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Diagnosis of *Pasteurella Multocida* and *Mannheimia Haemolytica* Infections in Cattle Using Lateral Flow Immunochromatographic Assay

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ABSTRACT

This study was mainly designed to advance and recover the control measures of Bovine respiratory disease (BRD) via accelerating the field identification of some BRD causing pathogens. A simple rapid field test was developed mainly to identify *Pasteurella multocida* and *Mannheimia haemolytica*, both are highly associated with BRD. A full identified *Pasteurella multocida* and *Mannheimia haemolytica* cultures were earned from Microbiology Department, Faculty of Veterinary Medicine, Cairo University, Egypt. Species-specific bacterial antigen of outer membrane protein of each bacterial strain was prepared separately for *M. haemolytica* and *P. multocida.* Specific polyclonal antibodies were prepared by amplifying each one in guinea pigs and rabbits. Pathogens-specific lgG were purified using caprylic acid technique. Pathogens-specific lgG titers were measured by ELISA. The colloidal gold nanoparticles were conjugated to the rabbit mono specific polyclonal lgG antibody and did not conjugate to guinea pig specific polyclonal lgG. The particles were then utilized to develop the Lateral flow kits (LFK) which is a rapid assay for detection for *P. multocida* and *M. haemolytica* in clinical samples when Compared to the conventional Bacteriological tests. Results: Percentage of accuracy, specificity and sensitivity of LFK kits recorded 93, 94.4 and 90.4%, respectively, for *P. multocida* and 91.5, 94.3and 84.7%, respectively, for *Mannheimia haemolytica*.

Key words: Lateral flow kit (LFK), *P. multocida*, *M. haemolytica*, bovine respiratory disease (BRD).

INTRODUCTION

Bovine respiratory disease complex (BRDC) is a well-known disease causing huge economic losses for both cattle feedlot because of high mortality rates, expensive costs of prevention, treatment and low carcass value (Confer 2009; Fulton 2009; Mcvey 2009; Mohiuddin et al. 2022). In North American cattle industry, BRD induced were assessed to be about one billion dollars annually (Molaee 2022; Miles 2009). Two members of the Family Pasteurellaceae, namely, *Pasteurella multocida* and *Mannheimia haemolytica* serve as a main bacterial causative pathogen as cattle respiratory disease, which are highly accompanied by BRD (Rice et al. 2007; Griffin et al. 2010; Imran et al. 2022; Valeris-Chacin 2022).

In general, these bacterial species are commensals in wild and domestic animals like cattle, horses, dogs, cats and also birds. The BRD epidemiology is not understood and is complicated and multifactorial disease. It is believed that stress plays necessary factor to induce BRD in addition to the asymptomatic carriers (Griffin 2010; Taylor et al. 2010; Crosby 2022; Andrés-Lasheras et al. 2021) can overcome host immune system, rapid proliferation of nasopharynx, pathogen replication in the lower part of respiratory tract with lung infections. Concerning *Pasteurella multocida*, it is known by its host range and ability to elicit the disease in different animals other than cattle (Alarawi and Saeed 2021). For example, in pigs, it causes atrophic rhinitis, rabbits snuffles and birds fowl cholera (Boyce et al. 2006; Harper et al. 2006; Pesapane et al. 2022; Akalu et al. 2022).

The Lateral flow immunochromatographic strip tests are rapid tests as it decreases the time spent for results to be just minutes instead of hours which is lost when apply conventional methods. It needs less technical training, no special tools, and has decreased device cost in addition to

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its rapidity and simplicity (Sayed et al. 2019; Abousenna et al. 2020). Moreover, its suitability to qualitative, semi quantitative investigation and mass screenings also the findings are recorded for about 5 to10min by the naked eye (Kong et al. 2017; Xinghua et al. 2022; Sayed et al. 2022).

The objectives of the study: The above mentioned work was designed to conduct a lateral flow immunochromatographic kits to be a simple field rapid test method to detect *Pasteurella multocida* and *Mannheimia haemolytica* for cattle. Accuracy, specificity with sensitivity to the developed kits was determined using bacteriological examination as a master gold test.

MATERIALS AND METHODS

Growth condition for Bacterial strains: *Pasteurella multocida* and *M. haemolytica* reference strains were obtained from Microbiology Department-Faculty of Veterinary Medicine-Cairo University. *P. multocida* and *M. haemolytica* strains identification was confirmed using bacteriological and molecular techniques according to Quinn et al. (2002).

Procedure for outer membrane proteins Preparation: *Pasteurella multocida* with *M. haemolytica* OMPs were prepared according to Roier et al. (2012) with modification of disrupted cells which was homogenization by 0.1mm glass beads and a Power LyzerTM 24 for three cycles of 1min, for 3400rpm were applied for 1min intervals in-between on ice. The concentration of protein of the prepared OMPs was determined using photometric measurements by absorbance at 260nm by DU730 spectrophotometer within a Tray Cell using the equation of Warburg–Christian.

Preparation of specific polyclonal antibodies: Rabbits immunization: Four female New Zealand rabbits at age 9-11 weeks were immunized for preparation of *M. haemolytica* and *P. multocida* specific polyclonal antibodies: All rabbits were acclimated for 1 week before any procedure. Each rabbit was inoculated with OMPs (1000µg/time) at different age (0, 14, 28, 42 and 52) day. OMPs adjuvanted with complete Freund's adjuvant were used in the initial dose and incomplete Freund's adjuvant was used in booster doses whereas the route of initial dose was subcutaneous and for the booster doses intramuscular (Elke et al. 2008).

Guinea pigs immunization and preparation of *P. multocida* and *M. haemolytica* specific polyclonal antibodies: It was done according to Sayed et al. (2022).

Goat anti-rabbits' polyclonal antibodies: It was supplied by (EMD Millipore), Catalogue No. (AP132).

Collection of blood samples from immunized animals and separation of serum: All immunized rabbits and guinea pigs were scarified at two months post immunization and blood was collected for serum separation after centrifugation for 10min at 1500rpm followed by storage at -20ºC.

Purification of rabbit and guinea pig Polyclonal antibodies by Caprylic acid: Twenty-five mL of individual serum samples were centrifuged for 30min at 10000xg then discard the formed pellet to add 0.06M sodium acetate buffer pH 4.6 to Twice serum volume of and placed on a magnetic stirrer. Slowly add 2.02mL of caprylic acid drop wise with 30min stirring at room temperature followed by centrifugation for 20min at 10000xg. Discarded the pellet but keep the supernatant to be retained for dialyzing at 4˚C with PBS buffer overnight with three buffer changes; the final purified IgG was 1.005g/dL (Da Silva et al. 2019; Sayed et al. 2022).

Preparation of Colloidal gold nanoparticles: The Colloidal gold nanoparticles were adjusted to be 40nm (diameter size). Purified water (50mL) containing 0.1%HauCl4 boiled by vigorous stirring then 1mL of 1% (w/v) sodium citrate was rapidly poured until solution colored into red (2min), the mix was extra boiled for 10min then left for cooling. Later, 0.02% (w/v) of sodium azide was poured and the papered nanoparticles diameter was scanned using spectrophotometer of 400 to 600nm. The prepared Colloidal gold was adjusted to be pH8.5 by 0.02M K2CO3. Gentle stirring was done for 0.5mL of the purified rabbit IgG.1mg/mL was mixed by 50mL of adjusted Colloidal gold with gentle mixing for 10min. (20000 1%m/v final conc.) PEG was used for blocking by gentle stirring about 15min with centrifugation for 30min at 10.000g. Suspend the conjugated CG in 1mL diluted buffer (3% (w/v) sucrose, 20m M Tris contain 1% (w/v) BSA and 0.02% (w/v) sodium azide) then kept at 4˚C (Herizchi et al. 2016).

Development and evaluation of lateral flow immunochromatographic kits for rapid detection of *M. haemolytica* and *P. multocida* in clinical samples

Sample pad Preparation: glass fiber was pretreated by the sample pad treated solution of pH8.5 purified water containing 2% (w/v) titronX100, 1%(w/v) PVP, 0.15% (w/v) SDS, 0.5% (w/v) sodium cholate, 0.02% (w/v), 0.1% (w/v) casein sodium salt, 3.81% (w/v) Borax, sodium azide and dried at 37°C (Guo et al. 2015; Shaimaa et al. 2018).

Conjugation pad Preparation: the glass fiber pretreated by conjugation pad- treated solution at pH7.4 (2.5% (w/v) sucrose, 20mM PBS contained 2% (w/v) BSA, 1 % (w/v) triton x100, 0.3% (w/v) PVP and 0.02% (w/v) sodium azide) and dried at 37ºC then stored in dry condition. The pad was saturated by the conjugated CG and dried at 37°C for 1hr then stored in dry condition.

Nitrocellulose membrane (NC) Preparation: dispense 2 lines on Nitrocellulose (25 mm × 300 mm) by Iso flow dispenser. The previously purified bacterial species-specific polyclonal IgG Guinea pig antibodies (1mg/1mL) dispensed around the bottom of test line (1µL/1cm line) and the goat anti-rabbit antibodies (0.5mg/mL) dispensed at the control line upper position as (1µL/1cm line). 5mm was a distance in-between the bottom and upper lines. The loaded Nitrocellulose membrane was dried at 37ºC for 2hrs and left in dry condition.

Treated pad, loaded NC membrane, treated conjugate pad and absorption pad stuck down in PVC card. Later, PVC card was collected and cut into test-strips of 3.9mm width using an automated cutter. Separate Strips for detection of both pathogens were prepared.

Validation and Evaluation of LFT for detection of *Pasteurella multocida* and *Mannheimia haemolytica* bacterial pathogens

It was done according to (Thrusfield 2007) where the clinical samples examined with the prepared LFIK were also examined by bacteriological examination (gold standard test) and the result was compared from which the accuracy, specificity and sensitivity of the conducted kits were determined. One hundred nasal swab samples collected from suspected cattle in cattle farms were included in the study.

RESULTS

Accuracy, Sensitivity and specificity of LFIT in detection of *P. multocida* as compared with bacteriological examination

Duplicated nasal swab from each cattle were collected from 200 cattle showing respiratory distress. These samples were examined using both the developed LFIT and conventional bacteriological method. By comparing and analysis of the obtained results, specificity, sensitivity accuracy was determined Table 1. When compare LFIT to bacteriological investigation, the sensitivity, specificity and accuracy test were 90.4, 94.4 and 93%, respectively. The least number of *P. multocida* bacteria that could be detected by the developed kits reached 10^3 bacteria/mL (Fig. 1). Also Fig. 2 shows the specificity of the developed kits.

Accuracy, Sensitivity and specificity of LFIT to detect M. haemolytica as compared with bacteriological examination

As shown in Table 2, Fig. 3 and Fig. 4, the examination of 200 nasal swaps from BRD clinically suspected cattle by the developed M. haemolytica LFIT as compared to bacteriological examination the accuracy, sensitivity and specificity, of the kits reached to 84.7, 94.3 and 91.5%, respectively.

DISCUSSION

Bovine respiratory disease complex is known as a main cause of economic losses, especially in countries where the cattle industry is continuing to intensify and shielding of animal is applied. *P. multocida* and *M. haemolytica* are usually responsible for economic losses in cattle industry represented as cattle death and huge loss in meat and milk production. The standard diagnostic method of both *P. multocida* and *M. haemolytica* should have the ability to detect these pathogens in the clinically suspected animals in the shortest possible time. Also, it should be inexpensive, sensitive, simple and specific. Moreover, it has to be applicable in field and laboratory also can be easily applied on large samples scale. Therefore, the main target of the present study was the development and application of the LFK as a detection method for *Pasteurella multocida* and *Mannheimia haemolytica* of clinical specimens for suspected cattle.

Table 1: Validation results of LFIT for detection of *P. multocida*

New Diagnostic Test (LFIT)	Gold Standard Test (Bacteriological Examination)			Sensitivity test	Specificity test	Accuracy test
	Diseased (+)	Non- Diseased (-)	Total			
Positive (+)	66(T+)	7 (F+)	73			
Negative (-)	7(F-)	120 (T-)	127	90.4%	94.4 %	93%
Total	73	127	200			

True positive (LFI + Gold standard test +); false positive (LFI+ Gold standard test −); true negative (LFI − Gold standard test −); false negative (LFI − Gold standard test +).

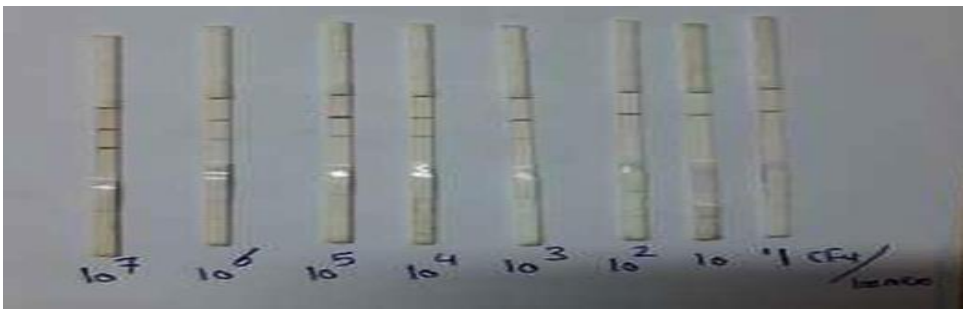

Fig. 1: The minimal count of *P. multocida* that gives positive results in LFIT (10^3 CFU/0.1mL).

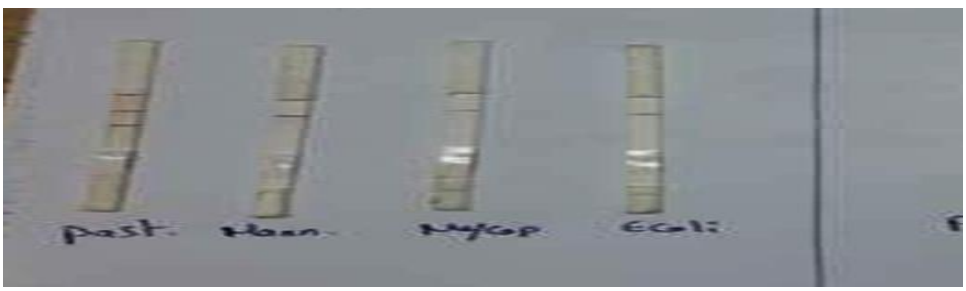

Fig. 2: LFT gives positive result against *P. multocida* and negative results against M. haemolytica, Mycoplasma gallisepticum and E. coli.

Table 2: Validation results of LFT for detection of *Mannheimia haemolytica*

New Diagnostic test (LFT)	Gold Standard Test (Bacteriological Examination)			Sensitivity test	Specificity test	Accuracy test
	Diseased (+)	Non Diseased (-)	Total			
Positive (+)	50(T+)	8 (F+)	58			
Negative (-)	9(F-)	133 (T-)	142	84.7%	94.3%	91.5%
Total	59	141	200			

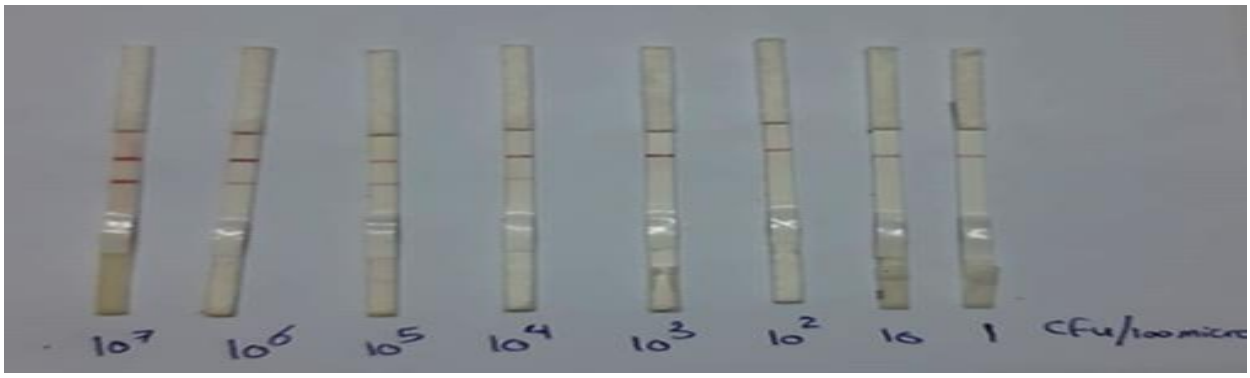

Fig. 3: The minimal *Mannheimia haemolytica* bacterial count that gives positive results in LFT was (10^4CFU/0.1mL).

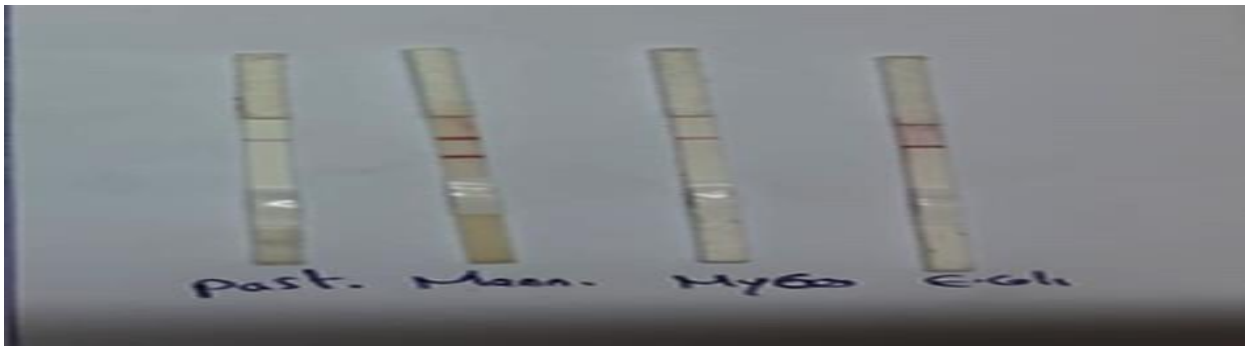

Fig. 4: LFT gave positive result with *Mannheimia haemolytica* and negative results with *P. multocida*, *Mycoplasma Gallisepticum* and *E. coli*.

The LFK is low- cost tests, fast, do not need qualified persons, easily applied and results in accurate results within only 5 min. Moreover; it is very helpful for screening of large cattle flocks for detection of *M. haemolytica* and *P. multocida*. Sensitivity or minimal amount of *P. multocida* detected by the developed LFK was 10^3 CFU/0.1mL. While the minimal count of *M. haemolytica* that gave positive results in LFK was 10^4CFU/0.1mL (figure 3). It was recorded a sensitivity of 10 CFU/mL for leptospira by lateral flow devices (Chirathaworn et al. 2011). Whereas it was demonstrated that the developed lateral flow immunochromatographic was sensitive for detection of S. aureus for bronchoalveolar lavage samples were 10^6 CFU/0.mL (Wiriyachaiporn et al. 2013). It was showed that the sensitivity of Listeria monocytogenes was 10 CFU/25µl in dairy products by using lateral flow Devices (Blaskoza et al. 2009). furthermore it was mentioned that the lateral flow devices sensitivity for E. coli O157: H7 bovine feces reached 10^5CFU/g (Jung et al. 2005). Also, another study was investigated an increase in LFD strips sensitivity for detection of Vibrio harveyi of 1–10CFU/mL in the tested sample (Sithigorngul et al. 2007; Kumar et al. 2022; Huda et al. 2022). The prepared *P. multocida* LFK when compared to conventional bacteriological tests were analyzed and were recorded as sensitivity, specificity and accuracy test were 90.4, 94.4 and 93%, respectively, while M. haemolytica kits showed 84.7, 94, and 91.5%, respectively. Interestingly, it was reported that the sensitivity and specificity of lateral flow device was 93 and 95%. When applied to detect Candida albicans in samples of woman vagina compared to fungus isolation (Dan et al. 2010). On the other hand, it was reported the sensitivity, specificity and accuracy rates of LFD as 90.6, 95.8 and 94.0%, respectively. When used to detect Helicobacter pylori of infected children. Comparing the old conventional *P. multocida* and *M. haemolytica* diagnostic approach (Kato et al. 2004) the new developed LFK is not only a fast as it takes just 5 min, but also is very easy, applicable, with long shelf time and able to be applied by unqualified non-skilled personnel in cattle farms with no need for any additional tools or equipment like Polymerase Chain Reaction or bacteriological tests. Additionally, this technology is highly appreciated and supported for future improvement of its sensitivity and specificity.

Conclusion

The developed LFK test is an alternative method for the currently used laboratory methods, especially PCR

and bacteriological tests. It provides a rapid, easy and on-site diagnosis for BRD.

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Author Contributions

Conceptualization: RH Sayed, R Soliman, MS Abousenna. Data curation: RH Sayed, SA Elsaady. Formal analysis: RH Sayed, R Soliman, MS Abousenna. Investigation: RH Sayed, R Soliman, MS Abousenna Methodology: RH Sayed, MS Abousenna, SA Elsaady, FA Shasha, H Mahmoud, SM Saber, HM Soliman, AA Mohamed Supervision: RH Sayed, R Soliman, MS Abousenna. Validation: H Mahmoud, SM Saber, HM Soliman, AA Mohamed Visualization: RH Sayed, MS Abousenna, SA Elsaady, FA Shasha, Writing – original draft: RH Sayed, MS Abousenna, H Mahmoud. Writing-review and editing: RH Sayed, MS Abousenna, H Mahmoud.

Conflicts of Interest

The authors certify that he has no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

Ethical Statement

All methods in the study were performed according to relevant guidelines and regulations. All experiments were carried according to ARRIVE 2.0 guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC) in the Faculty of Veterinary Medicine, Cairo University under the code (VetCU01102020217).

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