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Development of Solid – Based Paper Strips for Rapid Diagnosis of Pseudorabies Infection

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ABSTRACT

Pseudorabies (Aujeszky's disease) is an economically significant disease of swine known to cause central nervous disorders, respiratory disease, reproductive failure and mortality in infected pigs. In attempts to eradicate the disease from becoming endemic, early detection is important to prevent further economic losses and to allow for detection and removal of infected pigs in domestic herds. Thus, a rapid and sensitive technique is necessary for the detection of the virus. For rapid and simple examination, an immuno – chromatographic lateral – flow assay system based on immunologic recognition of specific pseudorabies virus antigen was developed by utilising, as signal generator, colloidal gold conjugated to secondary antibody to detect primary or sample antibody in the sera of pseudorabies infected animals. The pseudorabies virus used as a capture antigen in the test strip was first cultivated in VERO cell culture and then purified by sucrose gradient separation to produce the viral protein concentration of 3.8 mg/ml. The standard pseudorabies antigens reacted well with the hyperimmune serum (HIS). The antibody detection system is basically composed of colloidal gold – labelled antibodies fixed on a conjugate

pad, and the complementary pseudorabies antigen immobilised onto a nitrocellulose membrane forming capture zone. If the target antibody is present in a specimen, the colloidal gold-labelled antibody will form a complex with the antibody sample. Subsequently, the formed complex will migrate to the capture zone and is then bound to the solid phase via antigen – antibody interaction. As a result, a signal marker is generated by the accumulation of colloidal gold for detection confirmation. The results obtained demonstrated that the optimum combination of pseudorabies antigen needed as the capture reagent and gold conjugate as secondary antibody recognition marker was at a concentration of 0.38mg/ml and at 1:10 dilution factor respectively. The sensitivity of the solid – based test strip towards pseudorabies antibodies was high with a detection limit of 1 to 10,000 – dilution factor. The specificity of the assay was 100% with no cross – reaction being observed with other sera or antibodies. Accurate reading time needed for confirmation of the assay can be completed in 5 min with a whole blood sample of 25 µl. The colloidal gold – labelled antibody is stable at room temperature for 6 months or more (data not shown). Findings from this study indicated that the solid – based test strip assay system provided high sensitivity and specificity for the detection of pseudorabies at low levels of antibody concentration. The assay was rapid, simple, cheap, and does not require any sophisticated equipment. Thus, the solid based test strip will be a useful serological screening technique or for rapid diagnosis of an infectious disease in target populations of animals characterised by heterogeneous antibody responses.

INTRODUCTION

Pseudorabies (Aujeszky's disease) is a highly contagious, widespread and economically significant disease of swine characterised by a range of clinical signs, including central nervous system disorders, respiratory diseases, reproductive failure and death, depending on age, reproductive status and immune status of the infected swine (Kluge *et al*., 1998). In geographic areas where pseudorabies virus (PRV) infection is enzootic, control and eradication programs often employ the use of vaccines and diagnostic tools as aids in the attempt to control the economic losses. However, attempts for total eradication of the virus are still unsuccessful as carrier animals spread the infective virus without showing any signs of the disease themselves. In Malaysia, despite vaccination, outbreak of the disease has been reported frequently in different parts of the country (Too, 1995). Due to the expanding population, the demand and consumption of swine products are expected to increase substantially. The disease has emerged to be one of the most important problems affecting the swine industries and serious steps must be taken to prevent severe losses. Rapid and accurate diagnosis of pseudorabies infection would definitely pave for a higher effectiveness in eradication of the disease. As an alternative to the use of current diagnostic tools, the immuno – chromatography test strip assay has become a new approach for detecting many veterinary diseases. With the advent of immuno – chromatography based techniques, numerous reports describing the advantages and functions in diagnosing diseases were published attesting to the perceived importance of this new diagnostic tool (Eliades *et al*., 1998; Kim & Choi, 2000).

Thus, the employment of immuno – chromatographic test strip will provide an easy mean for detection of pseudorabies virus. The use of labelling substances like gold and the immobilisation of biological components (antigen / antibody) makes it possible to facilitate a convenient and relatively inexpensive approach to obtain rapid analytical results due to the elimination of washing steps and faster antigen – antibody interaction. We attempted to develop a highly sensitive and rapid solid based paper strip assay, using colloidal gold as an indicator, for the detection of antibodies in serum specimen. For this approach, pseudorabies antigen and antibodies were prepared and screened out for their application to immunoassay system. In this paper, we describe the different characteristics of developing the immunoassay system and compare the detection sensitivity together with specificity of the developed solid based paper strip assay with that of ELISA screening test kit.

MATERIALS AND METHODS

Antigen Preparation

VERO (African green monkey kidney cells, ATCC, CCL81) were infected with pseudorabies virus (10^6 p.f.u./cells) and grown in Leibovitz – 15 medium (L15M) supplemented with 1% (v/v) antibiotic – antimycotic (1000U/ml penicillin, 1000mg/ml streptomycin), 5% (v/v) foetal bovine serum (FBS). The infected cells were collected 72 h after infection and viruses were purified by sucrose gradient centrifugation. Virus suspension (1.0 ml) was layered on top of

10 – 60% sucrose solution (in TNE buffer) linear density gradient and was ultra – centrifuged a 40,000 rpm for 2 h at 4 ºC (Beckman, Ultra centrifuge LB – 70M SW 41 rotor). The virus pellet obtained was inactivated by incubation of the virus suspension at 56 ºC for 30 min and used as an antigen in test strip immobilization.

Mouse Hyper Immune Sera

Mice were immunized with 1 mg of inactivated pseudorabies virus in Freund's complete adjuvant (FCA) by multiple intramuscular injections into the muscle of the hind leg. The mice were given a second boost 2 weeks later. A week later the serum of the immunized mouse was tested by ELISA. When the titre of anti – pseudorabies in the serum is reached more than 1/5000, the serum of the immunized rabbit collected.

Pseudorabies Antigen Immobilization

Approximately 5 µl of pseudorabies antigen and control line using mouse sera was immobilized by the use of Pasteur pipettes with minor modification to 'direct blotting' simply onto the desired area of nitrocellulose membrane (5 x 25 mm^2) in a 1 mm wide band 15 mm from the bottom of the sheet. The antigen-coated membranes were air dried for hydration under desiccated temperature in (VWR Scientific Model 2100 CO_2 Incubator) incubator at 37 ºC for 1 h. After hydration period, the membrane strip was immersed in the blocking agent [1.0% (w/v) skim milk, ddH_2O] for 10 min with gentle agitation followed by washing

with washing buffer [5 mM Na_2PO_4, 0.01% (w/v) SDS, ddH_2O] and air dried under desiccated temperature before use.

Preparation of Pseudorabies Solid – based Test Strip

Colloidal gold conjugate solutions (Arista Biological Inc., US) were prepared using diluents (0.01M KH_2PO_4 / K_2HPO_4, 0.15 NaCl and 0.01% NaN_3 in PBS, pH 7.4). Untreated glass – fibre membrane conjugate pads were cut in a size of 5 x 5 mm^2 and saturated in the conjugate solutions, before being air – dried at 37°C for 2 h. The sample pad (5 x 15mm^2), which affects the migration of gold – antibody, was treated with sample pad buffer containing 0.02% (v/v) Tween – 20, 0.5% (w/v) skim milk 0.05% (w/v) SDS and completely air – dried. Absorption pad (5 x 15mm^2) used had no treatment. The pre – treated sample pad, colloidal – gold antibody saturated conjugate pad, antigen – coated nitrocellulose membrane and absorption pad were assembled and attached to a plastic backing.

Test Strip Assay Procedure

A portion (min. 25 µl) of serum sample was transferred directly onto the sample pad. The immuno – strips were incubated laterally for 5 minutes, and the sera solutions absorbed from the sample padding of the strips. The medium immediately dissolved the gold conjugate, the reaction between the conjugate and antibody took place, and this complex was carried forward into the nitrocellulose membrane containing the immobilised binder. The second antigen – antibody interaction takes place forming an immune complex at the solid surface and the

unbound reagent was subsequently separated by the flow of the medium and washing. When two purple lines were observed after incubation at test line and the control line, sample evaluation was defined as positive. In the case of observation of only one purple line at the control line, sample evaluation was defined as negative.

Detection and Quantitation

Responses of the system at the capture zone were determined by the presence of a band measured optically. The intensity of band discoloration was determined by using BioRad Gel Doc Imaging System with Quantity One® Quantitation software. This result allows the intensity be read using optical density (OD) of 255 nm and converted into percentage of relative intensity in OD compared with the control, that is, assigning a value of 100% to the control.

RESULTS

Blocking Agents for the Nitrocellulose Membranes

Initial testing involved the use of varying concentrations of different blocking agents prepared in deionised and distilled water (ddH_2O) and Phosphate buffered saline (PBS). Blocking agents involving non – fat skim milk, skim milk, Bovine serum albumin (BSA) and gelatin were tested at 0.1, 0.5, 1, 5 and 10%. Tween – 20 was tested at 0.002, 0.01, 0.02, 0.05, 0.1 and 0.5%. Among the blocking agents tested (Figures 1 and 2), skim milk with 1.0% concentration diluted in ddH_2O, provided the best protection from dust and blockage from non

– specific binding without interfering the signal intensity as indicated with the highest signal obtained the antigen capture zone. The use of saline solution causes losses in signal intensity in comparison to that of ddH_2O. Listed in order of decreasing effectiveness in preservation of nitrocellulose and dehydrated antigens are the blocking agents at their individual optimal concentrations and the respective signal intensity: 1.0% skim milk (100% intensity), 0.5% non – fat skim milk (80% intensity), 0.5% BSA (70% intensity), 0.1% gelatin (50% intensity) and 0.02% Tween – 20 (30% intensity). The use of unblocked membrane and blocking agents in other concentrations resulted in incomplete capillary flow of HIS and gold conjugates across the membrane and becomes embedded in the nitrocellulose membrane.

Effects of Drying Time of Blocked Membranes on Recovery of Gold Conjugates

Drying of the blocked membranes using the Mar Equipment Costech 400W desiccated dryer for more than 15 min (60 ºC) and overnight in an incubator (37 ºC) resulted in very dry membranes. During the migration of the mobile phase it was observed that the liquid migrated preferentially along the strip edges, which reduced the chances of gold, conjugated HIS binding in the capture zone (Figure 3A). By decreasing the drying time and the temperature of the blocked membranes to 2 h at 37 ºC, the carrier solution migrated more rapidly and uniformly through the capture zone and the signal intensity of the antigen was improved (Figure 3B).

Effects of Antigen Concentration as Capture Reagent

The concentration of antigen has a dramatic effect on the specificity and sensitivity of the test strip assay. As seen from Figure 4, the highest signal intensity obtained (OD) was seen to be with undiluted antigen using gold particles conjugates. The intensity signals derived from the test strip are no significantly different from dilution of 1:1 – 100 µl (3.80 mg – 38.0 µg/ml) tested with undiluted positive sera. Relatively, the signal intensity of the test strip decreased as the concentration of both antigen and sera decreased. From the dilution, a cut off point of antigen concentration was determined to be at the ratio 1: 100 (38.0 µg/ml) with a final intensity signal of 8.5% OD of 255 nm similar to the results tested with negative sera. Dilution of antigen greater than 1:100 dilution factor failed to provide desired result. Therefore, in order for sufficient consideration of any positive results, at dilution of 1:10 (0.38 mg/ml), minimum value was determined with a relative signal intensity of 41.7% OD of 255 nm, as tested with 1:5000 sera dilution.

Effects of Antigen Purity as Capture Reagent

Results as in Table 1 demonstrated that the signal intensity generated in relative to control strip produced the descending flow of optical density, with the control strip marked as 100% followed by test strip immobilised with virus after sucrose gradient purification (97.51%), virus sample after sonication (58.69%) and finally virus sample after virus cultivation (41.70%). Test strip performed with VERO tissue culture gave no signal and rendered as negative control for non – specific binding. The test strips experimented with samples obtained before the

final sucrose gradient preparation produced intensity signals of less intensity as compared to that of the sample virus taken after further purification.

Determination of Optimal Concentration of Conjugate

As shown in Figure 5, OD relative to control strip (100%) decreased with dilution of hyper immune serum (1:1/ 100.00%, 1:5/ 93.20%, 1:10/89.35% and 1:50/80.89%). At dilution less than 1:50, OD changed rapidly in signal intensity in a declining manner with increasing sera dilution. Dilution beyond 1:50 provided no signal intensity and was classified as negative result similar to that produced by test strip immobilised with VERO tissue culture used as negative control. From the result obtained, a dilution of 1 to 10 was chosen which represent a significant amount to produce efficient signal generation due to low variables between the signal generated from the higher gold conjugate concentration and still able to provide rapid washing.

Sensitivity of Solid Based Test Strip for the Detection of Pseudorabies Antibody

Sensitivity of the test strip was compared with that of a conventional ELISA pseudorabies screening kit (Herdchek, IDEXX, USA) for the detection of pseudorabies antibody. The conventional ELISA test kit was able to detect HIS dilution factor of 1:10,000 but not at a dilution factor beyond 1:10,000 producing reading S/P ratio less than predetermined cut – off value of 0.4. Similar to that of the ELISA screening test kit, the solid based test strip was able to detect HIS sera at a maximum dilution of 1:10000 at a %OD of 255 nm of (35.36%) signal

intensity (Table 2). Dilution beyond 1:10,000 gave signal intensity that was considered too low for result confirmation. Therefore, the pseudorabies antibody detection cut – off value was determined to be at a minimum of OD 30% to ensure for absolute distinguishable result. Figures 6A and 6B shows the examples of positive and negative detection using the solid based test strip. Positive result was determined by the generation of signal band at the test line (capture zone) and control line at any intensity level. Negative result was determined with the generation of signal band at the control line with the absence of signal band in test line.

Specificity Determination

The specificity of the solid based test strip was tested by performing different trial run with antibodies obtained against infectious bursa disease virus (IBDV), rat cytomegalovirus (RCMV), chicken anaemia virus (CAV), VERO cell culture and uninfected mouse serum. As illustrated in Figure 7, all antibodies run against pseudorabies immobilised antigen did not show any signal of reactivity in comparison to that of the control test strip. This indicated that the pseudorabies antigen did not cross react with other common viral antibodies and thus it was highly specific to pseudorabies.

Sample Amount Determination

The performance of the test strip measurement system was examined by challenging the immuno assay system with different blood volumes (1 µl to 200 µl) and measuring the effects of serum separated from whole blood on the

production of band intensity. Figure 8 shows that sample amount of 50 – 100 µl whole blood was able to saturate the system. Sample amount beyond 100 µl resulted in constant sera recovery. Excess amount challenged with higher volumes (5.0 ml) produced similar performance, suggesting that there is no practical upper limit to sample volume. The minimum amount of sample volume needed to produce a valid result was seen as 25 µl, however, in this situation the sample amount would depend on the viscosity of the whole blood sample solution.

Reading Time Evaluation

To test the influence of reading time on the results, test strip was read minute by minute 5 min before and after the reading time proposed (5 min). The developed test strip was found to be influenced by the reading time of the test. Reading result 1 to 2 min earlier than expected greatly reduced the signal intensity of the band showed in the capture zone. Reading results after 1 to 2 min of the proposed time gave no significant changes to the signal intensity obtained suggesting colour stability of the gold conjugate. However, after prolonged period (1 – 2 h) the intensity of the signal generated began to decrease to an amount of less than those obtained earlier. No further decolourisation was observed after advance storage of the used test strip.

DISCUSSION

The function of the pseudorabies detection test strip depends on numerous factors including membrane flow rate, capture reagent (antigen), gold conjugate

and the amount of sample tested. Altering one of the above significantly changes the parameters of other factors. Thus, equilibrium is needed to obtain a functioning system. Solid phase antigen – antibody (Ag/ Ab) binding methods like the western blotting requires optimum Ag/Ab concentration for both element to bind at state of thermodynamic in equilibrium (Capitan – Vallvey e*t al.*, 2002). The design of rapid test strip on the other hand requires kinetic at a unequilibrium state where reactions taken place are not in equilibrium (Zarakolu *et al*., 2002). In this application, the primary parameter that controls the final result is the capillary flow rate. The slower the membrane flow rate, the higher the sensitivity. Further assessment of these factors and modifying the parameters were then executed to accommodate the required objectives.

At the current study, the kinetic at unequilibrium state formula for measurement of the test strip was given as R = k [Ag][Ab], where R is the reaction rate and k being the constant affinity between the antigen and antibody. When the flow rate doubled (for faster detection time) the amount of antigen and antibody concentration needed to produce similar results in generating signal intensity compared to slower flow rate increases by four fold. The reason why effective concentration of Ag and Ab increases with the increase in flow rate is that each component binding capabilities to have close proximity contact time decreases and therefore, higher amount of both components will be needed in concentration to generate the same intensity signal to compensate for the short exposure period. In this study, a high flow rate was chosen and fixed as a constant at 120 seconds for a distance of 4 cm. Thus, the determination of pseudorabies antigen was then evaluated in different concentrations to produce an

effective amount in detecting the complementary antibody. At the original stock concentration of 3.80 mg/ml, test strip was able to detect positive sera even at a serial dilution of 1:10000. Similar to other detection methods, as the dilution of the antigen protein increases, the amount of sera dilution detected will decrease (Figure 5). At a concentration from 3.80 mg/ml to 0.38 mg/ml, the declining manner in relation with different concentration showed no significant changes or sudden drop at the beginning of the serum dilution. The amount of antigen being reacted with the antibody amount in the positive sera was less than the total amount of proteins immobilised. Therefore, the decrease in protein amount in the serial dilution has not much effect in the generation of signal intensity. As the serum dilution increases however, the decrease in protein amount usage was clearly shown to have an effect in signal intensity with the lowering in signal intensity especially when low concentration of capture protein was used (76.0 µg/ml and 38.0 µg/ml) with the failure of the test strip to detect serum sample at a dilution of 1:5,000 at 38.0 µg/ml capture reagent. At a low concentration of antibody in the sample (1:1,000 to 1:5,000) the exposure of both components to each other did not generate complete binding of the entire antibody volume available due to short incubation period of flow rate and thus creating lower signal intensity level. The antigen concentration immobilised is proportionate to the amount of antibody detected. A concentration of 0.38 mg/ml (1:10 dilution) was chosen to be the suitable amount of protein used as capture reagent concentration.

Other than antigen concentration, gold conjugates used as recognition signals in the test strip was found to have an effect on the sensitivity detection of

pseudorabies antibody. In accordance to that of the antigen concentration, the concentration of the gold conjugates is also proportionate to the antibody detected with the same flow rate. The evaluation of gold conjugates optimisation shares similar results as the antigen optimisation; where undiluted gold detection marker provided higher signal intensity as compared to diluted ones (Figure 6). Antibodies in the sera samples and the gold conjugates interacts in a kinetic unequilibrium state which makes the time of exposure to each other essential in performing a complete binding. Due to the rapid flow rate, the concentration of the gold conjugate will have to be high in order to be sufficient for antibodies to be bound. Even in equilibrium state, the binding of the secondary and the primary antibodies to be completed is not instantaneous. The amount of incubation period needed is important for the two antibodies to form a complex. As the incubation period increases, the higher the chances of binding occur. In test strip detection, the format of kinetic state was used to elevate the time factor, thus in order for a high percentage of binding between the two antibodies to occur, the amount of gold conjugates will have to be high enough for higher exposure to primary antibodies to step in place for proper binding (Shyu *et al.*, 2002). A concentration of 1:10 dilution (~10^6 gold particles) was therefore chosen as the suitable gold conjugates concentration.

For the purpose of the prototype test strip preparation, the membrane parameters were investigated to ensure accurate and workable system was produced. At first, the blocking element for the membrane was tested as described in results (Figures 2 and 3). Skim milk at 1.0% provided the optimum blockage with no interference to the signal generated and low to none in non –

specific binding. Skim milk were able to enter the membrane pores and absorbed strongly enabling the formation of coating in preventing further immobilisation with ease of washing off excess skim milk after the blocking period. In addition, skim milk blocking agent has a lower viscosity compared to BSA, gelatin and Tween – 20, where high viscosity was discovered to generate the blockage of the membrane pores to rendering the flow rate of the solution migration. Blocking agents with high viscosity were also difficult to wash, as they were embedded into the membrane. High concentrations (>0.5% for BSA, skim milk, non – fat skim milk and gelatin; >0.05% for Tween - 20) of blocking agents were found to have the same effects as high viscosity in preventing uniform migration of sample solution to capture zone for complete detection. After blocking, the membrane of the test strip was then subjected to drying by several conditions, and found that the drying conditions used other than 37 °C for 2 h, created uneven migration of solution along the membrane (Figure 4A). One of the possible reasons is that the long hours of incubation (>3 h) or drying at high temperatures (>40 °C) resulted in very dry membrane that changes the electrostatic forces of the membrane. If this happens, the membrane builds up a static charge that will repel or deflect the reagents applied to the membrane, not to mention attraction of dirt particles and dust (Martorell *et al.*, 1999).

The use of buffers, detergents and alcohols in the treatment of the membrane for rapid diagnosis utilising membranes are considered natural and a necessity for long-term storage and optimised conditions of the reagents (i.e. capture protein, gold conjugates and sample). Considerable benefits in overall product performance may be obtained by making minor modifications in the

series mentioned above, which improves the performance of the system including assay sensitivity, reproducibility and stability. Vice versa, irregular usage of these reagents may also counter the effects of the test strip disabling its actual purpose. The usage of selective buffers, i.e. phosphate, tris, sodium chloride and glycine solutions often generate negative effects to the performance of the assay system. Glycine and tris buffers often contain amine groups, which has a tendency to interact between acidic residues of capture reagent and samples. This may have a deleterious effect on the capture reagent's ability to bind antibodies. Therefore, it was investigated that the use of buffers in the preparation of test strip was kept to a minimum with the dilution of most reagent solutions with deionised and distilled water as replacement. TNE buffer was only used in the storage and during the immobilisation of pseudorabies antigen at a low concentration. Salt containing solutions and alcohols like ethanol and methanol were able to change the membrane conditions from neutral to hydrophobic and solubilise the nitrocellulose membrane making uneven pore sizes respectively, thus was avoided in during treatment. Detergents like SDS were used at a low concentration (0.05%) to promote rewetting of the membrane and rehydration of the gold conjugates for uniform migration and was only treated at the sample and conjugate padding. Detergents are normally discouraged especially at high concentrations (>0.1%) as they are extremely effective wetting agents that would detach immobilised proteins and decreases assay sensitivity.

In the present work, the development of solid based test strip was described, an immunological assay system for antibody detection that entails the application of antigen onto nitrocellulose membrane and usage of gold conjugates

as recognition signal. In test strip, immobilised pseudorabies antigen retains the serological activity of its components. Thus, the test strip is well suited for developing serological assays for rapid diagnosis and detection of infected animals and other infectious diseases characterised by heterogeneous antibody responses (Lyahchenko *et al*., 2000).

The present investigation described a method for rapid detection in laboratory rather than actual field implementation. The established solid based test strip is both specific and sensitive. The high specificity of the method is achieved by utilising function of antigen – antibody recognition, where compliments of these two components showed high affinity against one another. Pseudorabies proteins used as the capture antigen by binding compliment pseudorabies antibody was highly purified to ensure any unwanted contaminants deriving from foreign proteins, cells and potential reactive substances. After assembly, the test strip was able to detect positive sera to pseudorabies and giving no immunogenic response to other antibodies tested (Figure 8), indicating to be highly specific towards pseudorabies antibodies. The test strip developed in this work proved to have the same sensitivity as the ELISA pseudorabies antibody screening test kit. Both detection methods were able to detect the positive sera up to 1:10000 serial dilutions. The minimum amount of whole blood sample needed was 25 µl to produce acceptable signal for result detection. Colourimetric detection of pseudorabies antibody by test strip was comparatively much more applicable for routine usage due to its rapid and simple to carry out capacity, taking just 5 min and not requiring any special equipment which enables simultaneous processing of bulk samples. The test strip could be stored at room

temperature for at least 6 months or more and the kit could be delivered and used at ambient temperature (data not shown). Another advantage is cost, where the gold conjugate used is considerably less expensive than enzyme conjugates or fluorescent conjugates with easy handling and simple to perform. For the reasons mentioned, the test strip lends itself better for making rapid large scale screening of field populations for Aujeszky's disease. The solid based test strip pseudorabies detection has an immediate application as a serodiagnosis tool to screen suspected field samples of pseudorabies infection. Once positive samples are identified, they can be processed further towards virus isolation and pathotype identification by laboratory methods. Thus, this will significantly reduce the usage of materials and labour help, as only those with pseudorabies positive samples tested would be processed further. This established method however, is not without its limitation. The test strips currently developed are unable to distinguish between vaccinated antibody and the field antibody, thus, it is not possible to differentiate the vaccinated and infected animals. Nevertheless, the differentiation of the animal stocks can be easily achieved by using recombinant glycoprotein antigens as capture reagent; for current vaccines used are glycoprotein deleted. On the whole, the solid based test strip is versatile and can be adapted for fast detection of other diseases which could be an advantage in the eradication with the help of early detection. By switching the antibodies or antigen and making small adjustments to the chemistry of the strip format, the same test design may be used for many applications.

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Legends for Figures

Fig. 1: Effects of Non – fat skim milk, skim milk, BSA and gelatin concentrations in blocking reagent on the recovery of gold conjugates dehydrated on the NC strips expressed as intensity signals of the pseudorabies antigen band produced by positive samples. NC strips incorporating 0.38 mg/ml with 1:10 gold labelled conjugates. Amounts of concentrations tested: 0.1%, 0.5%, 1.0%, 5.0%, and 10.0%

Fig. 2: Effects of Tween – 20 concentrations in blocking reagent on the recovery of gold conjugates dehydrated on the NC strips expressed as intensity signals of the pseudorabies antigen band produced by positive samples. NC strips incorporating 0.38 mg/ml with 1:10 gold labelled conjugates. Amounts of concentrations tested: 0.002%, 0.01%, 0.02%, 0.05%, 0.1%, and 0.5%

Fig. 3: Migration of carrier solution by capillary action. Different drying conditions in both incubation and temperature cause uneven migration of solution through the test strips (A). With some adjustments done, a uniform and even capillary action may be observed to provide better exposure of signal markers and antibodies to the capture zone (B)

Fig. 4: Optimisation of pseudorabies antigen. Samples of different antigen dilutions (1:1, 1:5, 1:10, 1:50 and 1:100 which corresponded to protein concentration of 3.80 mg/ml, 0.76 mg/ml, 0.38 mg/ml, 76.0 µg/ml and 38.0 µg/ml respectively) were reacted with positive and negative sera with 1:10 gold labelled conjugates. The results were plotted against the optical density at wavelength 255 nm.

Fig. 5: Optimisation of gold conjugate. Different conjugate dilutions (1:1, 1:5, 1:10, and 1:50) were reacted with positive and negative sera on test strip immobilised with 0.38 mg/ml pseudorabies antigen. The results were plotted against the optical density at wavelength 255 nm

Fig. 6: Examples of positive (A) and negative tests (B) for pseudorabies disease detection using solid based test strip

Fig. 7: Cross reactivity test performance with various antibodies. Capture zone was immobilised with 0.38 mg/ml pseudorabies antigen with dilution of 1:10 gold conjugate as detection marker. Antibodies used were diluted according to specified procedures and was tested with antigen counterpart for confirmation

Fig. 8: Sample amount determination. Capture zone was immobilised with 0.38 mg/ml pseudorabies antigen with dilution of 1:10 gold conjugate as detection marker. The results were plotted against the optical density at wavelength 255 nm

Figures:

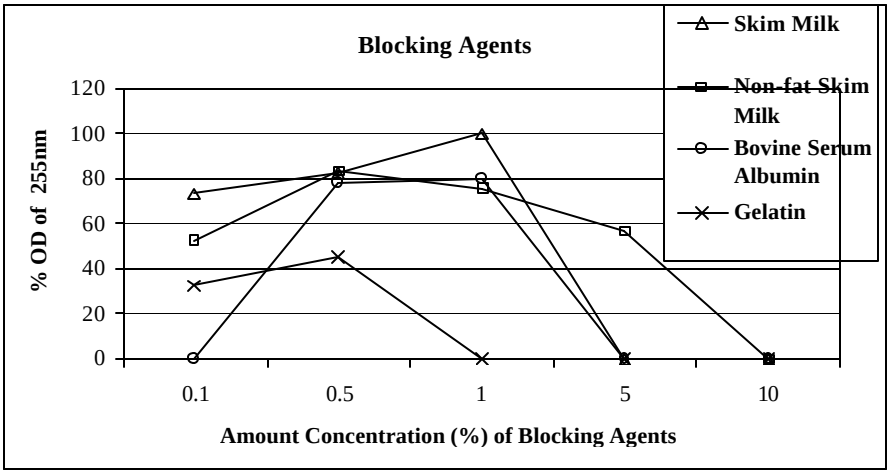

Fig. 1

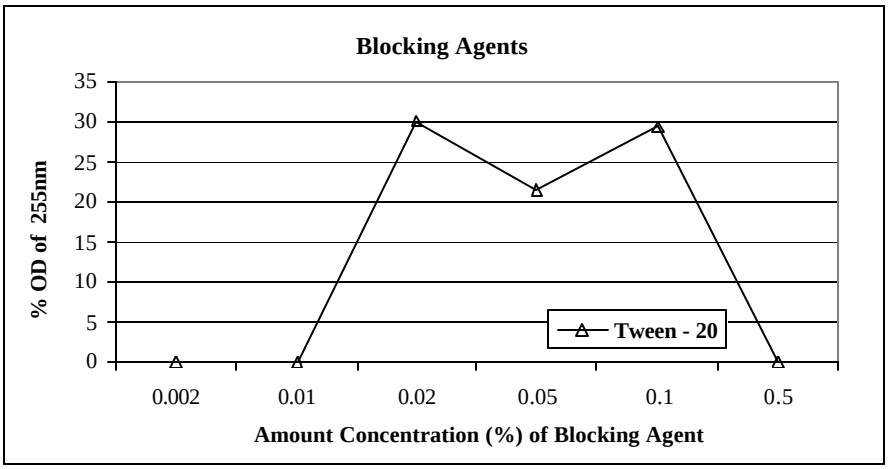

Fig. 2

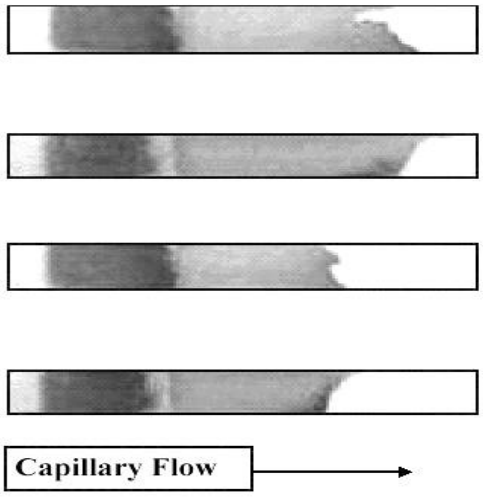

Fig. 3A

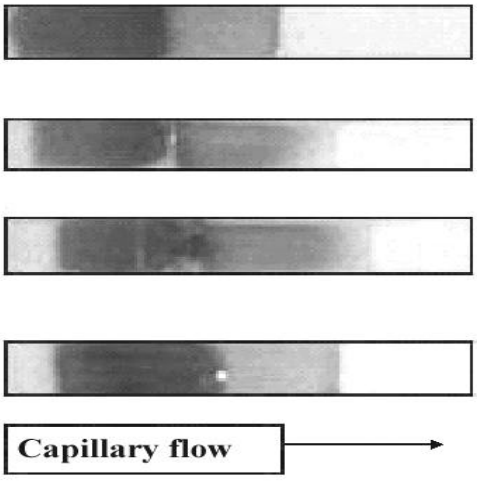

Fig. 3B

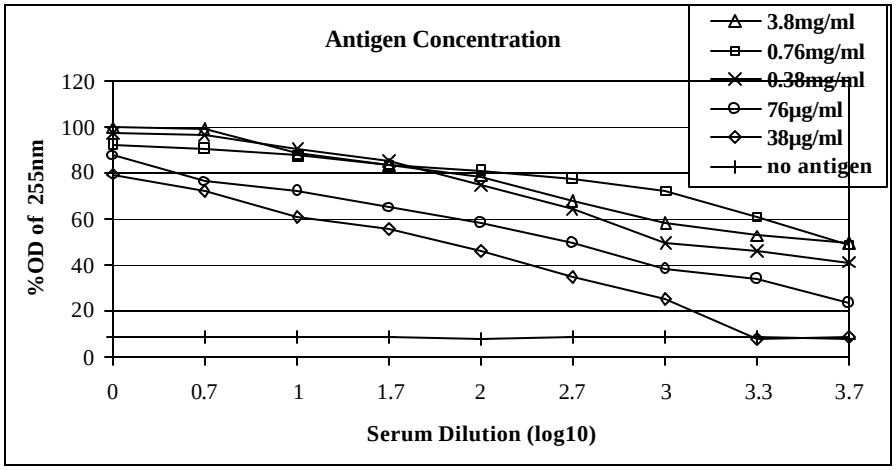
Antigen Concentration
3.8mg/ml
0.76mg/ml
0.38mg/ml
76µg/ml
38µg/ml
no antigen
%OD of 255nm
120
100
80
60
40
20
0
0
0.7
1
1.7
2
2.7
3
3.3
3.7
Serum Dilution (log10)

Fig. 4

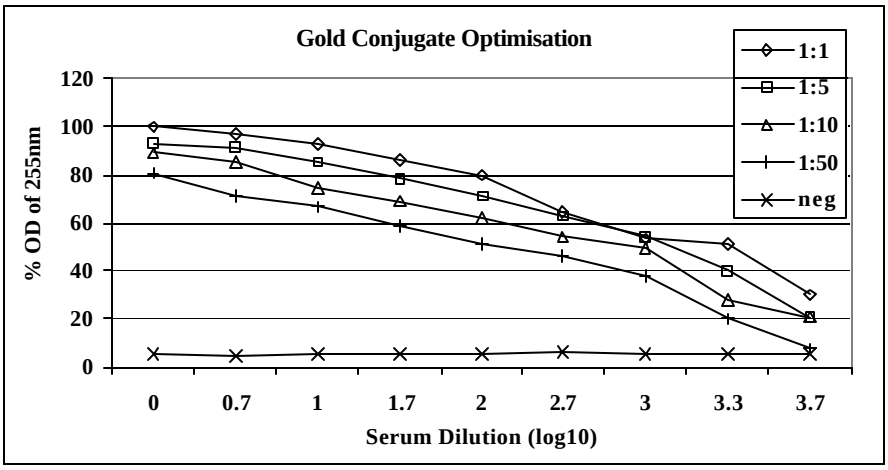
Gold Conjugate Optimisation
1:1
1:5
1:10
1:50
neg
% OD of 255nm
120
100
80
60
40
20
0
0
0.7
1
1.7
2
2.7
3
3.3
3.7
Serum Dilution (log10)

Fig. 5

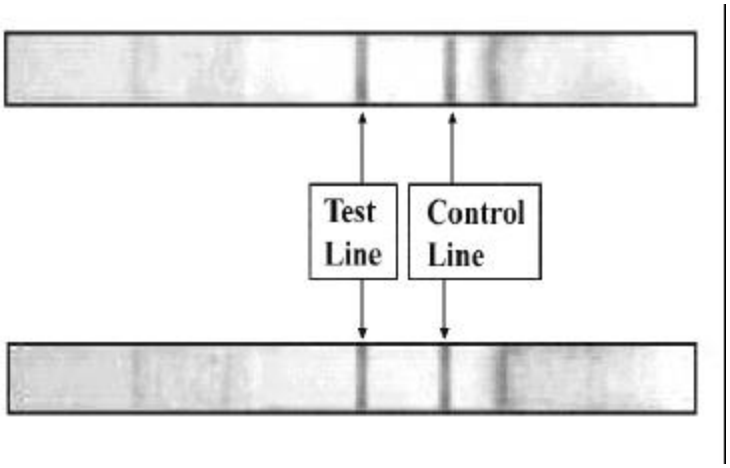

Fig. 6A

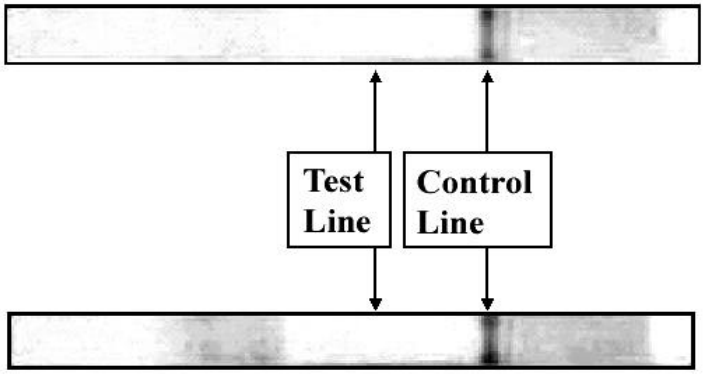

Fig. 6B

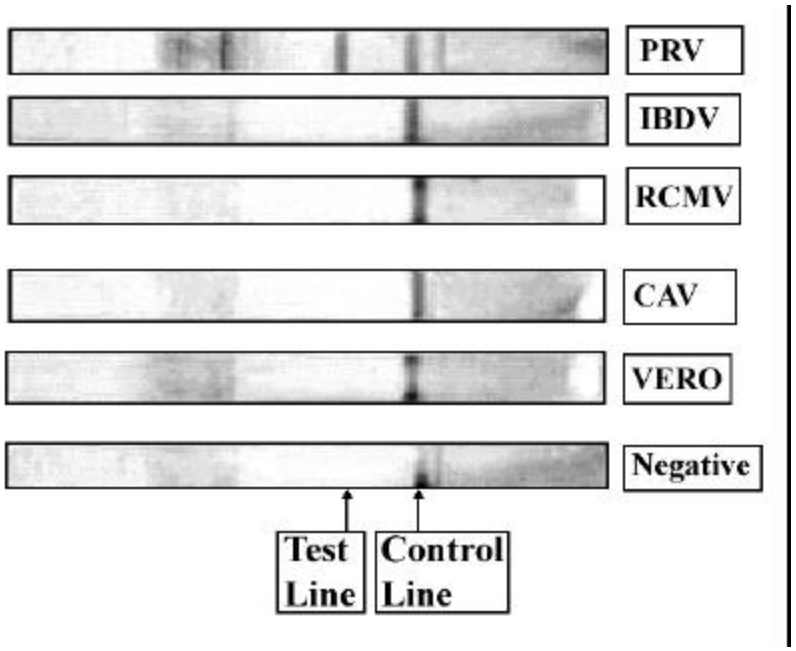
PRV
IBDV
RCMV
CAV
VERO
Negative
Test Line
Control Line

Fig. 7

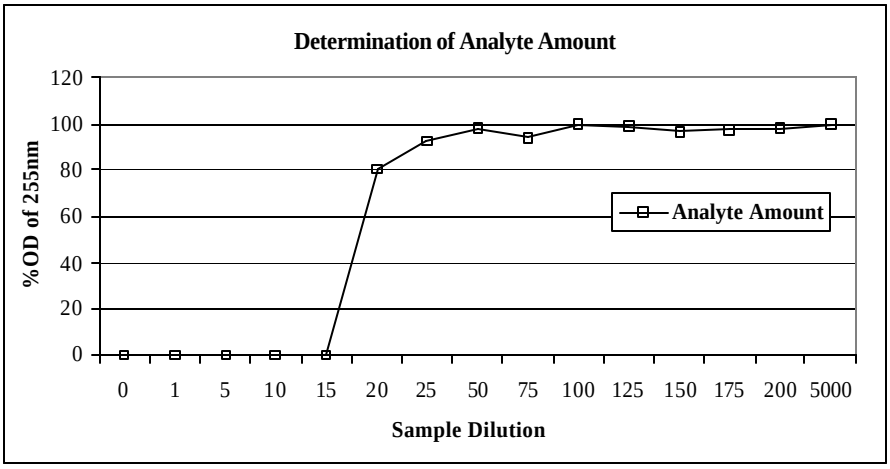
Determination of Analyte Amount
%OD of 255nm
120
100
80
60
40
20
0
0
1
5
10
15
20
25
50
75
100
125
150
175
200
5000
Sample Dilution
Analyte Amount

Fig. 8

Legend for Tables:

Table 1: Test Performance Using Different Purification of Virus Antigen as Capture Reagent. Samples were taken after each purification step and tested for its effects on test strip performance. Each of the sample used were adjusted to have the same concentration before applying as capture reagent (3.0 mg/ml)

Table 2: Comparison of Solid Based Test Strip Method and ELISA. Sensitivity range of the test strip was demonstrated by serial dilution of positive and negative sera. Test strip used was immobilised with 0.38 mg/ml virus antigen and 1:10 gold conjugates. ELISA results were determined against the optical density at wavelength 410 nm and test strip results were determined against the optical density at 255 nm

Tables:

Table 1

Effects of Antigen Purity as Capture Reagent (Test Performance)	
Strips	Signal Intensity % OD of 255nm
Control	100.00%
After sucrose gradient	97.51%
After sonication	58.69%
After virus cultivation	41.7%
VERO cell culture	8.5%

Table 2

Group Serum Dilution (log10) Positive Sera	Solid Based Test Strip		ELISA S/P Ratio	
0	100%	+	2.636	+
1.0	99.68%	+	2.193	+
1.7	94.87%	+	1.707	+
2.0	83.68%	+	1.641	+
2.3	74.40%	+	1.359	+
2.7	69.61%	+	1.173	+
3.0	65.44%	+	1.225	+
3.7	48.00%	+	0.896	+
4.0	35.36%	+	0.712	+
4.3	24.49%	-	0.319	-
Negative sera				
0	21.04%	-	0.152	-
1.0	20.90%	-	0.168	-
1.7	21.60%	-	0.157	-
2.0	21.04%	-	0.136	-
2.3	21.05%	-	0.205	-
2.7	21.69%	-	0.200	-
3.0	21.04%	-	0.194	-
3.7	20.94%	-	0.133	-
4.0	21.04%	-	0.184	-
4.3	20.90%	-	0.125	-

Interpretation of results
+ = positive result
- = negative result