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Analysis of an Inkjet Printed Strip Assay for Pregnancy Test in Cattle

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ANALYSIS OF AN INKJET PRINTED STRIP ASSAY FOR PREGNANCY TEST IN CATTLE

by

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Abstract

Using commercially available antibodies, we created a modified ELISA strip assay to determine pregnancy in cattle. The anti-progesterone antibody was printed on small membrane strips via an Inkjet printer. The strips were subsequently analyzed using varying concentrations of progesterone. The objective of this study was to assess this novel antibody printing process and to determine the binding activity of the anti-progesterone antibody. The long-term objective is to develop a pen-side pregnancy test that would cost less than $1.00. In this specific study, it was determined that the anti-progesterone antibody used did not provide adequate sensitivity for visualization required for pen-side analysis. Additional studies will assess the sensitivity of other anti-progesterone antibodies and the possible implementation of amplification steps.

Introduction

Detecting pregnancy in cattle can be a difficult process. Many methods are used – ultrasound, rectal palpation, and blood testing. However, there are problems with all of these methods. Ultrasound and rectal palpation aren't accurate until later in pregnancy, and both of those methods as well as blood testing require the service of a veterinarian. Creating a pregnancy test for cattle that utilizes milk or urine is ideal – the producer can then check for pregnancy accurately, quickly, and independent of a veterinarian.

Although these pregnancy test kits have been created, they are expensive. Other labs have been able to increase specificity of the antibody to the hormone. This means that

progesterone can be detected a lower levels, which will increase accuracy of the test and lower cost because less reagent is being used. However, this method needs some fine-tuning and that is my goal with this project. I am going to work on verifying antibodies to use and cleaning up the background color in the test while maintaining specificity.

Literature Review

Human Pregnancy Testing

This project, on a basic level, is an imitation of a human pregnancy test. Human pregnancy tests work by detecting presence of human chorionic gonadotropin (hCG) hormone which is present in blood as well urine (Senger, 2003). In 1928, the first idea of using hCG as an indicator for pregnancy was born (Speert, 1973). Over the next 40 years, various test preformed on rabbits (Fishbein, 1976) and other animals were used to determine presence of hCG and therefore a patients pregnancy status. Eventually, after the development of the immunoassay (KöHler et al., 1975) and monoclonal antibodies (Riechmann et al., 1988), which made testing very inexpensive, take home pregnancy tests were possible and became very popular.

Pregnancy Testing in Livestock

There are multiple hormones and proteins that can be used to detect pregnancy in livestock. A common protein used in pregnancy testing in cattle is pregnancy specific protein-B (PSP-B). PSP-B is found in blood when the animal is pregnant. In a study done by Romano et al., an ELISA pregnancy test was done that utilized this protein. They were able to detect pregnancy as early as day 28 with 94% sensitivity (Romano et al., 2010). This is indicative of a very successful test, however it is not able to be performed pen-side or with urine or milk.

Two common hormones used in commercially available pregnancy test kits for livestock are progesterone and estrone sulfate. Estrone sulfate is present is milk (Betteridge et al., 2012) and urine (Park et al., 2008) and is therefore a good hormone for a quick and easy pregnancy test. This hormone has been used by Dr. Keith Henderson of New Zealand to produce a pregnancy test for mares (Horsetalk, 2008). This test analyzes the estrone sulfate just like the test for this project would, with a micro-ELISA test strip. It allows horse owners to determine pregnancy status of their mare without the aid of a veterinarian.

The hormone that we are going to focus on in our test will be progesterone. Progesterone is also found in milk and urine (Rioux et al. 2004). Progesterone is the hormone of pregnancy, it is always found at high levels when and animal is pregnant (Senger, 2003). Many test kits are available for cattle that utilize progesterone and they can be found on product websites, however details of how they work are not available due to patent laws. Although there are many of these tests available, they all require samples be sent away for processing and cost more than one dollar per cow. Our hypothesis was that, using a validated anti-progesterone antibody obtained from an ELISA based progesterone kit, an accurate pen-side assay would be able to be designed for rapid pregnancy testing in cattle that would cost less than one dollar.

Micro-ELISA Method

ELISA stands for enzyme-linked immunosorbent assay. For this project, we will be using a Sandwich ELISA technique. Typically, a sandwich ELISA is just what it sounds like, a sandwich of antibodies. During an ELISA test, a test sample containing (or not) a certain antigen is placed in a 96-well plate (Lequin, 2005). Through a series of steps, if the antigen

is present, the primary antibody is added and if antigen is present, will bind to it. Following the primary antibody is the secondary antibody, which binds to the primary, and so forth. In the end, a visualizing agent is added and will bind to the final antibody. The visualization agent allows you to plainly see the results of the ELISA. If color change is observed, the antibodies were able to bind and therefore antigen was present and, in our case, the cow is pregnant (Thermo Scientific, 2012).

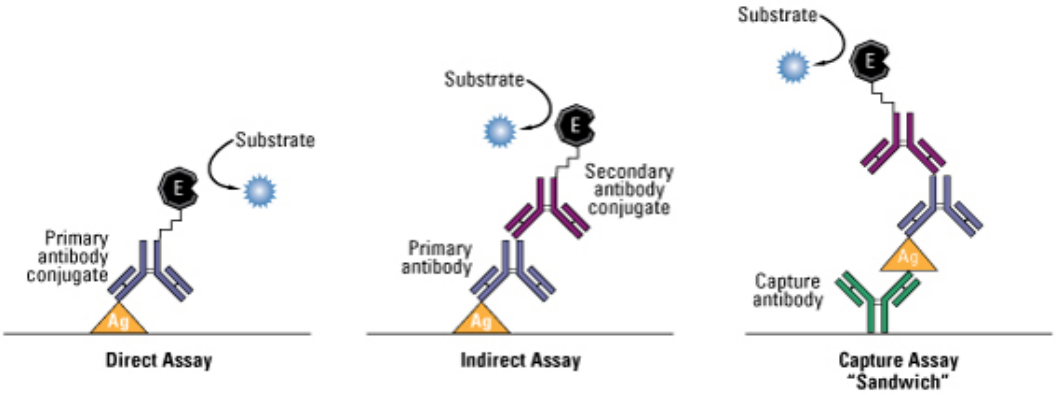

Photo courtesy of Thermo Scientific

We will be using a modified version of a typical sandwich ELISA, ours will be preformed on a smaller scale and not in a 96-well plate. This smaller, test-strip based ELISA is called a micro-ELISA. In a micro-ELISA, the antigen/antibody process remains the same, as well as visualization methods. The only difference is it is preformed on a strip instead of in a 96 well plate.

Materials and Methods

Materials

- Progesterone ELISA Kit
  - Progesterone Alkaline Phosphatase Conjugate, 6 ml.
  - Progesterone Monoclonal Antibody, 6 ml

- Streptavidin-AP conjugate (1:2000)
- Streptavadin t35 (1:1000)
- Poly DA (1:5000)
- FITC t20 (1:2000)
- Anti-FITC AP (1:2000)
- BCIP-NBT
- 12 well plates
- Membrane strips
- Filter pads
- Yellow dye
- Printer paper
- Printer
- PBS
- 70% Ethanol
- Casein

Methods

In order to maximize the amount of prints we could do and the amount of each reagent we would have, we opted to purchase a kit that supplied a few of the more pricey reagents, the Progesterone ELISA Kit from Enzo.

1. A verification control was performed to verify that the reagents were working (see Fig. 1).
2. A standard run of the ELISA kit was preformed following protocol directions supplied with the kit to ensure all supplied reagents were working.
3. After the reagents were verified, the initial anti-progesterone was printed via Inkjet printer onto the membrane (see Fig. 2). The printing protocol is as follows:
    I. The cartridge was purged with alcohol
        a. The bottom of the reservoir was filled with alcohol
        b. Cartridge lid was replaced (in proper orientation) and cartridge was inserted into printer (in proper orientation: copper strip in first)
        c. Blank paper was loaded into printer
        d. The "purge" powerpoint slide was printed
            i. I made sure I was able to see the whole line being printed, and the paper was slightly damp
                1. If paper was not damp, I repeated steps a-d
                2. If cartridge was clogged, I saw blotchiness while printing (I would clean and purge with alcohol and PBS before using antibodies)
    II. The cartridge was rinsed 3X with PBS
    III. The cartridge was purged with PBS
        a. The bottom of the reservoir was filled with PBS
        b. The cartridge lid was replaced (in proper orientation) and inserted into printer (in proper orientation: copper strip in first)
        c. Blank paper was loaded into printer
        d. The "purge" powerpoint slide was printed

i. I made sure I was able to see the whole line being printed, and the paper was slightly damp
1. If paper was not damp, I repeated steps a-d
2. If cartridge was clogged, I saw blotchiness while printing (I would clean and purge with alcohol and PBS before using antibodies)

IV. The most concentrated antibody solution was pipetted into bottom reservoir of print cartridge.
a. The cartridge lid was replaced (in proper orientation) and inserted into printer (in proper orientation: copper strip in first)
b. Blank paper was loaded into printer
c. The "bottom line" powerpoint slide was printed

V. Nitrocellulose strip was taped to this piece of paper
a. The nitrocellulose strip was taped so the scored line was on the left side
b. The printed line was used as a guide to make sure that when I printed this line again, it would be on the nitrocellulose
i. I was also sure to leave enough room to the left of the line for additional lines to be printed on the same nitrocellulose strip
c. I was sure to tape down the bottom corners (that entered the printer first) of the strip, so they would not snag

VI. The most concentrated antibody solution was printed onto nitrocellulose strip
a. The paper with nitrocellulose strip taped to it was loaded into printer (making it flush with the printer on the right side)
b. The "bottom line" powerpoint slide was printed

VII. Any left over antibody solution was removed from cartridge

VIII. The cartridge was rinsed 3X with PBS

IX. The cartridge was purged with PBS
a. The bottom of the reservoir was filled with PBS
b. The cartridge lid was replaced (in proper orientation) and inserted into printer (in proper orientation: copper strip in first)
c. Blank paper was loaded into printer
d. The "purge" powerpoint slide was printed
i. I made sure I was able to see the whole line being printed, and the paper was slightly damp
1. If paper was not damp, I repeated steps a-d
2. If cartridge was clogged, I saw blotchiness while printing (I would clean and purge with alcohol and PBS before using antibodies)

X. The 2nd most concentrated antibody solution was pipetted into bottom reservoir of print cartridge.
a. The cartridge lid was replaced (in proper orientation) and inserted into printer (in proper orientation: copper strip in first)
b. Blank paper was loaded into printer
c. The "middle line" powerpoint slide was printed

XI. The 2nd most concentrated antibody solution was printed onto nitrocellulose strip

a. The paper with nitrocellulose strip taped to it was loaded into printer (making sure it was flush with the printer on the right side)
b. The "middle line" powerpoint slide was printed

XII. Any left over antibody solution was removed from cartridge
XIII. The cartridge was rinsed 3X with PBS
XIV. The cartridge was purged with PBS
a. The bottom of the reservoir was filled with PBS
b. The cartridge lid was replaced (in proper orientation) and inserted into printer (in proper orientation: copper strip in first)
c. Blank paper was loaded into printer
d. The "purge" powerpoint slide was printed
i. I made sure I was able to see the whole line being printed, and the paper was slightly damp
1. If paper was not damp, I repeated steps a-d
2. If cartridge was clogged, I saw blotchiness while printing (I would clean and purge with alcohol and PBS before using antibodies)

XV. The least concentrated antibody solution was printed into bottom reservoir of print cartridge.
a. The cartridge lid was replaced (in proper orientation) and inserted into printer (in proper orientation: copper strip in first)
b. Blank paper was loaded into printer
c. The "top line" powerpoint slide was printed

XVI. The least concentrated antibody solution was printed onto nitrocellulose strip
a. The paper with nitrocellulose strip taped to it was loaded into the printer (making sure it was flush with the printer on the right side)
b. The "top line" powerpoint slide was printed

XVII. Any left over antibody solution was removed from cartridge
XVIII. The cartridge was rinsed 3X with PBS
XIX. The cartridge was purged with PBS
a. The bottom of the reservoir was filled with PBS
b. The cartridge lid was replaced (in proper orientation) and inserted into printer (in proper orientation: copper strip in first)
c. Blank paper was loaded into printer
d. The "purge" powerpoint slide was printed
i. I made sure I was able to see the whole line being printed, and the paper was slightly damp
1. If paper was not damp, I repeated steps a-d
2. If cartridge was clogged, I saw blotchiness while printing (I would clean and purge with alcohol and PBS before using antibodies)

XX. The cartridge was purged with alcohol
a. The bottom reservoir was filled with alcohol
b. The cartridge lid was replaced and inserted into printer
c. The blank paper was loaded into printer

   d. The "purge" powerpoint slide was printed
      i. I made sure I was able to see the whole line being printed, and the paper was slightly damp
         1. If paper was not damp, I repeated steps a-d
4. The membranes were then cut into strips and blocked with a 0.05% casein solution for at least 30 minutes.
5. The membranes were then briefly patted dry and taped tightly to filter pads.
6. They were then set in a well containing a PBS wash, followed by progesterone, another PBS wash, anti-progesterone, and a final PBS wash (see Fig. 3).
7. The concentrations of the progesterone and the detection anti-progesterone were determined using concentrations used in the verification control. Test concentrations were grouped as follows:
   a. No dilution of progesterone, no dilution of detection anti-progesterone
   b. 1:10 dilution of progesterone, 1:1000 dilution of detection anti-progesterone
   c. 1:10 dilution of progesterone, 1:2000 dilution of detection anti-progesterone
   d. 1:10 dilution of progesterone, 1:5000 dilution of detection anti-progesterone
   e. 1:100 dilution of progesterone, 1:1000 dilution of detection anti-progesterone
   f. 1:100 dilution of progesterone, 1:2000 dilution of detection anti-progesterone
   g. 1:100 dilution of progesterone, 1:5000 dilution of detection anti-progesterone
   h. 1:1000 dilution of progesterone, 1:1000 dilution of detection anti-progesterone
   i. 1:1000 dilution of progesterone, 1:2000 dilution of detection anti-progesterone
   j. 1:1000 dilution of progesterone, 1:5000 dilution of detection anti-progesterone
8. After the membranes ran through the last PBS wash, they were developed with BCIP-NBT. This was done by removing the filter pad and adding enough BCIP-NBT drop-wise to cover the membrane (see Fig. 4). They were allowed to develop until lines were visible, with a maximum development time of 90 minutes.
9. Development was stopped by rinsing with double distilled water. They were then set out to dry overnight.

Results

Three replicates of test strips were tested using the anti-progesterone antibody. Each time, there were no detectable levels of progesterone after the maximum development time of 90 minutes. Figure 5 is a picture of the first set of strips run after 90 minutes of developing.

Conclusion and Further Study

After the completion of this experiment, it was determined that the anti-progesterone antibody tested in this experiment did not provide adequate sensitivity for visualization required for pen-side analysis. However, the control 96-well ELISA assay performed as expected using the same antibody. Additional anti-progesterone antibodies will need to be screened to determine one that will provide the specificity and sensitivity required for this inkjet printed strip assay.

Antibody Sensitivity

A future study could assess the sensitivity of other anti-progesterone antibodies. Running the same test done in our experiment but incorporating multiple anti-progesterone antibodies of varying sensitivities could help us to assess the sensitivity. That same experiment could also determine the minimum sensitivity of anti-progesterone needed for detection.

Biotin –labeling

Another avenue for future research would be the implementation of biotin-labeled amplification steps. A biotin-label amplified the signal by binding to the detection antibody, in our case detection anti-progesterone, and allowing streptavidin-HRP to bind multiple times. The extra streptavidin molecules amplify the signal of the single capture antibody creating a more sensitive signal.

Figures

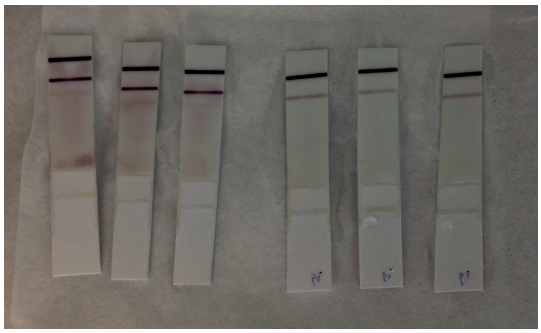

Figure 1. Positive verification control.

Figure 2. Printed line of anti-progesterone on membrane.

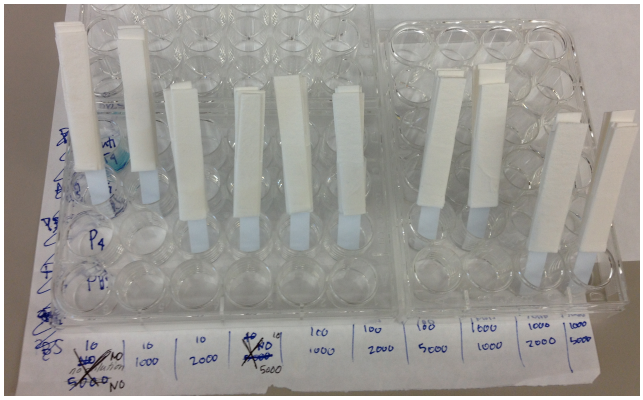

Figure 3. Run wells set up.

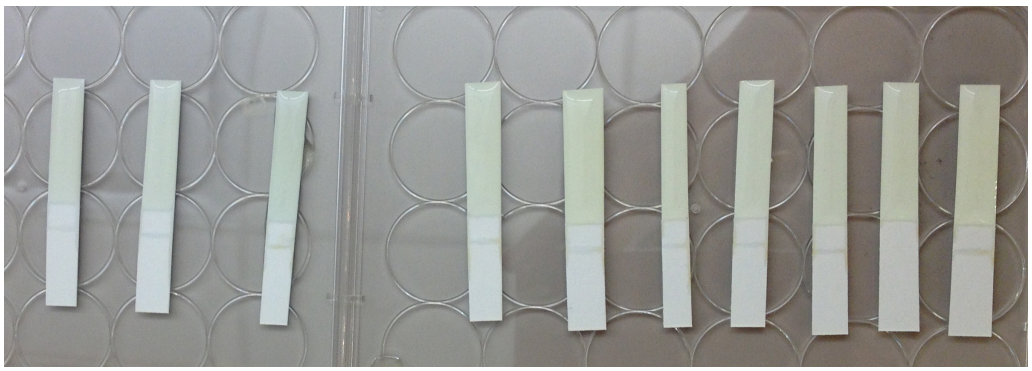

Figure 4. Membranes developing with BCIP-NBT.

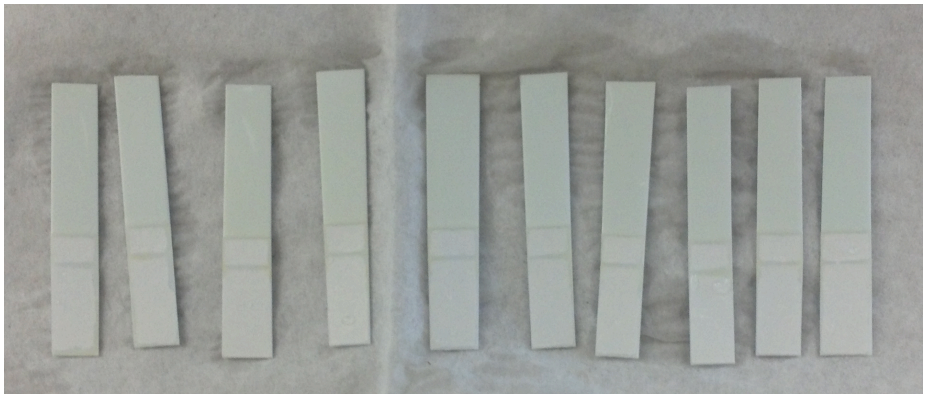

Figure 5. No development on test membranes.

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