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SIMULTANEOUS MULTIPLE DETECTION OF BACTERIAL CAUSES OF BOVINE MASTITIS USING LATERAL FLOW IMMUNOCHROMA TOGRAPHIC ASSAY

By

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ABSTRACT

Bovine mastitis continues to be the most economically important affection of dairy cattle. Several microbial agents alone or mixed are incriminated as a causative agent of bovine mastitis. *Staphylococcus auras, Streptococcus pyogens, Streptococcus agalactiae*, *Pseudomonas aeruginosa*, *K. pneumoniae*, *E. coli and Candida albicans are* among the most commonly isolated pathogens. The conventional bacteriological isolation and identification of these agents are not only time consuming, requiring full equipped laboratory but also it needs experienced personnel. In the present work a lateral flow device (LFD) was developed for simultaneous multiple identification of three of the causative agents of mastitis in few minutes. Rabbit polyclonal antibody (rPAb) specific to the antigenic components of the 3 of the abovementioned microbial agents, namely *S. aureus, E. coli, and K. pne*umoniae were conjugated with colloidal gold as the detector antibody. It was laid on the conjugate pad. Different Guinea pigs polyclonal antibodies (gPAb) specific to the different antigens tested were used as the capture antibody at the test line (T). Goat anti-rabbit IgG antibody (GAR) was used as the capture antibody at the control line (C) of nitrocellulose strip.

The ready-to-use strips were held in a device consists of 3 strip-holding channels each strip for detection of the 3 different bacterial antigens. The minimal microbial counts that gave positive result using the developed LFD were 100CFU/100µl. The sensitivity, specificity and accuracy of LFD for detection of *E. coli* as compared to bacteriological examination reached to 93.7%, 95.3% and 94.6% respectively. In detection of *S. aureus*it reached to 82.6%, 92.3% and 89.3%, respectively, while in case of *K.pneumoniae* it reached to 94.1%, 98.2% and 97.3%, respectively. It has been proved that treatment of the milk samples with a prepared

application buffer associated with pre-incubation of tested milk samples in TSB for 6 hr at 37°C increased significantly the sensitivity results of prepared LFD kits. The developed kit proved simple, convenient and results can be obtained in less than 15 min.

Key words:

Bovine mastitis, *S. aureus, E. coli, K. pneumoniae*, polyclonal antibodies, lateral flow device.

INTRODUCTION

Bovine Mastitis is one of the most devastating infectious diseases in the dairy industry, affecting the quality and the quantity of milk produced **(Fetrow 2000).** It is a multi-factorial disease and is one of the most difficult to control. Several bacterial species are incriminated as a cause of bovine mastitis; the most commonly isolated bacterial species includes *E. coli, S. aureus* and *K. pneumonia*, **(Aarestrup *et al.,* 1995)**. More attention has been given for diagnosis of clinical and subclinical mastitis using indirect tests, which depends upon the cellular reaction between reagent and certain protein factor in mastitis milk.

This test includes somatic cell count (SCC) according to **Zecconi *et al.,* (2002),** California mastitis test (CMT) according to **Schalm *et al.,* (1971)** and modified white side test (MWST) according to **Murphy and Hanson, (1941)**.

On the other hand, the PCR reaction is sensitive and specific for diagnosis of clinical and subclinical mastitis and can detect the pathogen in milk samples at species level in few hours **(Ghorbanpoor *et al.,* 2007)**. However, it is expensive and needs a special lab. For application. The bacteriological isolation of the causative microorganisms is the most accurate procedure, however, it is expensive and time consuming. The need for a simple quite sensitive, rapid and reliable test sufficient to be applied on large scale of animals is essentially required.

Lateral Flow Devices (LFD) are simple strip assays, which gain more and more popularity as faster analysis methods that are required for direct measurements at the production line or in the field. Besides its widely used fordetection of bacterial pathogens such as *S. aureus* **(Shyu, *et al.,* 2010),** *Vibrio cholera* **(Choo *et al.,* 2011)**, *Vibrio harveyi* **(Sithigorngul *et al.,*2007),***Yersinia pestis* in human **(Hong *et al.,*2010),***Leptospira* in urine **(Chirathaworn *et al.,* 2011)**, *Helicobacter pylori* in humans **(Pelerito *et al.,* 2006 and Trevisani *et al.,* 2007),** *Streptococcus suis* serotype 2 **(Ju *et al.,* 2010),** *Salmonella* enteric subsp.

Entericaserovar Typhimurium (*Salmonella* typhimurium) **(Fang *et al.,* 2009),** *Tilletiaindica* **(Singh *et al.,* 2010),** *and Candida albicans* in vagina **(Dan *et al.,* 2010).**

The aim of the present work was the development of a lateral flow immunochromatographic kits for simultaneous multiple identification of any of the following three common bacterial causes of bovine mastitis, namely, *Staphylococcus auras, K. pneumoniae* and *E. coli*.

MATERIAL AND METHOD

The bacterial strains: The following pathogens were used in the study; *E. coli* (ATCC#25922) *Staphylococcus aureus* (ATCC#25923) and *K. pneumonia* (ATCC#700603). These strains were kindly supplied from the Laboratory for Control and Evaluation of the Biological Preparations, Abbasia, Egypt and from the Microbiology Department, Faculty of Veterinary Medicine, and Cairo University, Egypt.

Preparation of specific bacterial antigens (Norris and Love 2000): The cultures of above mentioned strains were harvested into chilled micro-centrifuge tubes containing 400 ml of sterile phosphate buffered saline (PBS), and washed three times using PBS by centrifugation at 3000xg for 15 minutes. The harvested cells were disrupted by sonication, cell debris was removed by centrifugation and the supernatant fluid comprising the soluble whole cell antigen preparation was diluted with sterile PBS to a protein concentration of 2 mg/ml estimated using spectrophotometer.

Preparation of polyclonal antibodies against the specific antigen of the selected microbial strains:

Preparation of antigen-specific polyclonal antibodies in Guinea pigs (gPAB) according to **Gulbenkian *et al.* (1987).** The soluble whole cell antigens from each bacterial species were mixed with equal volume of complete Freund's adjuvant (200µg/ dose). The emulsion was originally injected subcutaneously. Four successive booster doses (100µg antigen/dose mixed with incomplete Freund's adjuvant) were injected S/C in the immunized guinea pig at 2 weeks intervals. After one week of the last injection, the serum containing the antigen-specific polyclonal antibodies was collected and tested.

Preparation of polyclonal antibodies in rabbit: (Han *et al.,* 2011). The soluble whole cell antigens of each strain were mixed with equal volume of complete Freund's adjuvant. The emulsion was originally injected intradermally at a dose of 0.5 mg/kg into male rabbit.

Four booster doses of antigen mixed with incomplete Freund's adjuvant were injected S/C in the preimmunized rabbits at a dose of 0.15 mg/kg at two weeks intervals. After 10 days of the last injection, rabbit serum containing polyclonal antibody specific to each of the microbial antigen was separated and tested.

Concentration and purification of microbial-specific guinea pig and rabbit polyclonal antibodies using the ammonium sulphate procedure: The prepared pathogen specific polyclonal antibodies prepared in guinea pig and rabbits was concentrated using the ammonium sulphate procedure according to **Donovan and Brown, (1995).**

Removal of cross-reactivity in the prepared antisera by adsorption procedure according to De Roe, *et al.* (1987): Each of the prepared microbial- specific antisera was absorbed by the other two microbial antigens to remove any cross reactive antibodies. The antiserum antigens mixture was incubated at 37 °C for 1 hour. The mixture was then centrifuged for 10 minutes at 1000xg and the serum was collected and preserved at 4 °C till used.

Preparation of immunochromatographic lateral flow assay:

Preparation of colloidal gold nanoparticles (Seema *et al.,* 2010): One ml solution of 1% (m/v) sodium citrate was added to 100 ml boiling deionized water. When the mixture was heated to boiling, another one ml solution of 1% (m/v) HAuCl4 was added rapidly by constant stirring. After the color of the solution changed to red (in about 2 min), the solution was boiled for another 10 min. After cooling, deionized water was added until the volume reached to 100 ml. The obtained gold colloidal was supplemented with 0.02 % (m/v) of sodium azide and stored at 4°C. The particle diameter was checked with transmission electron microscopy (TEM, H-7650).

Conjugation of the bacterial specific rabbit polyclonal antibodies and colloidal gold (Jiang *et al.,* 2008): The colloid gold solution was adjusted to pH 8.5 with 0.02M K_2CO_3. With gentle stirring, 0.1 ml of the rabbit polyclonal antibody (2 mg/0.1ml of 0.05% NaCl buffer) was added drop wise to 10 ml of pH-adjusted colloid gold solution.The mixture was gently mixed for 10 min, blocked with 1% (m/v) final concentration of polyethylene glycol (PEG - 20,000 kDa) with stirring for another 15 min and centrifuged at 10,000 g for 30 min. The gold pellets were suspended in 1 ml dilution buffer [20mM Tris/HCI buffer (pH 8.2) containing 1% (w/v) BSA, 3% (w/v) sucrose and 0.02% sodium azide], and stored at 4°C until used.

Preparation of immunochromatographic test device according to Yang *et al.*, (2011):

-Sample pad: It was made of glass fiber. It was saturated with PBS solution, pH 7.2, containing 0.3% Tween-20 and 0.5% (w/v) triton X100) and dried at 37◦C. it was kept under dry conditions at room temperature until used.

-The conjugate pad: It was prepared as follows; a glass fiber was treated with 0.1% Tween-20 for 10 min and dried at 60 °C. The prepared glass fiber was cut into sections (4cm×0.5 cm), and then saturated with 0.15 ml of colloidal gold probe. The conjugate pad was dried for 1 h at 37°C, and stored under dry conditions at 4 °C until used.

-Nitrocellulose membrane: BIODOT (XYZ-3) was used to dispense two lines on the NC membrane (25mm×300 mm). the bacterial species specific guinea pigs polyclonal antibody (1.5mg/0.1ml) was dispensed around the bottom as the test line (1µl per 1 cm line) while the goat anti-rabbit IgG (1 mg/ ml-)was dispensed at the upper position as the control line (1µl per 1 cm line).The distance between two lines was 5 mm. After applying of the test line, the membrane was dried for 2 to 6 hours in room temperature. Then it was blocked by immersing the membrane into the membrane blocking buffer. After the whole membrane was wetted it was washed by immersing it five times in the first PBS and 5 times in the second PBS solution. After that, the membrane was covered with top laminate and cut into 0.5-cm-width test-strips by using an automated cutter machine.

Specificity testing of the prepared bovine mastitis diagnostic LFD: Pure cultures of under test microbial pathogens, namely, *S. aureus, E. coli*, *K.pneumoniae,* were grown on trypticase soya agar (TSA) for 3 hours. The harvested culture was suspended in the application buffer and tested using the LFD.

Sensitivity testing of the prepared bovine mastitis diagnostic LFD: Each of the three microbial pathogens was tenfold serially diluted (10^1 to 10^8) with the application buffer and bacterial suspension at each dilution was tested by the prepared diagnostic LFD.

Determination of the sensitivity of the developed LFD after pre-enriched of bacterial samples:

Various adjustable concentrations from 10^1 to 10^6CFU/ ml were added in trypticase soy broth (TSB) at 37°C at different incubation periods 1/2hr, 1hr, 2hr, 3hr and 6hr, then immediately heat killed at 60°C for 30 min and stored at 4C before testing with the prepared LFD diagnostic kits.

Evaluation of the efficacy of prepared diagnostic LFD device for rapid simultaneous detection of any of these 3 microbial pathogens causing bovine mastitis:

For evaluation of the efficacy of the developed LFD and determination of its sensitivity, specificity and accuracy in detection of bacterial causes of bovine mastitis, 112 bovine milk samples collected from clinically suspected mastitis were examined bacteriologically (gold standard test), and also by the developed LFD.

Milk samples:

A total number of 112 milk samples were collected aseptically from dairy cattle for bacteriological examination. The samples were collected from cases either with atrophied quarter, dried or suffering from severe mastitis according to the procedures of **Andrews *et al* (2004) and Radostits *et al*. (2007).** From each quarter, three milk samples were collected; 15-20 ml milk samples were collected in a clean sterile screw capped bottle then labeled. The milk samples were kept in an ice container till delivered to the laboratory. One of the three samples was examined for somatic cell count (SCC) (this sample was kept on formalin 10 % if it will not examined for the somatic cell count at the same day). The second sample was subjected for lateral flow test according to the results of CMT and SCC. The third sample was subjected to bacteriological examination after being pre-incubated for 24 hours.

Determination of the effect of sample preparation before testing on the sensitivity and specificity of the developed LFDs diagnostic test:

The tested milk samples were examined untreated and after being treated by different methods to determine the best pre-testing preparation protocols. The milk samples were tested in the following forms:

A.Untreated milk samples.

B.Milk sample treated with application buffer.

C.Untreated Milk whey.

D.Milk whey treated with application buffer.

Preparation of milk whey (Farjam *et al.*, 1991): A low pH sample treatment buffer was formulated in a matrix of standard buffers such as PBS or tris buffer (pH 4.3) and containing detergent or surfactant as triton X-100 at 2% a concentration of 2%.

Treatment of tested milk samples with the application buffer: Each of the milk samples was mixed with the application buffer and incubated for 15 minutes just before being tested

(Sithigorngul, *et al.,* 2007).The Application buffer composed of: 30mM Tris,336mMNaCl, 9mM EDTA, 1% tween 80 and the pH was adjusted to pH 9.3.

Statistical analysis and evaluation of diagnostic kits (testvalidity): Evaluation of diagnostic kit by using direct bacteriological isolation (gold standard) and determined the sensitivity, specificity and accuracy by using the Statistix® (1996) package**.**

RESULTS

Specificity testing of the developed lateral flow device (LFD) kits: The LFD kits prepared against the 3 tested microorganisms showed clear positive results when tested against these microbial species and were negative when tested against other different microbial species, namely, ***Streptococcus agalactiae and S. dysgalactiae, Candida albicans and Pseudomonas aeruginosa***.

Sensitivity testing of the developed lateral flow device (LFD):

The lowest concentrations of the bacterial cultures prepared from the under testing bacterial strains (*E. coli, S. aureus, and K. pneumonia)* that gave unambiguous color Fig. (1), (Table 1) at the test line were as follows:

- at bacterial count of 10^3CFU/ml = The color degree was suspected (-/+),
- at bacterial count of 10^4CFU/ml = Weak positive (+)
- at bacterial count of 10^5 CFU/ml = Positive ++
- at bacterial count of 10^6CFU/ml = Strong positive (+++)

Results of examination of the bovine milk samples using the conventional procedures, namely, CMT and SCC: The collected milk samples were examined for presence of mastitis using California Mastitis Test (CMT) and Somatic Cell Count (SCC). Using CMT the percentage of apparently healthy, subclinical mastitis and clinical mastitis milk samples were 34 samples(30.36%),63samples(56.25%)and 15 samples (13.39%),respectively. Examination of the 63 milk samples of subclinical mastitis cases using SCC test showed the following

results: 8 samples were at the range of < 10^5 SCC/ml, 25 samples were in the range of >10^5 - 3 x10^5 SSC/ml and 30 samples in the range of >3 x10^5 - 5 x10^5 SCC/ml.

On the other hand examination of 15 milk samples from clinical mastitic cases using SCC test revealed the following; 13 cases showed SCC in the range of >5 x10^5 – 10^6 SCC/ml and 2 cases showed SCC in the range of >10^6 SCC/ml (Table 2).

Results of bacteriological examination of the positive CMT milk samples:

Bacteriological examination of the positive CMT milk samples (78) revealed that 96.15% of the tested samples were bacteriologically positive, while three samples (3.85%) were negative. The bacteriologically positive samples (75 samples) were divided into single bacterial infections samples (60.2%) and mixed bacterial infection (35.8%) as shown (Table 3).

The number of cases associated with single infection, mixed infection and bacteriological free samples were 47 (60.2%), 28 (35.8%) and 3 (4%), respectively.The identified bacterial agents related to milk samples with single infection were as follow; *E. coli* (25.5%), *S. aureus* (14.8%), CNS (12.7 %), *St. agalactiae* (12.7%), *St. pyogenes* (10.6 %), *K. pneumoniae* (8.5 %), *Salmonella* spp.(4.2 %), *Proteus sp*. (4.2%), *Ps. aeruginosa* (4.2 %) and *C. albicans* (2.1%). Meanwhile in case of the mixed infection the bacterial agents were *S. aureus* plus *E. coli* (17.8%), *E. coli* plus *K. pneumoniae,*(14.2 %), *S. aureus* plus *K. pneumoniae,*(14.2%),*CNS* plus *E. coli,*(14.2 %), *S. aureus* plus *St. agalactiae*plus *E. coli,*(7.1%), *S. aureus* plus *E. coli* plus *K. Pneumoniae* (7.1%),*St. agalactiae* plus *E.coli,*(7.1%), *St. pyogenes*plus *E. coli, (*7.1%), *CNS*plus *K. pneumoniae,*(7.1 %) and *Proteus spp*plus *S. aureus (*3.5%) as shown in (Table 4).

Effect of the method of treatment of the milk samples before testing on the sensitivity of the established diagnostic procedures:

The examined milk samples were tested in different forms including untreated fresh milk samples, milk sample treated with application buffer, untreated milk whey and milk whey treated with application buffer. The differently treated milk samples were examined with the developed LFD kits and best results were recorded in milk samples treated with the application buffer Fig. (2).

Determination of sensitivity, specificity and accuracy of the developed LFD as compared to bacteriological examination (the gold master test):

The bacteriologically positive samples (75 samples) were examined by the prepared LFD kits. Fifty five samples were positive using the prepared LDF, of which 16 with single bacterial

infection and 39 with mixed bacterial infections (Table 5). LFD positive milk samples with single infection were *E. coli* (21), *S. aureus* (11), *K. pneumoniae* (7).The LFD positive milk samples with mixed infections were *S. aureus* and *E. coli* (6) *E.coli* and *K.pneumoniae* (4) *S.aureus* and *K.pneumoniae* (5) and *S. auras, E. Coli and K. Pneumoniae* (1),the sensitivity, specificity and accuracy of the developed LFD as compared to bacteriological examination were calculated and was found to be for *E. coli* 93.7%, 95.3% and 94.6%, respectively; for *S. aureus*, 82.6%, 92.3% and 89.3%, respectively, and for *K. pneumoniae* 94.1%, 98.2% and 97.3%**,** respectively, (Table 6).

Effect of pre-incubation time of tested microbial cultures on pre-enriched media on the sensitivity of lateral flow device kits: To determine the minimum incubation time needed to achieve the highest sensitivity of the developed bovine mastitis diagnostic tools, the counted bacteria of each microbial species was incubated in pre-enriched (TBS) media at different intervals ½ hour, 1 hour, 2 hours, 3 hours and 6 hours before being tested. (Table 7) show that the minimum incubation time exhausted to get the suspected positive reading with the CFU/ml of 10^1, 10^2 and 10^3 for the following microorganisms to be as follow: *E. coli* it was 3hr, 2hr and 1hr, respectively**,** for *S. auras* it was 6hr, 6hr and 1/2hr, respectively, and for *K. pneumoniae* it was 3hr, 2hr and 1 hr., respectively**.**

Table (1): Sensitivity of the prepared lateral flow device kits in detection of.

Count*	**0**	**10^1**	**10^2**	**10^3**	**10^4**	**10^5**	**10^6**	**10^7**	**10^8**
E. coli	-	-	-	-/+	+	++	+++	+++	+++
S. aureus	-	-	-	-/+	+	++	+++	+++	+++
K. pneumoniae	-	-	-	-/+	+	++	+++	+++	+++

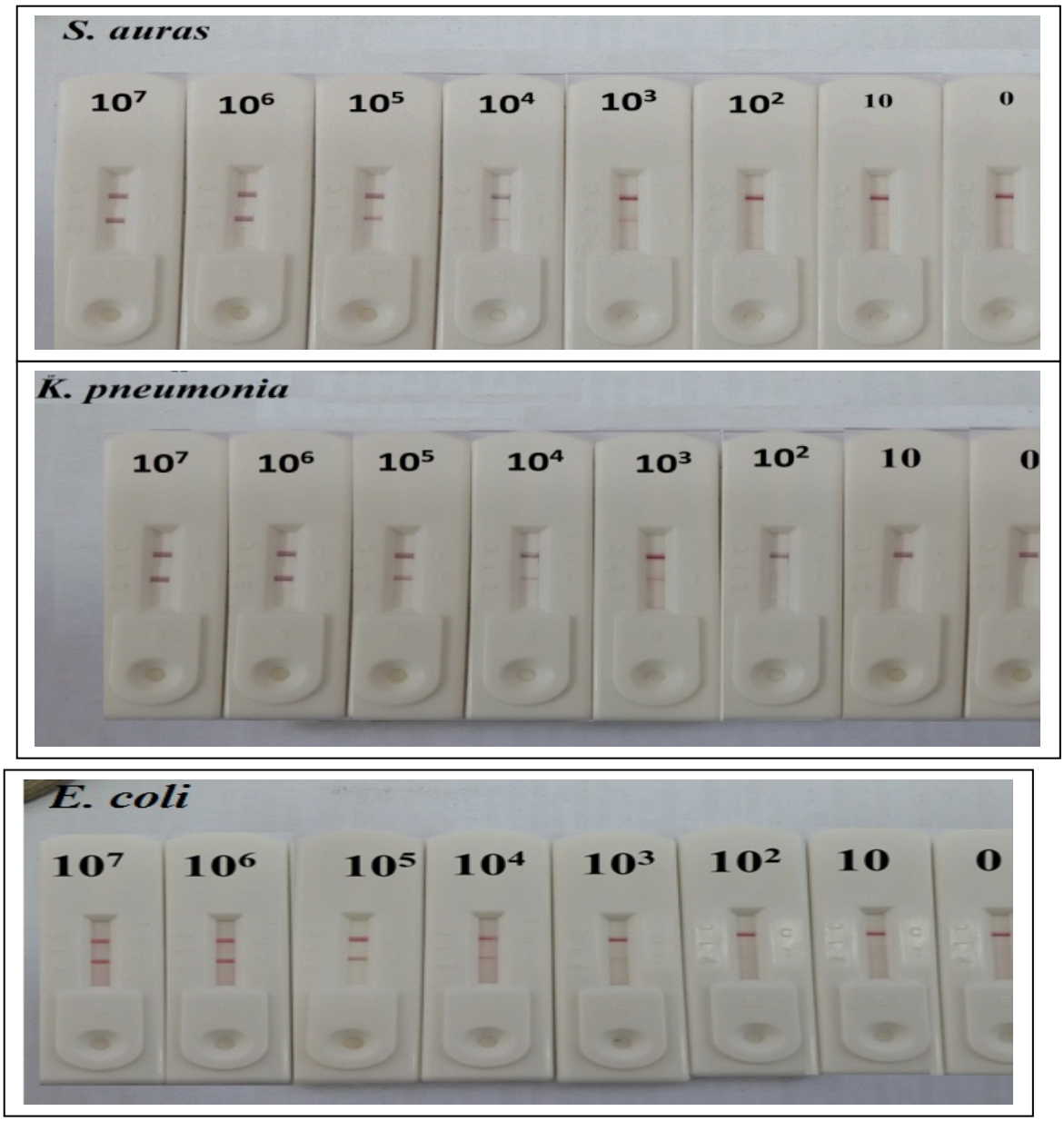

Fig (1): Sensitivity of the prepared lateral flow device kits in the detection of the bacterial species causing bovine mastitis.

*** In testing using the prepared Lateral flow device kits the culture dilutions were double fold diluted with the application buffer before application, and then 100 µl were added per device.**

Table (2): Results of examination of bovine milk samples using CMT and SCC.

	Milk samples			
The test Interpretation	**CMT**		**SCC/ml**	
	No. of samples	**%**	**ranges**	**No.**
Healthy	**34**	**30.3**	**$<10^5$**	**34**
Subclinical mastitis	**63**	**56.3**	**$< 10^5$**	**8**
			$>1 \times 10^5 - 3 \times 10^5$	**25**
			$>3 \times 10^5 - 5 \times 10^5$	**30**
Clinical mastitis	**15**	**13.3**	**$>5 \times 10^5 - 1 \times 10^6$**	**13**
			$>10^6$	**2**
Total	**112**			**112**

Table (3): Results of bacteriological examination of the positive CMT bovine milk samples.

Total no. of bacteriologically examined milk samples	**Bacteriologically positive samples**						**Bacteriologically negative samples**	
	Single infection samples		**Mixed infection samples**		**Total**			
78	**No.**	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**
	47	**60.2**	**28**	**35.8**	**75**	**96**	**3**	**4**

Table (4): The incidance of bacterial agents recovered from the CMT positive milk samples.

Single bacterial infection				Mixed bacterial infection			
No. of milk samples	**Bacterial species**	**No. of isolates**	**%**	**No. of milk samples**	**Bacterial species**	**No. of isolate**	**%**
47	***E. coli***	**12**	**25.5%**	28	***S. aureus & E. coli***	**5**	**17.8%**
	S. aureus	**9**	**14.8%**		***S. aureus, St. agalactiae & E. coli***	**2**	**7.1%**
	Coagulase negative Staphylococci (CNS)	**6**	**12.7%**		***S. aureus ,E. Coli & K. pneumoniae***	**2**	**7.1%**
	St. agalactiae	**6**	**12.7%**		***E. coli & K. pneumoniae***	**4**	**14.2%**
	St. pyogenes	**5**	**10.6%**		***S. aureus & K. pneumoniae***	**4**	**14.2%**
	K. pneumoniae	**4**	**8.5%**		***CNS & E. coli***	**3**	**14.2%**
	Salmonella spp	**1**	**4.2%**		***St. agalactiae & E. coli***	**2**	**7.1%**
	Proteus spp	**1**	**4.2%**		***St. pyogenes & E. coli***	**2**	**7.1%**
	Ps. aeruginosa	**2**	**4.2%**		***CNS & K. pneumoniae***	**2**	**7.1%**
	C. albicans	**1**	**2.1%**		***Proteus spp& S. aureus***	**1**	**3.5%**
Total		**47**		**Total**		**58**	

Fig. (2): Effect of methods of milk samples preparation on the Test results of the lateral flow procedure (*E. coli* LFD).

Table(5): The number oF LFD kits positive samples among the bacteriological positive samples.

Single bacterial infection		Mixed bacterial infection		
Bacterial sp.	**No. of LFD positive**	**Bacterial sp.**	**No. of LFD positive**	
E. coli	**21**	***S. aureus & E. coli***	**6**	
S .aureus	**11**			
K. pneumoniae	**7**	***S. aureus , E. coli & K. Pneumoniae***	**1**	
		E. coli & K. pneumoniae	**4**	
		S. aureus & K. pneumoniae	**5**	
Total	**39**	**Total**	**16**	**55**

Table (6): Sensitivity, specificity and accuracy results of lateral flow kitfor detection of microbial causing mastitis.

		Bacteriological examination			Sensitivity test	Specificity test	Accuracy test
		+ ve	- ve	Total			
E. coli	+ ve	30	2	32	93.7%	95.3%	94.6%
	- ve	2	41	43			
	Total	32	43	75			
S. aureus	+ve	19	4	23	82.6%	92.3%	89.3%
	-ve	4	48	52			
	Total	23	52	75			
K. pneumoniae	+ve	16	1	17	94.1%	98.2%	97.3%
	-ve	1	57	58			
	Total	17	58	75			

Table (7): Results of examination of bovine milk samples using LFD after incubation of the tested samples in pre-enriched medium.

	10^1		10^2	10^3	10^4	10^5
	Time	**reading**	**reading**	**reading**	**reading**	**reading**
E. coli	**½ hr**	-	-	-	+	++
	1 hr	-	-	-/+	+	++
	2hr	-	-/+	+	++	++
	3hr	-/+	+	+	++	+++
	6hr	+	++	+++	+++	+++
S. aureus	**½ hr**	-	-	-/+	+	++
	1 hr	-	-	-/+	+	++
	2hr	-	-	-/+	+	++
	3hr	-	-	+	++	++
	6hr	-/+	-/+	+	++	+++
***K. pneumonia*e**	**½ hr**	-	-	-	+	++
	1 hr	-	-	-/+	+	++
	2hr	-	-/+	+	++	++
	3hr	-/+	+	+	++	+++
	6hr	++	++	+++	+++	+++

Note: In lateral flow strips the culture was diluted into half concentration with application buffer before application then100 µl were added per strip.

DISCUSSION

The laboratory diagnosis of bovine mastitis depending upon the bacteriological isolation of the causative microorganisms is the gold master test.However,it is expensive, time consuming and requires experienced staff. Also the molecular diagnosis using polymerase chain reaction (PCR) is sensitive and specific for diagnosis of clinical and subclinical mastitis and could be used in detection of the pathogens in milk samples at species level in few hours **(Ghorban poor *et al* ., 2007 and Taponen *et al*., 2009)**. However and again it is expensive, not suitable as field test and like the bacteriological examination requires a special laboratory and experience. The ideal diagnostic tool of bovine mastitis should be able to detect the causative agent 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 animals. Such

diagnostic approach when developed will facilitate the application of the correct method of treatment in the proper time and reduces the complication of mastitis.

As shownin (Table 1) the sensitivity (minimal CFU that give positive result) of the prepared diagnostic LFD kits were determined, where the minimal microbial counts,

These results are similar to those reported by **Humar*et al.,* (2008)** who recorded a sensitivity of 100 CFU/100 µl of *Salmonella enterica*serovar*typhi* using plate ELISA**.**

Also, **Chirathaworn *et al.,* (2011)** demonstrateda sensitivity 10 CFU /ml of leptospires using lateral flow devices. **Blaskoza *et al.,* (2009)** estimated a sensitivity of 10 CFU/ 25 µl of *Listeria monocytogenes*in dairy products using the lateral flow Devices. On the other hand **Wiriyachaiporn *et al.,* (2013)** showed that, the lateral flow immunochromatographic devices sensitivity for *S. aureus form* Broncho alveolar lavage samples was 10^6 CFU/ml, 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/ gm.

Also in the present work the specificity, sensitivity and accuracy of the developed diagnostic LFD kits in examination of bovine milk samples from bovine mastitis cases as compared with conventional bacteriological methods (gold test) was done. The collected 112 bovine milk samples were examined first by CMT and SSC. Then the CMT positive samples were subjected to bacteriological examination. Examination of the collected bovine milk samples with CMT revealed a subclinical mastitis and clinical mastitis rates of 56.3% and 13.3%, respectively, as shown in (Table 2).These findings are relatively similar to those recorded by several authors **(El-Rashidy *et al.*, 1988; Awad, 1999;Karimuribo *et al.,* 2008 and Kivaria *et al.,* 2007)** who reported a subclinical mastitis prevalence rates ranging from 62.08 % to 78% among the microbiologically examined dairy cattle. On the contrary, The CMT recorded rate of subclinical mastitis in the present work disagree with that reported by **Abou-Zaid and Bahout (1993)** who reported prevalence rates of 20.6 % and 21.2 %. Also **Lakew *et al.,* (2009)** reported a rate of 32.7 % of subclinical mastitis among dairy cows. Such variation in the recorded rates of subclinical mastitis might be attributed to many factors, mainly the hygienic standards in the tested farms**.** Regarding the prevalence rate of clinical mastitis in the tested samples, it reached to 13.3%. Similar results reporting clinical mastitis prevalence rates ranging from 3.6 % to 14.8 % were reported by several authors **(Ahmed, 2000; Hanaa, 2001 and Petrovski *et al.,* 2009).** On the other hand, our findings were not supported by results

obtained by **Tarek(2006)and Lakew *et al.*,(2009)** who reported a clinical mastitis prevalence of up to 26.5 %. (Table 3) shows the result of bacteriological examination of the SCC and CMT positive samples, where 96% of total positive cases were bacteriologically positive. This agrees with what stated by **Rysanek *et al.*, (2009)** that there is clear correlation between infection and number of SCC.Meanwhile, **Seleim *et al.*,(2002)**reported that,the bacteriological positive samples were 92.36 %. Also **Char *et al.*, (1993)** reported that, the bacteriologically positive samples reached to 86.7%.The incidence of *E. coli* recovered from the tested quarter milk samples as a single infection reached to was 25.5%, representing the highest prevalence rate among the recorded microbial pathogens. **Vaarst and Enevaldsen (1997)** reportedan *E. coli* prevalence of 36 % and **Akram *et al.*, (2013)** reported that *E. coli* was the most common pathogen that could be isolated from the milk samples as an environmental pathogen. Concerning *S. aureus* it was isolated at a rate of 14.8 %, which is correlated with findings obtained by **Sampimon *et al.* (2009),** who recovered *S. aureus* was at a rate of 17.03 %. Meanwhile, **Tufani *et al.*, (2012)** recorded 66.67% isolation rate of Staphylococcus spp. and 15.87% of *E. coli*.

As shown in (Table 6), the sensitivity, specificity and accuracy of developed LFD as compared to bacteriologically examination were calculated and was found to be 93.7%, 95.3% and 94.6% respectively, for *E. coli;* 82.6%, 92.3% and 89.3%, respectively for *S. aureus* and 94.1 %, 98.2% and 97.3 %, respectively for *K. pneumonia*. These results are similar to those recorded by **Fang *et al*., (2009)** who found that the sensitivity and specificity of LFD for detection of Salmonella spp. compared to bacteriological Examination were 100% and 99 %, respectively. Also **Bautista *et al.*, (2002),** however**,** reported a sensitivity rate of 12.3% and a specificity of 100 % of the lateral flow strips developed for detection of *Salmonella typhimurium* in chicken. The variation in the sensitivity and specificity of the LFD can be attributed to many factors, the most important of which is the quality and specificity of the prepared pathogen specific antibodies. The increase of the sensitivity of our prepared kits are attributed to the use of two types of specific polyclonal antibodies for each pathogens, the first one (primary antibodies) was prepared in guinea pigs for catching the pathogen antigens present in the tested sample. The second antibodies (secondary antibodies), however, was prepared in a different animal species, namely, rabbit and this was conjugated with the nano-gold chloride particles. The two types of antibodies prepared in a different

animal species might recognize different epitopes on the same antigen molecules, which stand behind the increasing of the LFD kits sensitivity. These facts were described by **O'Keeffe *et al.,* (2003), Sithigorngul *et al.,* (2007) and Rudolf *et al.,* (2009).**

The effect of time of pre-incubation of the tested samples in TSB medium on it is efficacy was determined. As shown in (Table7) the minimal time required for the pre-enrichment to get the suspected positive reading with the 10 CFU / ml sample was 6 hr in TSB, as the positive results were observed with the tested samples. **Humar *et al* .,(2008)** proved that pre-incubation of tested samples for 4 hr in brain heart infusion broth increase the LFD sensitivity for detection of salmonella in water at least 10 times. Also, **Seo *et al.,(*2003)** reported a *Salmonella enteriditis* sensitivity of 100 % in raw egg pools inoculated with 10 *Salmonella enteriditis* cells per ml of egg and incubated in buffered peptone water (BPW) or tetrathionate brilliant green broth (TBG) for 24 h at 37 C.**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 pretesting treatment of the milk samples with of application buffer associated with its pre-incubation in TSB for 6hr at 37C increased significantly the sensitivity results of the three prepared diagnostic LFD kits.

Compared with the conventional bovine mastitis diagnostic tools The LFD kits are not only very rapid (5 minutes) but also are simple, convenient, has long shelf time and can be used by untrained personal at dairy farm site without requirement of additional equipment. Moreover the LFD preparation 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.

REFERANCES

Abou-Zaid, A.A. and Bahout, A.A. (1993): Studies on subclinical mastitis in cattle. J. Egy. Vet. Med. Ass. 53(1&2):251-259.

Ahmed, M.A.M. (2000): Epidemiological studies on mastitis in Friesian cattle. M.V.Sc. Thesis (Infectious diseases). Fac. Vet. Med. Cairo Univ**.**

Andrews A H, Blowey R W, Boyd H, Eddy RG. (2004): Bovine Medicine Diseases and Husbandry of Cattle 2nd edition, Blackwell Science, p. 328 - 329.

Awad, W. S. (1999): Some studies on subclinical mastitis in cattle and buffaloes. PH.D. Thesis, Fac. Vet. Med., Cairo Univ.

Bautista, D.A.; S. Elankumaran, J. A. Arking, and R. A. Heckert (2002): Evaluation of an immunochromatography strip assay for the detection of *Salmonella* sp. from poultry. J Vet Diag Invest 14:427 - 430 (2002).

Blaskoza M, Koet M, Rauch P, and Amerogen AV. (2009): Development of a nucleic acid lateral flow immunoassay for simultaneous detection of *Listeria spp*. and *Listeria monocytogenes* in food. Eur Food Res Technol., 229:867-874.

Char, N. L., M. R. K. RaoandK. R. Rajeswari (1993): Clinical mastitis in cows: bacterial and anti-microbial activity. Ind. Vet. J., 70 (4): 378 - 379.

Chiratha worn, C.; Chantaramalai, T.; Sereemaspun, A.; Nipaporn - Kongthong, N. and Suwancharoan, D. (2011): Detection of leptospira in urine using antileptospira-coated nanoparticles. Comp. Immunol*, Microbiol and Infect. Dis.,* 34:31-34.

Choo, Y.; Yong, A.; Lim, C. E.; Husni, T.; Su, Y.L.; Gustavo, F.; Oscar O. I. ; Fatima, R.; Armando, A.; Maria, E. S.; Santanu, G.; thandavarayan R.; Chan, Y.; pathabhiraman, L. and Manickam, R. (2011): Dry-reagent gold nanoparticle-based lateral flow biosensor for the simultaneous detection of Vibrio cholera serogroups O1 and 139. *J Microbiol meth,* 86:277-282.

Dan, M.; Yael Leshem, Y. and Yeshaya, A. (2010): Performance of rapid yeast in detection Candida Spp. in vagina*. Diag Microbiol and in fect. Dis.,* 67; 52-55.

De Roe, C.; Courtoy, P. J. and Baudhuin, P. (1987): A model of protein colloidal gold interactions. *J. Histochem. Cytochem.* 35:1191-1198.

Donovan, J and Brown, P. (1995): Current Protocols in Immunology (1995) 1.0.3 - 1.0.6.

EL-Rashidy,A.A.;Amin,A.;Mostafa, A.S.and Tawfik,M.S. (1988): Evaluation of mastitis screening test in diagnosis of subclinical cases compared with the conventional bacteriological methods. Vet. Med. J. 36 (1): 91-100.

Fang, S.; Tseng, W.; Lee, H.; Tsai, C.; Huang, J.; Hou,S. (2009): Identification of *Salmonella* using colony-print and detection with antibody-coated gold nanoparticales*. J. Microbiol Meth.,* 77:225-228.

Farjam, A.; De-Vries, R.; Lingeman, H. and Brinkman (1991): Immunopercolums for selective on-line sample pertrearment of aflatoxins in milk prior to column liquid chromatography. *J. Environ. Anal. Chem.* (44) 175 -184.

Fetrow, J. (2000): Mastitis: an economic consideration. Proceeding of the 29th annual meeting of Natl. Mast. Coun. Atlanta, GA, Natl. Mast. Coun. Madison, WI. P. 3 - 47

Ghorbanpoor, M.; Seyfiabadshapouri, M.; Moatamedi, H.; Jamshidian, M. and Gooraninejad, S. (2007): Comparison of PCR and Bacterial culture methods for diagnosis of dairy cattle's subclinical mastitis caused by Staphylococcus aureus. *J. Vet. Res.,* 62. (4): 87-91.

Gulbenkian, S.; Wharton, J. and Polak,J.M.(1987): The visualization of cardiovascular innervations in the guinea-pig using an antiserum to protein gene product. *J. Auton. Nerv. Syst.,* 18, 235-247.

Han, Y.; Zhang, X.; Chen, Y.; Li, Y.; Hao Chen, H. and Fang, L. (2011): High-level expression, purification, polyclonal antibody preparation against recombinant OprD from *Pseudomonas aeruginosa. Afri J Biotech,* 10, (7): 1246 -1251,

Hanaa Abdel-MonemAsfour (2001): Microbial and other factors associated with bovine mastitis. M.V.Sci. Thesis (Microbiology) Fac. Vet. Med., Cairo Univ.

Hong, W.; Lihua, Huang., Haoran, W.; Jianfeng, Q.; Zhaobiao, G.; Chengke X.; Ziwen, Z.; Youbao, Z. Zongmin, D. ; Yanfeng, Y.; Yan, Z.; Huijie, H.; Ruifu, Y. and Lei, Z. (2010): Development of an upconverting phosphor technology-based 10- channel lateral flow assay for profiling antibodies against Yersinia pestis. *J Microbiol meth,* 83: 133 - 140.

Humar,S.; Balakrishina, K and Batra, H.V.(2008): Enrichment-ELISA for Detection of Salmonella typhi From Food and Water Samples. *Biomed. Environ. Sci.,* 21, 137-143.

Jiang,Z.;Wenchao,C.;Yitong, L.andGuohua,C.(2008): Development of an immunochromatographic assay for the rapid detection of bromoxynil in water. *Environ Poll,* 156: 136-142.

Ju, Y.; Hao, H.; Xiong, G.; Geng, H.; Zheng, Y,;Wang, J. Cao, Y.; Yangm, Y; Cai, X. and Jiang, Y. (2010): Development of colloidal gold based immunochromatographic assay for rapid detection of *Streptococcus suis* serotype 2. *Vet Immunol. Immunopath,* 133:207-211.

Jung BY, Jung SC, Kweon CH. (2005): Development of a rapid immunochromatographic strip for detection of *Escherichia coli* O157**.** J Food Prot.; 68 (10):2140-3.

Karimuribo, E.D.,J.L.Fitzpatrick,E.S.Swai,C.Bell,M.BryantJ,N.H.Ogden,D. M. Kambarageand N. P. French (2008): Prevalence of subclinical mastitis and associated risk factors in smallholder dairy cows in Tanzania. Vet Rec. 5 163 (1): 16 -21.

Kato, S.; Kyoko Ozawa, Masumi Okuda, Yoshiko Nakayama, Norikazu Yoshimura, Mutsuko Konno, Takanori Minoura and KazuieIinuma (2004): Multicenter Comparison of Rapid Lateral Flow Stool Antigen Immunoassay and Stool Antigen Enzyme Immunoassay for the Diagnosis of *Helicobacter pylori* Infection in Children . *Helicobacter*, 9, 669 - 673

Kivaria, F .M, J .P. Noordhuizenand M. Nielen (2007): Interpretation of CMT scores using *Staphylococcus aureus* culture results for screening of subclinical mastitis in low yielding small holder dairy cows in the Dar Es-Salam region, Tanzania. Prev. Vet. Med., 78 (3 - 4):274 -285.

Lakew, M., T. Tolosa and W. Tigre (2009): Prevalence and major bacterial causes of bovine mastitis in Asella, South Eastern Ethiopia. Trop Anim Health Prod.

Murphy, J. M. and Hanson, J. J. (1941): A modified Whiteside test for detection of bovine mastitis . Cornell Vet., 31:47-55.

Norris, J.M. and Love, D.N. (2000): Serum antibody responses of cats to soluble whole cell antigens of fline Porphromonas gingivalis. *Vet Microbiol.* 73:37- 49.

O'Keeffe, M.; Crabbe, P.; Salden, M.; Wichers, J.; Van Peteghem, C.; Kohen, F.; Pieraccini, G. and Moneti, G. (2003): Preliminary evaluation of a lateral flow immunoassay device for screening urine samples for the presence of sulphamethazine. J Immunol Meth., 278:117- 126.

Pelerito, A.; Monica, O.; Ana, I. L.; Paulo, R.; Josa, C. and Lurdes, M. (2006): Evaluation of rapid test assures *Helicobacter pylori* for diagnosis of H. pylori in pediatric population. *J. Microbiol. Meth, 66:331-335.*

Petrovski ,K. R., C. Heuer, T. J. Parkinson and N. B. Williamson (2009): The incidence and aetiology of clinical bovine mastitis on 14 farms in Northland, New Zealand. N. Z. Vet J. 57 (2): 109-15.

Premchand, Berhra, G.D. and Chakravarty, A.K. (1995): Comparative incidence of mastitis in relation to certain factors in cattle and buffaloes. *Ind. J. Anim. Sci*., 65: 12-14.

Premchand, Berhra, G.D. and Chakravarty, A.K. (1995): Comparative incidence of mastitis in relation to certain factors in cattle and buffaloes. *Ind. J. Anim. Sci.,* 65: 12-14.

Radostits, O .M, C. C.Gay, K .W .Hinchcliff and P. D .Constable (2007): A text book of the diseases of Cattle, Sheep, Pigs, Goats and Horses. Veterinary Medicine, 10th Ed.

Rysanek, D., M. Zouharova and V. Babak (2009): Monitoring major mastitis pathogens at the population level based on examination of bulk tank milk samples. J. Dairy Res., 76 (1): 117-23.

Sampimon ,O .C., H. W. Barkema, I .M. Berends, J. Sol and T .J. Lam, (2009): Prevalence and herd-level risk factors for intramammary infection with *coagulase-negative staphylococci* in Dutch dairy herds. Vet Microbiol., 16; 134 (1-2):37 - 44.

Schalm, Q.W.; Carroll, E.J. and Jain, N.C. (1971): Bovine mastitis. Lea and Febiger, Philad, USA.

Seema, N.; Vinay, T.; Harpal, S. and Tulsidas G. S. (2010): Colloidal gold probe based rapid immunochromatographic strip assay for cortisol. *Analytica Chimica Acta,* 682: 66 -71

Seo, K.; Peter S. Holt, Henry D. Stone, and Richard K. Gast (2008): Simple and rapid methods for detecting *Salmonella* enteritidis in raw eggs. Biomed. Environ. Sci., 21, 137-143.

Shyu, R.; Tang, S.; Chiao, D. and Hung, Y. (2010): Gold nanoparticle-based lateral flow assay for detection of staphylococcal enterotoxin B. *Food chemistry* 118: 462-466.

Singh, S. Singh, M.; Varuan, V. and kumae, A. (2010): An attempt to develop surface Plasmon resonance based immunosensor for karnalbunt (Tilletia inidica) diagnosis based on the experience of nano-gold based lateral flow immune-dipstick test. *Thin Solid film* 519:1156-1159.

Sithigorngul, P.; Rukpratanporn, S.; Pecharaburanin, N; Suksawat, P.; Longyant, S. and Sithigorngul. W. (2007): A simple and rapid immmunochromatographic test strip for detection of pathogenic isolates of Vibrio harveyi. *J. of Micro. Meth.* 71: 256 -264.

Taponen, S. Salmikivi, L.; Simojoki, H; Koskinen, M.T. and Pyörälä. S. (2009): Real-time polymerase chain reaction-based identification of bacteria in milk samples from bovine clinical mastitis with no growth in conventional culturing. *J. Dairy Sci.* 92:2610-2617.

Tarek E.M. (2006): Clinico-pathogical studies on mastitis in dairy buffaloes and cattle.M.V Sc. thesis Fac. of Vet. Med. Cairo Univ.

Trevisani, L.; Cifala, V.; Fusetti, N.; Gilli, V.; Tombesi, P.; Torchiaro, M.; Boccia, S. and Abbas Ciano, V. (2007): Diagnostic accuracy of a rapid fecal test to confirm *H. pylori* eradication after therapy: Prospective comparison with a laboratory stool test. *World J. Gastroenterol.* 13 (33): 4484 - 4488.

Tufani N A, D. M. Makhdoomi and A. Hafiz (2012): Epidemiology and therapeutic of bovine mastitis. Ind. J. Anim. Res., 2012; 46 (2): 148 - 151.

Vaarst, M. and C. Enevaldsen (1997): Patterns of clinical mastitis manifestation in Danish organic dairy herds. J. Dairy Res. 64 (1): 23-37.

Wiriyachaiporn, S.; Howaeth, P.H.; Bruce, D.K. and Daily, AN. (2013): Evaluation of a rapid lateral flow immunoassay for *Staphylococcus aureus* detection in respiratory samples. *Diag Microbiol and infect Dis.,* 75: 28-36.

Yang, W.; Li, X.; Liu, G.; Zhang, B.; Zhang, Y.; Kong, T.; Tang, J.; Li, D. and Wang, Z. (2011): A colloidal gold prob-based silver enhancement immunochromotographic assay for the rapid detection of abrin-a . *Biosensors and bioelectronics* 26: 3710 -3713.

Zecconi, A.; Casirani, G.; Binda, E. and Piccininni, R. (2002): The importance to assess the effects of voluntary milking system on teat tissues, intramammary infections and somatic cell counts. Dept. Anim. Pathology-Infectious diseases laboratory, Univ. of Milan. Delaval Hygiene, Technology Center, Inaugual Symposium, May 15-16.