SE bacterial strains No K482/9A that had been inoculated in tryptone soy broth at 37C for 24hrs. About 1ml of TSB was put in Eppendorf tube 1.5 ml then centrifuged at 13000 rpm for 10 min and supernatant was discarded, the bacterial pellet was suspended in 1ml PBS and re centrifuged at 13000 rpm for 10 min and supernatant was discarded, the bacterial pellet was resuspended in 200µl PBS. The DNA extraction was carried out according the manufacturing instruction of DNA extraction kit **(**Qiagen Hilden, Germany**)**. The DNA extract was stored at -20 C until use.

The DNA extract was tested by multiplex PCR using Biometra personal thermocycler using a primer and as enumerated in Table (1) and used PCR kit iNtRON, Korea. The amplification conditions were adjusted to 1 cycle at 94°C for 1 min, 35 cycles at 94°C for 30 s, 56°C for 1 min 30 s, 72°C for 30 s followed by 1 cycle at 72°C for 10 min.

Sensitivity test for LFIT and PCR:

SE bacterial strain No K482/9A was tenfold diluted (10^{9} to 10^{-1}) / 0.1ml serially diluted with PBS and bacterial suspension at each dilution was applied to two diagnostic test.

Sensitivity testing with pre-enriched media for LFIT

Various adjustable concentration from 10^{-1} to 10^{5}CFU/0.1 ml were added in trypticase soy broth (TSB) at 37C at different incubation periods 1/2hr, 1hr, 2hr, 3hr and 6hr and immediately heat killed at 60C for 30 min and stored at 4C before testing LFIT.

Specificity test for LFIT and PCR:

Standard bacteria of the same group of *Salmonella* such as *Salmonella* Pullorum (SP) other group of *Salmonella* such as *Salmonella* Typhimurium (ST) were tested by used the two diagnostic methods. *Mycoplasma gallisepticum* (MG), and *Escherichia coli* were also tested by LFIT.

Validity test of LFIT and PCR for detection of SE (group D) Bacteria compared to the direct bacteriological examination (gold test)

One hundred fecal samples were collected from a poultry farm. The samples were tested with LFIT, PCR and conventional bacteriological examination.

Statistical analysis for determined the sensitivity, specificity and accuracy of the two diagnostic kits (LFIT&PCR) compared with bacteriological examination:

Descriptive statistics: Mean, Standard error of mean (SE Mean) and Standard deviation of LFIT and PCR were determined (Thrusfield, 2007).

Determination of sensitivity and specificity and accuracy (Thrusfield, 2007).

Sensitivity (True positive rate): The ability of LFIT and PCR to correctly identify the percentage of the sample contains SE:

$$\text{Sensitivity} = \frac{T+}{(T+)+(F-)} \times 100 (\text{Stated as } \%)$$

Specificity (True negative rate):

The ability of LFIT and PCR correctly identify the percentage of the sample no contain SE:

Accuracy (validity):

Described the degree to which measurement reflects the

$$\text{Specifity} = \frac{T-}{(T-)+(F+)} \times 100 (\text{Stated as } \%)$$

$$\text{Accuracy} = \frac{(T+)+(T-)}{\text{Total number (n)}}$$

Fig. 1: Biochemical identification of SE using API 20 E

Table 1: Primer sets for *Salmonella* strains PCR

Primer set	*Salmonella* strain	Target gene	Primer sequence 5..3`	Length	Amplicon fragment	Reference
ST11	*Salmonella* Spp.	Randomly cloned chromosomal fragment	AGCCAACCATTGCTAAATTGGCGCA	25	429	Ibrahim *et al.* (2016)
Tym	*Salmonella*	Sef A gen	ACT CTT GCT GGC GGT GCG ACT T	22	312	
Sef167	Enteritidis		AGG TTC AGG CAG CGG TTA CT	20		

Table 2: Evaluation of LFIT and PCR by using direct bacteriological examination (gold standard):

		Bacteriological examination		
		Positive (Diseased)	Negative (Not diseased)	Total
LFIT or PCR	+	A (T+)	b (F+)	a + b
	-	C (F-)	d (T-)	c + d
	Total	a + c	b + d	a + b + c + d (n)

Gold Standard: The means by which one can detect SE whether A is truly present or not. In this study bacteriological examination is the *gold standard*. *False positive* (F+): when the LFIT or PCR indicated that the sample contains SE but in fact it does not contain this SE. *False negative* (F-): when the LFIT or PCR indicated that the sample does not contain SE but in fact it contains the SE. *True positive* (T+): when the LFIT or PCR indicated that the sample contains SE and indeed contains the SE. *True negative* (T-): when the LFIT or PCR indicated that the sample free from SE and indeed it is free.

RESULTS

Biochemical identification of SE: The results of biochemical identification using API 20E for SE was enumerated in Table 3 and Figure 1.

Molecular identification of SE using multiplex PCR: Molecular identification was carried out using multiplex PCR with primers can differentiate between *Salmonella* species. As shown in Fig 2 the SE gave two bands (lane: 1) and the other *Salmonella* strains (*Salmonella* Typhimurium and *Salmonella* Pullorum) gave single bands (lanes: 2 &3), while *E. coli* did not give any band (lane: 4 this reflected the high specificity of PCR test).

Sensitivity test of LFIT and PC: The minimal SE bacterial count that gave positive results in LFIT and PCR were 100 CFU/ 0.1ml and 10 CFU / 0.1ml respectively as shown in Figures 3&4.

Effect of pre-incubation time of tested SE cultures on pre-enriched media on the sensitivity of LFIT: The minimum incubation time exhausted to get the suspected positive reading with the CFU/ml of 10^{-1}, 10^{1}, 10^{2}, 10^{3} for SE was 6hr, 3hr, 1hr and ½ hr, respectively.

Specificity of LFIT and PCR for detection of SE: LFIT gave positive for the same group (SP) and gave negative results for other group of salmonella (ST) also give negative for MG and *E. coli* as shown in Figure 5. PCR, in case of SE gave two band but in case of ST and SP gave one band only, for *E. coli* no band was found as shown in Figure 2.

Validation for LFIT and PCR for detection of Salmonella compared with bacteriological examination: One hundred poultry fecal sample were collected and tested by LFIT, PCR and bacteriological examination. The results were compared and analyzed to determine the sensitivity and specificity and accuracy. As shown in Table 5. When LFIT compared with bacteriological examination, the (T+), (F+), (F−) and (T−) were 82, 2, 8 and 8 respectively and the sensitivity, specificity and accuracy test were 91%, 80% and 90% respectively. When PCR compared with bacteriological examination the (T+), (F+), (F−) and (T−) were 82, 1, 3 and 14 respectively and the sensitivity, specificity and accuracy test were 98.8%, 82.3% and 96% respectively.

DISCUSSION

Avian salmonellosis continue to cause economic losses, particularly in those parts of the world where the poultry industries are continuing to intensify and where open sided housing is common. Salmonellae are responsible for considerable losses in the poultry industry through the death of birds and loss in production. The ideal diagnostic tool of salmonellae should be able to detect SE in the shortest possible time, simple, sensitive, specific and inexpensive. Also it should be suitable as field test or laboratory test and can be applied on large scale of samples. Although this was the main aim of the present work, development of the LFIT and conventional PCR for detect SE in Poultry.

The LFIT was low cost, fast, no need for skilled persons, applicable in field condition and gives results within 5 min that is helpful in large poultry flock *Salmonella* screening. Unfortunately the prepared LFIT wasn't able to differentiate between same group (D) that

Table 3: Biochemical identification of SE using API 20 E

Bio-chemical test	ONPG	ADH	LDC	ODC	CIT	H2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	OXY	NO2
	-	+	+	+	+	+	-	-	-	-	-	+	+	-	+	+	+	+	-	+	-	+

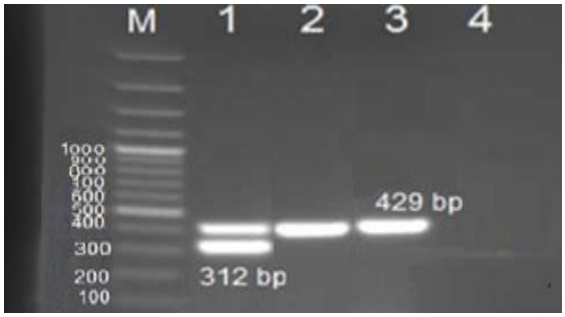

Fig. 2: Molecular identification of SE using multiplex PCR. Lane M: 100 bp DNA ladder (Fermentas). Lane 1: showed two bands at 429 and 312 bp specific for *Salmonella* Enteritidis, Lane 2: showed band at 429 bp specific for *Salmonella* Typhimurium, Lane 3: showed band at 429 bp specific for *Salmonella* Pullorum (SP) and Lane 4: showed no band was formed among *E. coli* strain.

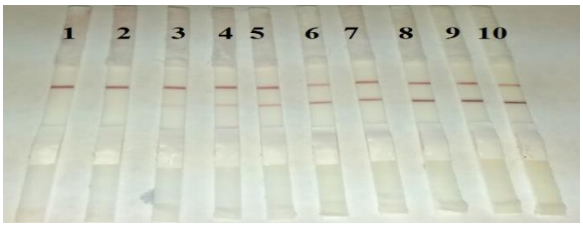

Fig. 3: Sensitivity test of LFIT for detection of SE. Strip 1: 1 CFU /0.1ml, Strip 2: 10 CFU /0.1ml, Strip 3: 10^2 CFU /0.1ml, Strip 4 : 10^3 CFU /0.1ml, Strip 5: 10^4 CFU /0.1ml, Strip 6: 10^5 CFU /0.1ml, Strip 7: 10^6 CFU /0.1ml, Strip 8: 10^7 CFU /0.1ml, Strip 9: 10^8 CFU /0.1ml and Strip 10: 10^9 CFU /0.1ml.

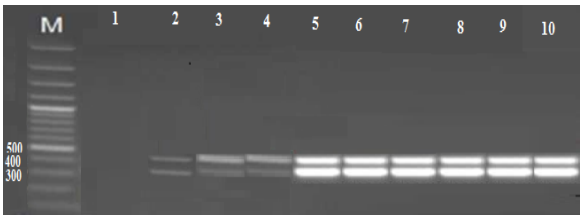

Fig. 4: Sensitivity test of PCR for detection of SE. Lane M: 100 bp DNA ladder (Fermentas), Lane 1: 1 CFU /0.1 ml, Lane 2: 10 CFU /0.1ml, Lane 3: 10^2 CFU /0.1ml, Lane 4: 10^3 CFU /0.1ml, Lane 5: 10^4 cfu/0.1ml, Lane 6: 10^5 CFU /0.1ml, Lane 7: 10^6 CFU /0.1ml, Lane 8: 10^7 CFU /0.1ml, Lane 9: 10^8 CFU /0.1ml and Lane 10: 10^9 CFU /0.1ml.

Table 4: Results of LFIT after using pre-enriched medium.

		10^{-1}	10^1	10^2	10^3	10^4
	Time	reading	reading	reading	reading	reading
SE	½ hr	-	-	-	+	+
	1 hr	-	-	+	+	+
	2hr	-	-	+	+	+
	3hr	-	+	+	+	+
	6hr	+	+	+	+	+

gave positive results in case of SP. On the other hand the PCR can differentiate between SE and SP these results agree with Ibrahim *et al*. (2016).

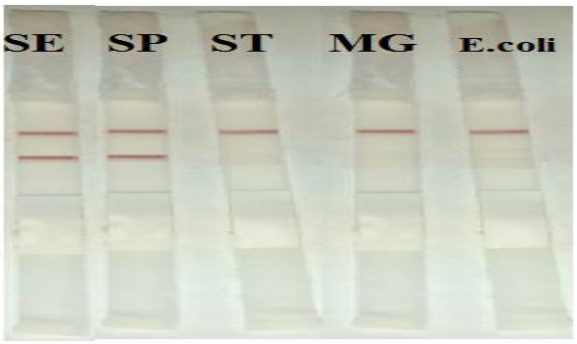

Fig. 5: Specificity test of LFIT.

Table 5: Validation test for LFIT and PCR for detection of SE as comparing with bacteriological examination

Test	Bacteriological isolation			Sensitivity test	Specificity test	Accuracy test
	+ve	-ve	total			
LFIT				91%	80%	90%
+ve	(T+)82	(F+)2	84			
-ve	(F-)8	(T-)8	16			
total	90	10	100			
PCR				98.8%	82.3%	96%
+ve	(T+)82	(F+)3	85			
-ve	(F-)1	(T-)14	14			
total	83	17	100			

The sensitivity or the minimal amount of SE for LFIT was 10^2 CFU / 0.1 (Figure 3) but the PCR method can detect 10 CFU/0.1 (Figure 4). Also, Chirathaworn *et al.* (2011) demonstrated a sensitivity 10 CFU /ml of leptospira using lateral flow devices. Wiriyachaiporn *et al.* (2013) showed that the lateral flow immuno-chromatographic devices sensitivity for *S. aureus* form bronchoalveolar lavage samples was 10^6 CFU/0.1ml. Blaskoza *et al.* (2009) estimated a sensitivity of 10 CFU/ 25 µl of *Listeria monocytogenes* in dairy products using the lateral flow Devices. While Jung *et al. (*2005) reported that the sensitivity of lateral flow devices for *E. coli* O157: H7 in bovine feces was 10^5 CFU/ g.

The suspected sample with SE associated with its pre-incubation in TSB for 6 hr at 37C increased significantly the sensitivity results of LFIT that detected 1CFU / sample as shown in Table 4, also Sithigorngul *et al.* (2007) recorded an increase in the sensitivity of LFD strips for detection of *Vibrio harveyi* to 1–10 CFU/ml of the test sample, if these samples were pre incubated in tryptic soy broth (TSB) for 6 hr before application to the strip. Such sensitivity is comparable to that of PCR.

The sensitivity, specificity and accuracy of LFIT as compared to bacteriological examination were calculated and were found to be 91%, 80% and 90% respectively, on the another hand the PCR method found to be 98.8%, 82.3% and 96% respectively, Dan *et al.* (2010) reported lateral flow device sensitivity and specificity of 93% and 95%, respectively in detection of *Candida albicans* in woman vagina compared with fungus isolation. Also, Kato

et al. (2004) recorded LFD sensitivity, specificity and accuracy rates of 90.6% 95.8% and 94.0%, respectively for detection of *Helicobacter pylori* in children. Compared with the conventional SE diagnostic tools The LFIT are not only very rapid (5 minutes) but also are simple, convenient, has long shelf time and can be used by untrained personal at poultry farm site without requirement of additional equipment like as PCR or bacteriological examination. Moreover, the technology has been strongly improved, which will be reflected on its sensitivity and specificity. These tools are badly required for routine diagnosis in the laboratory and under field conditions.

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