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Advanced characterization and analyses of *Lawsonia* sp and *Brachyspira* sp

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Lawsonia sp

Lawsonia intracellularis, the etiologic agent of porcine proliferative enteropathy (ileitis), is an obligately intracellular, vibroid-shaped bacterium that is found in the apical cytoplasm of affected enterocytes. The disease has been reported in various other animal species, including hamsters, horses, ferrets, foxes, rabbits, deer, some avian species, and non-human primates. In fact, equine proliferative enteropathy is an emerging disease of horses, with individual reports and outbreaks increasingly being reported. Although isolates of *L intracellularis* from pigs and other host species show a high degree of genetic and phylogenetic similarity, more sensitive molecular subtyping techniques suggest that porcine isolates are distinct from those obtained from other animal species.

Current diagnostics

L intracellularis must be propagated in a cell culture system with a specific atmosphere, which has led to non-cultivation methods for the diagnosis of proliferative enteropathy. Laboratory confirmation of a clinical diagnosis may be obtained by demonstration of *L intracellularis* in feces by PCR assays using specific primers. However, fecal PCR analysis is not sufficiently sensitive for the diagnosis of all infections, especially subclinical or mild chronic forms. Methods described for the serologic diagnosis of proliferative enteropathy have employed whole bacterial antigen incorporated into an immunoperoxidase monolayer assay or several ELISA-based assays. These assays are more sensitive than PCR when used for cross-sectional or longitudinal monitoring of pig herds, especially for mild or subclinically affected herds. For definitive diagnosis of proliferative enteropathy, necropsy followed by specific identification of *L intracellularis* in the lesions by immunohistochemistry continues to be the gold standard.

Advanced characterization

Proliferative enteropathy affects pigs worldwide and occurs in weaned pigs kept in all types of management systems. The disease in pigs displays several acute and chronic clinical manifestations, including proliferative hemorrhagic enteropathy, an acute hemorrhagic diarrhea with sudden death of pigs close to market age, and porcine intestinal adenomatosis, a chronic mild diarrhea with reduced performance of growing pigs. *L intracellularis* has been detected by PCR in the feces of a variety of wild animals including skunks, opossums, jackrabbits, and coyotes, and numerous other host species continue to be discovered. No epidemiological evidence supports transmission of *L intracellularis* from domestic pigs to horses. Clinical disease, with severity depending on the dose, is consistently reproduced by transmission of isolates within species, but neither clinical disease nor characteristic lesions have been observed in cross-species infection studies. This dichotomy of results suggests that *L intracellularis* isolates may be somewhat species-specific.

Little information is available on the source or spread of *L intracellularis* within or between affected species and outbreaks because of the lack of isolate differentiation techniques. Isolates from various animal species are antigenically indistinguishable, and standard phenotypic and genomic subtyping methods have not provided an acceptable level of discrimination between isolates within species. Preliminary investigations do suggest that isolates from pigs and horses may represent different genotypes and therefore may be species-specific.

Recently, the whole genome of a porcine *L intracellularis* isolate was sequenced and, not surprisingly, was found to contain numerous uncharacterized genes unique to the organism. This genome was subsequently analyzed for the presence of tandem repeats and this analysis identified very few (four) genomic regions containing variable number tandem repeat (VNTR) sequences, further confirming the monotypic nature of this organism. Comparison of these VNTR sequence profiles provided a sensitive method for analysis of the genetic relatedness of *L intracellularis* isolates obtained from various time points, geographical locations and animal species. This provided insight into the phylogenetic relatedness of these isolates. Furthermore, the technique is applicable to PCR-positive feces and tissue without the necessity of cultivating the bacteria, allowing sub-specific identification of field isolates.

VNTR sequence profiles of *L intracellularis* isolates from outbreaks occurring in pigs, horses, ostriches, spider monkeys, ferrets and hamsters have been analyzed to

determine the sources and phylogenetic relatedness of field isolates. VNTR sequence types obtained from pigs with proliferative enteropathy were very different from those obtained from horses or other non-pig species. Little or no genetic variation was found between isolates from within an outbreak, regardless of the clinical form of the disease, or even in multiple temporal samples taken from the same outbreak site. Slight variations between isolates obtained from outbreaks at different geographic locations were found, but variations among isolates from different continents were no more pronounced than those among isolates from neighboring states.

In conclusion, although *L intracellularis* is antigenically conserved, its genome sequence contains VNTR markers useful for the demonstration of variance among isolates and may even be used to identify species-specificity of certain strains. The utility of this epidemiologic profiling method lies in tracking different isolates of *L intracellularis* obtained from varying animal species, geographical locations, and proliferative enteropathy field outbreaks.

Brachyspira sp

Swine dysentery caused by *B hyodysenteriae* continues to be of global significance in grow-finish pigs. Although this disease has been largely absent from US swine herds for the last 20 years, a number of outbreaks of severe bloody diarrhea and hemorrhagic colitis caused by this pathogen have recently been reported in the US and Canada. At this time, it is not clear whether re-emergence of dysentery is a major economic concern in the US However, considering these recent reports in the US and their impact on pig production globally, better diagnostic and surveillance methods are needed.

The genus *Brachyspira* consists of several gram-negative, anaerobic intestinal spirochete species that colonize the large intestine of a wide range of animals including swine. While some of these species cause intestinal disease, others are typically considered to be nonpathogenic commensals in pigs. *B hyodysenteriae* is a strongly hemolytic species that causes a severe mucohemorrhagic diarrhea in pigs with high morbidity and low to moderate mortality. The other officially recognized swine *Brachyspira* sp. are weakly hemolytic and appear to cause either a milder colitis (ex. *B pilosicoli* and *B intermedia*) or no apparent disease at all (ex. *B murdochii* and *B innocens*).

Current diagnosis

Several PCR-based assays have been developed for the diagnosis and identification of clinical colitis caused by *Brachyspira* pathogens. Perhaps the most useful of these tests is a duplex PCR that can identify a *Brachyspira* isolate as *B hyodysenteriae* or *B pilosicoli*. These tests are limited, however, in that they are not able to identify many of the other *Brachyspira* isolates recovered from animals. For example, over 70% of the *Brachyspira*-positive cultures of clinical swine samples submitted to the Minnesota Veterinary Diagnostic Laboratory from 2008-2011 are "non-typable" using the duplex PCR mentioned above. This is particularly concerning because several of these non-typable isolates are both strongly hemolytic and associated with enteric disease.

Advanced characterization

For this reason, we have developed a diagnostic test that can be used to speciate otherwise non-typable *Brachyspira* isolates from clinical swine samples. This test involves amplifying a highly variable region of the *Brachyspira* NADH Oxidase (*nox*) gene with PCR and then sequencing this PCR product for species identification. The *nox* gene was selected as a target for this assay because it is highly variable in nucleotide sequence between species and therefore has considerable discriminative phylogenetic value.

Seventy-nine non-typable *Brachyspira* isolates obtained from clinical samples across five states submitted to the MVDL during 2009-2011 were characterized by *nox* sequencing. All of the isolates from these pigs tested negative for *B hyodysenteriae* and *B pilosicoli* during the course of their routine clinical diagnostic analysis using the species-specific duplex PCR. After PCR and sequencing of the *nox* gene, sequences were compared with those available in the NCBI databank for species identification. In addition, six isolates were subjected to 16S rDNA sequencing, which is the standard locus targeted for phylogenetic analyses of bacteria.

Of these 79 non-typable *Brachyspira* isolates, 56 were identified as *B murdochii*, four were *B innocens*, and 19 matched an unidentified "*Serpulina* sp" in the NCBI database. All 19 unidentified isolates were strongly hemolytic and recovered from animals presenting with clinical gastrointestinal signs. These isolates most likely represent a new species of *Brachyspira*. Bioinformatic and phylogenetic analyses of the 16S rDNA sequences were consistent with the findings from the *nox* sequences. These data further suggest that the *nox* sequencing assay can be used to accurately identify *Brachyspira* isolates to the species level.

Pathogenesis

The 19 non-typable *Brachyspira* isolates are strongly hemolytic and were recovered from pigs presenting with enteric signs (bloody diarrhea and colitis) consistent with virulent *Brachyspira* infection, which suggests that they are potentially virulent and associated with clinical disease in the field. We are reporting these isolates as 'Novel Strongly Hemolytic (NSH) *Brachyspira'* species and are continuing to characterize them at the phenotypic and

molecular levels. In addition, we have begun to analyze the case histories associated with these isolates in the hope of gaining insight into their pathogenesis and epidemiology.

Summary

Lawsonia sp. A preliminary epidemiological investigation into the relationship between *L intracellularis* isolates from pigs and those from other species including horses suggests that they represent different strains; therefore, the bacteria may exhibit some species-specificity. The ability to type isolates of *L intracellularis* will further enhance our understanding of the transmission dynamics and epidemiology of proliferative enteropathy in pigs, horses, and other species. Advancements such as this will assist in identifying the natural ecology of this unique pathogen.

Brachyspira sp. Many of the *Brachyspira* isolates recovered from pig intestines since 2008 have been non-typable using current diagnostic methods. Sequencing of the *nox* gene for *Brachyspira* sp. identification allowed the classification of 60 of 79 non-typable isolates to officially recognized *Brachyspira* species. Furthermore, a new *Brachyspira* species has been identified that did not match any of the previously known swine *Brachyspira* species by either *nox* sequencing or 16S rDNA analyses. The 19 isolates of this species are strongly hemolytic and were recovered from pigs presenting with enteric signs consistent with virulent *Brachyspira* infection, which suggest that they may be associated with clinical disease in the field. We are continuing to identify the pathogenesis and epidemiology of these isolates by considering associated case histories, as well as characterizing these isolates at the phenotypic and molecular levels.