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Respiratory disease in the growing pig: selecting materials for submission and making the diagnosis

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Introduction

Swine respiratory disease is often complex with multiple viral and bacterial pathogens involved. The pig and sample selection process is very important in obtaining and making the diagnosis. Also, as more is known about swine respiratory pathogens and their various serovars, serotypes, strains, virulence genes, and mutations, it may now be necessary to delve more deeply into the diagnosis than before.

Pig selection

The selection of pigs for testing, whether for serology or necropsy, is the most important step in obtaining an accurate and timely diagnosis. The majority of the veterinarian's time should be spent observing pigs, taking temperatures, and obtaining complete histories prior to collecting samples or performing euthanasia.

Sampling pigs that have been treated for days or weeks with antibiotics will be unrewarding if your goal is to isolate pathogenic bacteria. Likewise, sampling only pigs that are chronically ill will skew the diagnostic picture, resulting in isolation only of common secondary or opportunistic pathogens and finding histopathological lesions masked by scarring or regeneration rather than the inciting lesion.

In some situations, sample selection is straightforward – such as in outbreaks of diarrhea due to TGEV. In other, more complex situations such as porcine respiratory disease, many agents may be involved. Diagnostic sample selection may involve testing pigs at recurring intervals in order to define all the agents involved. Sampling carefully to include acutely affected pigs will be the greatest help to the producer, the pathologist, and the veterinarian seeking treatment or prevention strategies.

Why do pigs cough?

Coughing, as defined by Ettinger and Barrett, is an expiratory effort producing a sudden, noisy expulsion of air from the lungs, usually in an effort to free the lungs of foreign material (real or imagined). This expiratory effort is accomplished against a closed glottis. Causes of coughing include inflammation, allergies, trauma or physical irritation, parasites, cardiovascular disease, and, less likely, neoplasia. Coughing is a result of mechanical or chemical irritation of the pharynx, larynx, tracheobronchial tree, or the smaller airways. Additionally, lesions in the pleura, pericardium, diaphragm, nose and nasal sinuses may also contribute to or cause coughing.

Therefore, thorough examination and sample collection of not only the entire respiratory system (from snout to diaphragm) but also the heart and pericardium may be necessary to diagnose porcine respiratory disease if your initial examinations of the lung result in no significant findings.

The following are some of the more common pathogens involved in porcine respiratory disease, some brief discussion and background regarding each pathogen, diagnostic technologies available and samples to collect.

Porcine reproductive and respiratory syndrome virus (PRRSV)

Diagnosis of PRRSV is through culture of the virus, detection of nucleic acid by reverse transcriptase (RT)-PCR, demonstration of the antigen in tissues by immunohistochemistry, fluorescent antibody, or in-situ hybridization, genetic sequencing, and antibody detection. Methods routinely used at the Minnesota Veterinary Diagnostic Laboratory (MVDL) include serology using ELISA and immunofluorescent antibody (IFA), RT-PCR, virus isolation, and genetic sequencing.

The IDEXX HerdChek ELISA is the most common test used for detecting PRRSV antibodies. Care must be taken not to over interpret the magnitude of the s/p ratio as there is considerable animal to animal immune response and test to test variations. The test is meant as a herd screening tool and as such the results obtained from single animal tests should also be reviewed with caution. Finally, the IDEXX HerdChek PRRSV ELISA cannot differentiate between field virus or vaccination exposure. Positive ELISA results that are unexpected can be further investigated by repeating the ELISA, performing IFA tests and re-bleeding. IFA has high specificity, but the sensitivity can be influenced by type of PRRS virus used in the assay or technician experience.

RT-PCR for PRRSV is very commonly used on serum, semen, and tissues. Currently the MVDL employs one RT-PCR TaqMan test for the simultaneous detection of

most European-like and North American-like strains. The advantages are high specificity, high sensitivity, and rapid return of results, often in less than 12 hours. Once virus is detected by RT-PCR, attempts to culture the virus or genetically sequence the virus can be initiated. Since PRRSV is an RNA virus, changes in the nucleotide sequence in the areas targeted by RT-PCR primers may yield false-negative results. The use of multiple tests to confirm or refute a diagnosis of PRRSV is advisable to overcome any individual test's shortcomings.

Genetic sequencing of PRRSV at the MVDL is performed on the ORF 5 gene, a highly variable envelope gene. Based on the genetic sequence of ORF 5, some assumptions on relatedness of strains and changes between strains can be made (Hennings, et al.). Sequencing is also very helpful in determining if the virus is of vaccine origin or field-virus. To qualify two viruses as identical, entire genome sequencing is necessary and thus is often only used as a research tool. Sections of the viral genome associated with virulence are unknown (Hennings, et al.).

Often provided with ORF 5 gene sequencing information is the restriction fragment length polymorphism (RFLP) pattern of a strain. The RFLP is determined by examining three restriction enzyme cut sites within ORF 5. Since ORF 5 itself represents a small part (4%) of the overall PRRSV genome, the RFLP provides little information on strain relatedness or virulence. Furthermore, the RFLP cut pattern can change as a result of a single nucleotide base pair substitution (Hennings, et al.). RFLP is rarely an accurate measure of relatedness and there exists great diversity within viruses of the same RFLP cut pattern.

Swine influenza virus (SIV)

Once predictable, SIV has continued to evolve and combine with human and avian strains so that now three predominant subtypes of SIV are causing disease in pigs – H1N1, H3N2, and H1N2. Within each of these subtypes, there is increasing genetic diversity in the outer membrane genes (hemagglutinin HA and neuraminidase NA) and internal genes (especially polymerases PA and PB2). Accurate assessment of SIV in a herd is necessary and can be attempted by several antigen detection methods and serological assays.

Diagnostic tests for the detection of SIV include immunoassay, virus isolation, histopathology, immunohistochemistry, fluorescent antibody, and RT-PCR (Gramer, et al.). The commercially available immunoassay is the BD-Directigen Flu A test. It is designed for the rapid detection of influenza virus in human throat swabs. It has worked very well for pigs, birds, or horses infected with influenza A viruses. This test is highly specific and rapid. However, the sensitivity of the test varies between laboratories with the MVDL reporting a sensitivity of 77% (Gramer, et al.). This test is also fairly expensive and does not allow for subtyping. Virus isolation (VI) is considered the gold standard test for most laboratories. The advantages of VI are its high specificity, the production of a live virus that can later be characterized or used to make vaccines. However, VI takes approximately 5 days and requires viable virus. Once the virus is isolated, the virus is serotyped to determine HA type or NA type. Infection with swine influenza virus causes characteristic changes in the lungs resulting in bronchopneumonia with bronchial epithelial necrosis early in the disease, severe purulent inflammation, and eventual epithelial regeneration. SIV by itself causes little permanent lung damage (Rossow and Gramer). While the above describes an uncomplicated case of SIV, all of the above changes are obscured and more difficult to interpret when the pig is infected with other viruses and bacteria resulting in chronic disease. Immunohistochemistry (IHC) can be conducted on formalin-fixed lung. IHC is specific, does not require live virus, can be used when only fixed tissue available, and helps localize the virus. IHC has its disadvantages which include high cost and a turnaround time of at least 2 days from collection of lung to reporting of result. Also, variations in IHC results occur because of timing of lung sample collection, sample location, number of lung sections examined, and reader subjectivity. Furthermore, IHC does not allow for subtyping. The fluorescent antibody test (FA) has similar benefits and drawbacks to IHC but is less expensive and conducted on sections of frozen lung (Collins, et al.). RT-PCR that detects the nucleoprotein gene of all influenza A viruses is routinely used at the MVDL and has a 98% sensitivity and specificity on diseased lung tissues. The advantages of this test are that it is rapid, does not require live virus, and is cost effective (Gramer, et al.). The results are reported as presence or absence of type A influenza virus (positive/negative). RT-PCR of SIV lends itself to subsequent virus characterization by PCR to determine the HA and NA type of the virus.

SIV characterization of the HA and NA genes can be attempted by PCR tests or genetic sequencing. The advantages of a PCR test for HA and NA typing is that they are more specific than serotyping, are faster, do not require live or cultured virus and can be automated (Rossow and Gramer). Influenza virus subtyping tests using the TaqMan format allow for higher throughput, more objective data, and faster turnaround than gel-based multiplex typing tests. Nucleotide sequencing of swine influenza virus is becoming more and more important as new and changing swine influenza viruses continue to emerge. Information provided by sequencing the HA, NA, PA, and PB2 genes has shown increased diversity in swine H1 genes and the emergence of several SIV subtype variants including H1N1 viruses with avian internal genes and H1N1 viruses with human external genes (Webby). The genetic comparison of the first 600 base pairs of the HA gene can usually provide a great deal of information on virus evolution and some insight into relatedness. Genetic sequencing of the HA gene should be employed for the

following reasons: to examine multiple swine influenza virus isolates from a single herd or system; to compare isolates of apparently increased virulence to influenza isolates from humans, birds, and pigs; when routine serotyping of the virus by hemagglutination inhibition (HI) is inconclusive; when pigs have less than expected convalescent titers; or when pigs have failed to be protected by properly administered SIV vaccines.

Serotyping of SIV by HI is routinely used by all laboratories. Occasionally, an isolate is untypeable using the SIV reference antisera. In these cases, additional antisera can be obtained to expand the HI panel, PCR typing can be done, or nucleotide sequencing can be attempted. Determining the NA type by neuraminidase inhibition test is performed at reference laboratories such as the National Veterinary Services Laboratory in Ames, IA. Both the HA and NA inhibition tests are reliable if there is a range of antisera available to all subtypes of interest. Disadvantages of the HI test include the possibility of nonspecific reactions from products that are naturally occurring in sera. Additionally, it is necessary to standardize all parts of the test each time and have a person with much expertise read the results of the tests every time.

Seroreactions of viruses with available antisera can be used as an indicator of antigenic differences between isolates. Antigenic information coupled with genetic analyses can reveal a great deal about the epidemiology and origin of SIV. However, they do not provide us information as to how swine influenza viruses with different genetic sequences will react in the pig or will be prevented by administering the usual vaccine. Some work in Europe has shown that even though there were both antigenic and genetic differences in several of the swine influenza viruses circulating, vaccination with one type of SIV vaccine was able to protect against all of the various types of SIV if the pigs had sufficiently high antibodies (Van Reeth, et. al.). It is important now more than ever to take these different viruses that are found and do cross-protection and challenge studies to determine if the vaccines we have in place will continue to be effective in the US swine population.

Mycoplasma hyopneumoniae (Mhyp)

Mhyp is difficult to culture from lungs due to its fastidious nature, slow growth, requirement of special media, and constant worry of contamination with commensal respiratory Mycoplasmas such as *M. hyorhinis*. PCR testing has been valuable in diagnosing Mhyp and is routinely used. Detection of Mhyp should be correlated with presence of clinical signs, lung lesions, and eventual seroconversion as the possibility for false positive results exists and there is debate on whether there is a "colonization threshold" in the lung necessary to cause pneumonia. PCR conducted on lung tissue or lavage is more sensitive than from samples taken from the nasal cavities or upper respiratory tract (Thacker).

Serum antibody detection for Mhyp can be frustrating as seroconversion is delayed and variable, reported to be from 2 to 8 weeks following infection (Thacker). Three Mhyp ELISA serology tests exist. The most common tests are the Tween 20 ELISA, IDEXX ELISA and the DAKO ELISA. The tests differ either in the antigen that is employed in the test or in the method. The Tween 20 uses the whole cell membrane proteins as antigen and because of this is subject to cross-reactions with non-pathogenic *M. flocculare*. The IDEXX ELISA is an indirect ELISA in which purified Mhyp antigen is coated onto the solid phase and an anti-swine IgG horseradish perioxidase conjugate is used for detection (Velek, et. al.). The DAKO is a competitive ELISA uses a monoclonal antibody to a species-specific epitope of a Mhyp 74 kD protein, thus imparting a slightly higher specificity to the test. All serological tests can have unexpected/false positive results that should be confirmed with repeated serological tests, observation of clinical signs, and presence of lesions at necropsy or slaughter.

Actinobacillus pleuropneumoniae (APP)

APP is diagnosed by bacterial culture from swabs or tissues, polymerase chain reaction (PCR), or through serum antibody detection. APP strains are characterized as biotype I or II based on their NAD growth requirement (Frey). Most APP strains are biotype I and are dependent on NAD for growth in culture. There are 16 serovars (1, 2, 3, 4, 5a, 5b, 6, 7, 8, 9, 10, 11, 12, 13, 14, and 15) as determined by the type of capsular polysaccharides and cellular lipopolysaccharides present. Serotypes 9, 11, 13, 14, and 15 have not been isolated in North America to date. The structure of these capsular and cellular sugars can be similar, which causes serological cross-reaction between serovars 1, 9, and 11; serovars 3, 6 and 8; and serovars 4 and 7. APP produces very potent cytotoxins - ApxI, ApxII, and ApxIII – whose genes can also be used to identify or characterize the strains. ApxII is produced by all APP serovars except APP 10, which only possesses ApxI. APP 1, 5a, 5b, 9 and 11 possess both ApxI and ApxII toxins. Serovars 2, 3, 4, 6, and 8 possess both ApxII and ApxIII. Serovars 7 and 12 possess only the ApxII toxin (Frey). An APP specific toxin -ApxIVa – has been characterized and is detected in all APP serotypes (Schaller, et al.).

APP can be hard to isolate from diseased swine lung tissues, tonsils or nasal swabs. There exist PCR tests for ApxIVa toxin gene detection that were shown to be more sensitive than routine culture, detecting APP in 94% of the necrotic lung lesions compared to only a 76% recovery of APP from culturing the same lesions (Schaller, et al.). Biochemical characterization of APP isolates is not straightforward. Once APP is cultured, the confirmation of the isolate is done by further serotyping either by use of monoclonal antibodies or by PCR tests that detect the

Apx toxins. If there is ambiguity in any of the phenotypic results, genotyping or gene sequencing is recommended to confirm the isolate as APP and attempt to characterize its biotype and serovar.

IDEXX has acquired and made available an ELISA kit, CHEKIT-APP-ApxIV for the rapid, simple, sensitive and specific method for detecting antibodies against ApxIV, the APP specific toxin. This test will detect antibodies against any APP serotype. More serotype specific serological assays for APP are the ELISAs developed by Dr. Marcello Gottschalk of Université de Montréal, Québec, Canada. Using the capsular sugars, there are ELISA tests available to screen pigs for antibodies to Serotype 1 (9 and 11), Serotype 2, Serotype 3 (6 and 8), Serotype 5 (a and b), Serotype 7 (Oliveira, et al.), Serotype 10, and Serotype 12.Antibodies can be detected by ELISA at approximately 10 to 14 days post-infection and maternal antibodies can be detected for 2 months or longer (Burkhardt, et al.). Serotype specific ELISAs can produce false positive results as the specificities are sometimes lower than optimal (73.3% for APP 5 ELISA) and there is also the possibility for false negative results [e.g., the sensitivity for the APP 1 (9,11) ELISA is 89.9%]. Also, there is no APP 13, 14, and 15 ELISA tests currently available. Therefore, the CHEKIT-APP-ApxIV ELISA may be the best choice in screening herds for antibodies to APP.

When dealing with unexpected APP culture or serology results from herds with no clinical signs of disease, keep in mind that these extensive tests exist and can be done to help confirm the APP diagnosis.

Haemophilus parasuis (HPS)

HPS is diagnosed by bacterial culture or PCR. Detection of HPS antibodies in sera by ELISA is affected by the type of strain used in the ELISA test, so false negative results are very likely. Thus, serology for HPS is not routinely used as a diagnostic tool (Oliveira, et al.). Culture of HPS can be very difficult. HPS does not survive well in post-mortem tissues, and isolation attempts should be made by culturing tissues or swabs from euthanized pigs. In one study, culturing euthanized pigs yielded HPS in 75% of the attempts compared to only 25% of the isolations from swabs and tissues of pigs found dead (Oliveira, Galina, and Pijoan). In cases where typical lesions of HPS-induced polyserositis are discovered yet isolation is unsuccessful, PCR on swabs of the affected organs has proved very useful in detecting the pathogen.

HPS isolation from non-respiratory sites is encouraged as the systemic isolates are more likely to be the pathogenic strain responsible for disease in the herd. In cases where there is no polyserositis and an HPS is isolated from the lung only, this lung HPS is not likely to be the cause of disease in the herd. It has also been found that in cases where pigs have both meningitis and arthritis, the HPS from the brain is sometimes different from the HPS isolated from the joint, so culturing both meninges and joints is encouraged. For these reasons, characterization of the systemic HPS isolate is recommended.

HPS is characterized by serovar and genotype. There are 15 serovars of HPS; however many HPS strains are nontypeable and there is tremendous genetic diversity within and between serovars. Therefore serotyping HPS isolates will provide some antigenic information regarding the isolate, but is not an efficient predictor of whether vaccination with a specific serotype will be efficacious or cross-protective. Genotyping HPS isolates has revealed information on the epidemiology and prevalence of HPS in the herd (Oliveira, et al.). Genotyping information can be used to identify the prevalent strains in the herd that can be used in various control strategies.

Rhinitis

Rhinitis is caused most commonly by toxigenic bacteria (*Pasteurella multocida* or *Bordetella bronchiseptica* alone, in combination with each other, or in combination with other pathogens) or porcine cytomegalovirus. A diagnosis of progressive atrophic rhinitis caused by *P. multocida* requires not only demonstration of the lesion grossly or microscopically, but also presence of clinical signs, isolation of the bacteria, and detection of the toxin gene in the bacteria. Several snouts or nasal swabs need to be submitted to confirm or refute the diagnosis of an infectious cause of rhinitis. Samples should come from many ages of pigs, with the majority of the sampling targeted towards late nursery or early finisher aged pigs. Non-infectious causes of snout deformities include metabolic bone disease, physical irritation or trauma, or may be phenotypic variations in different breeds of pigs and may be difficult to distinguish from each other without exhaustive diagnostic efforts.

Bacterial pneumonias

Aerobic cultures of lungs yield many bacteria including, but not limited to, *Pasteurella multocida Streptococcus suis, Haemophilus parasuis, Bordetella bronchiseptica, Arcanobacterium pyogenes, Salmonella sp., Actinobacillus suis,* Beta-Hemolytic Streptococcus sp., *Actinobacillus pleuropneumoniae, Staphylococcus sp.,* other *Pasteurella sp.*, and other *Actinobacillus sp.* As many are normal inhabitants of the respiratory tract of swine, their isolation from lung tissue must be correlated with lesions, clinical signs, and lack of other causes of pneumonia before they are considered the primary cause. Anaerobic bacteria are unlikely to be significant causes of pneumonia in swine and are likely restricted to cases of septic pleuritis secondary to esophageal puncture or trauma to the thoracic cavity.

Verminous pneumonia

Verminous pneumonia and visceral larval migrans due to *Ascaris suum* can cause high mortality, especially when

pigs are placed into sites that are heavily contaminated with *Ascaris suum* eggs. Since the larvae hatch in the small intestine and can reach the lungs within 4 to 6 days after ingestion, the coughing, pneumonia can begin within the first week of placement onto an infected site. A review of the lifecycle of *Ascaris suum* is available in the 9th edition of Diseases of Swine and is paraphrased below (Bonner Stewart and Hoyt).

Pigs ingest the eggs containing the infective second stage (L2) larvae. The eggs hatch in the small intestine, releasing the L2s which then burrow into the intestinal wall. The L2s then enter the hepatic portal system and are carried to the liver within 24 hours of infection. In the liver and hepatic portal system, the first parasitic molt occurs (L2 to L3) and these third stage larvae (Le) continue their migration from the liver to the lungs via the venous system, right heart, and pulmonary arteries, reaching the lungs by 4 to 6 days after infection. The larvae then break out of the alveolar capillaries and migrate up the bronchial tree to the pharynx and are swallowed. The final two parasitic molts (L3 to L4 to immature adult worms) are completed in the small intestine by 3 to 4 weeks after infection. Mature gravid females begin to lay eggs approximately 6 to 8 weeks after infection.

Histopathological diagnosis is usually confirmatory, as in severe cases larvae are visible in the airways. Severe lung lesions from verminous pneumonia are usually accompanied by the pathognomonic "milk spots" on the livers; however, these grossly visible white areas on the liver fade after death when the liver is removed from the pigs abdomen and exposesd to the environment or as postmortem autolysis ensues. The histologic lesions will persist making the diagnosis rather straightforward.

PCV2-associated pneumonia

Porcine circovirus type 2 (PCV2) is ubiquitous (Segales, et. al.) and detection and localization of PCV2 in the lung by virus isolation, PCR, and immunohistochemistry is a common occurrence. At the MVDL, detection of other respiratory pathogens occurs in the majority of cases of PCV2-associated pneumonia, with the exception of chronically infected pigs or pigs receiving antibiotic therapy. Lungs in pigs infected with PCV2 may be enlarged, non-collapsing, and resilient with either the distribution being either patchy or diffuse (Segales, et. al.). Peribronchial lymphoid cuffing is usually prominent and reminiscent of Mhyp infection. Bronchiolitis is also a common feature and may be suggestive of prior influenza virus infection.

Serological detection of PCV2 antibiotics by Synbiotics ELISA (Lopez, et. al.) was recently introduced in the US and may prove helpful in determining seroconversion and timing of vaccination.

Summary

Porcine respiratory disease is commonly caused by multiple pathogens and rarely is a single agent the only cause of disease. Co-infections are the norm, and the determination of primary versus secondary infection is best determined by careful consideration of not only the diagnostic laboratory findings but also clinical signs, signalment, history, environmental conditions and response to treatment.

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