RESEARCH REPORT
SWINE HEALTH

Title: Application of an Epidemiologic Survey Tool for *Lawsonia intracellularis* – **NPB #07-053**

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Industry Summary:

The objective of this study was to develop a database of *L. intracellularis* genotypic types from various proliferative enteropathy outbreaks. This database will provide bioinformatics data and tools for applying genetic typing more widely and will further enhance our understanding of the transmission dynamics and epidemiology of ileitis in pigs.

New knowledge regarding infection and transmission of *L. intracellularis* was obtained using the variable number tandem repeat (VNTR) genetic typing technique. Though Lawsonia is phenotypically and antigenically conserved, there is genetic variation that exists between isolates. Thus far, no variation was observed between isolates obtained from various clinical types of proliferative enteropathy within herds (barns), including acute (proliferative hemorrhagic enteropathy – PHE), chronic (porcine intestinal adenomatosis – PIA), and subclinical samples. Slight variation between isolates from different geographic locations was detected, though those variations were no greater between isolates from different continents than between isolates from different Midwestern U.S. pig farms. Marked variation exists, however, between isolates from pig and non-pig sources. These variations may be used to track outbreaks occurring in pigs, horses, or other animals.

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Scientific Abstract:

The whole genome of *Lawsonia intracellularis* (strain PHE/MN-00) was analyzed for the presence of tandem repeats and this analysis identified four genomic regions containing putative variable number tandem repeat (VNTR) sequences. These regions consisted of two sequences of ATA_n and two sequences of CA_n nucleic acid repeats. Specific primer sequences were then designed, using Primer 3 software program, upstream and downstream of these four respective regions and used to generate VNTR profiles for isolates of *L. intracellularis* from diverse sources.

L. intracellularis isolates of geographic and temporal diversity, including the type strains PHE/MN1-00, VPB4, 15540D, and 963/93, were used to determine if there was variability of VNTRs among isolates of *L. intracellularis*. The results of our analysis show that each available pure culture *L. intracellularis* isolate examined herein had a unique VNTR profile and these data demonstrate that there are genetic differences between *L. intracellularis* isolates as reflected by VNTR typing.

To assess if the VNTR profiles were conserved and stable in a specific isolate, we tested an isolate prior to cultivating in cell culture, after low- and high- passage cell culture, and after serial passage through a pig. The results also showed that the VNTR profiles of a sample obtained directly from a diseased pig intestine was identical to that obtained after purification and inoculation into cell culture, after low passage, and after serial passage through a pig. These results suggest that the VNTR profiles are relatively stable during laboratory culture and over short periods of time and produce repeatable, unique profiles.

In this project we further used the VNTR method of typing *L. intracellularis* to analyze the genetic relatedness of field outbreaks obtained from pigs with various forms of proliferative enteropathy (PE; ileitis), from different geographical locations, and from various animal species.

During evaluation of a larger number of samples, patterns emerged that provide some insight into the sources and phylogenetic relatedness of field *L. intracellularis* isolates.

We have now extended our database of *L. intracellularis* VNTR types to include isolates from 58 proliferative enteropathy outbreaks occurring in pigs, 20 horses, and 7 other animal species which include ostrich, spider monkey, ferret, hamster, skunk, and rabbit. For this effort, we have performed numerous Lawsonia serologies and fecal PCRs on horses and other species submitted to the University of Minnesota Veterinary Diagnostic Laboratory for the purpose of identifying these outbreaks.

We continue to find that VNTR types obtained from pigs with proliferative enteropathy are very different from those obtained from horses or other non-pig species. From the data analyzed so far, little or no genetic variation (using a 70% cut-off value) was found within clinical types, including proliferative hemorrhagic enteropathy (PHE), porcine intestinal adenomatosis (PIA) and subclinical forms of proliferative enteropathy within a pig barn. Slight variation between isolates from different geographic locations was found, but this difference was no more pronounced between Lawsonia isolates from Minnesota herds than between Minnesota herds and European herds. Marked variation in VNTR types were found between isolates from pig sources and those obtained from non-pig sources.

ntroduction:

Lawsonia intracellularis (LI) is an intracellular bacterium and causative agent of proliferative enteropathy (PE; ileitis) in pigs as well as a variety of other species. Though PE is the most common enteric disease of grow-finish pigs, little is known of the epidemiology of this disease because of the lack of strain differentiation techniques. Isolation and cultivation of LI is extremely difficult due to the intracellular nature of this organism, making the identification of LI subtypes by traditional methods not feasible.

Unlike repetitive sequence polymerase chain reaction (Rep-PCR) and amplified fragment length polymorphism (AFLP) typing methods, PCR-based VNTR analysis does not require pure culture and cultivation of Lawsonia isolates, or molecular manipulation techniques beyond PCR. In VNTR analysis, specific genomic regions containing VNTRs are amplified. The number of repeats is then calculated for each locus, and a profile is generated.

We have recently identified VNTRs in the Lawsonia genome and have determined their utility as markers for differentiating laboratory strains of Lawsonia by the PCR-based VNTR analysis technique. Analysis of VNTR profiles appears to be a useful tool for distinguishing between strains of *L. intracellularis*. The assay proved to be robust and gave identical results on repeat analysis.

We evaluated the VNTR profiles of various samples from four field outbreaks of PE, both acute and chronic forms of the disease. All samples from within an outbreak produced a similar profile and profiles were unique between epidemiologically different outbreaks. It is our hypothesis that through the evaluation of a larger number of samples, a pattern will emerge that provides some insight into the sources and phylogenetic relatedness of these Lawspmoa isolates.

This Lawsonia VNTR database can then be easily mined for field isolate information based on year of isolation, source, geographic location, VNTR pattern, and other epidemiologic and demographic traits. Furthermore, we may be able to determine whether the source of Lawsonia infection in these outbreaks originates from the pig flow (system) or from the site of outbreak (environmental).

Objectives

In a previous Minnesota Pork Board-funded study, we successfully determined the utility of variable number tandem repeat (VNTR) sequences in the *Lawsonia intracellularis* genome as markers for differentiating strains of *L. intracellularis*. The objective of this study was to develop a database of Lawsonia VNTR types from various proliferative enteropathy (PE; ileitis) outbreaks. This database will provide bioinformatics data and tools for applying VNTR typing more widely and will further enhance our understanding of the transmission dynamics and epidemiology of PE in pigs.

Materials and Methods:

One to five PCR positive samples from each of 58 field outbreaks of proliferative enteropathy in pigs were typed by VNTR analysis. Herds for analysis were selected based on results of cross-sectional and/or serial Lawsonia serology and fecal PCR assays. Our objective expanded to include development of a database of *Lawsonia* VNTR types from various proliferative enteropathy outbreaks from other animal species. For this effort, we have performed numerous *Lawsonia* serologies and/or fecal PCRs on horses, ostriches, ferrets, ponies, rabbits, hamsters, opossums, skunks, and primates. In addition, 20 Lawsonia isolates from equine proliferative enteropathy outbreaks from various locations in the U.S. were obtained and VNTR typed.

For all samples, genomic DNA was extracted using the Qiagen extraction kit. Each DNA sample was then subjected to amplification by PCR using the primer sets specific for the four VNTR regions. Amplicons from the PCR products were sequenced and sequence data was then used to calculate the number of repeats at each VNTR loci. The number of tandem repeats for each loci was calculated, creating a VNTR profile for each sample. This VNTR profiles were compared and individual strains identified (Table 1).

All VNTR patterns were analyzed using the BioNumerics software package. Resultant data allowed us to construct a phylogenetic tree using Dice coefficients of similarity and cluster analysis with the unweighted pair group method with arithmetic averages (Figure 1).

Results:

In total, we have collected and analyzed samples from each of 58 unrelated proliferative enteropathy outbreaks in pig herds, from 14 horse herds, and from outbreaks in 5 other animal species (Table 1). VNTR typing profiles as well as isolate description, source, year of isolation, origin, and disease status of *L. intracellularis* isolates used for determining genetic relationships are listed. An investigation into the epidemiological relationships between the samples analyzed is shown in Figure 1.

These data demonstrate that a single VNTR type of *L. intracellularis* is found from all samples obtained from an individual pig barn. Therefore, we have been able to test a larger number of samples from a wider range of animal species and geographical sources than originally planned. We added VNTR typing of *L. intracellularis* isolates obtained from horse and other species for the purpose of phylogenetic and epidemiological comparisons.

From these data analyzed, no genetic variation was found within clinical types of proliferative enteropathy (PHE, PIA, or subclinical) within a pig barn. Using a 70% similarity cut-off, slight variation between isolates from different geographic locations was found, but this difference was no more pronounced between Lawsonia isolates from Midwestern U.S. herds than between Midwestern herds and European herds. Pig isolates formed two separate, distinct clusters. Most non-pig isolates (equine, spider monkey, ferret, hamster, skunk, and rabbit) formed a cluster distinct from all pig isolates.

Marked variation in VNTR types were found between isolates from pig sources and those obtained from non-pig sources. However, there were three interesting exceptions to this pattern. Two equine isolates (417-E and 412-E) clustered with the upper cluster of pig isolates in the VNTR dendogram. The only ostrich isolate tested (102-O) clustered with the lower cluster of pig isolates. All European pig isolates (3-P and 141-P from Denmark; 4-P from Scotland; and 434-1,2,3P from England) were found in the lower pig cluster, but appeared unrelated to each other except for two Danish isolates, which appear almost identical. Year of isolation or source of isolate (pure culture, feces, or intestine) appeared to have no influence on the VNTR type.

Discussion:

Variable number tandem repeats in the genomes of prokaryotes are often associated with a high level of polymorphism and enable bacterial strain differentiation with substantial discriminatory power. The results of our previous investigations demonstrate that the analysis of VNTR profiles is a useful tool for distinguishing between isolates of Lawsonia from field outbreaks of PE. This method of rapidly detecting Lawsonia and tracing specific isolates may allow rapid identification of the source and transmission pattern through epidemiological investigations, and help reduce transmission rate of PE through the implementation of management strategies that will help break the transmission chain. In addition, determination of the VNTR profiles of vaccine isolates will allow differentiation of vaccine and field isolates obtained from pigs shedding Lawsonia.

This VNTR database can be easily mined for field isolate information based on year of isolation, source, geographic location, VNTR pattern, and other epidemiologic and demographic traits. Furthermore, the data suggests that the Lawsonia isolates from pig and non-pig sources are phylogenetically unrelated. An interesting exception to this conclusion is the clustering of the ostrich isolate with the pig isolates. The significance of this is unclear and suggests that Lawsonia isolates from other ratite or avian species should be located and typed to determine if there is any epidemiological connection between these and the pig isolates.

Table 1. Host, description, source, year of isolation, origin, VNTR profile, and disease status of *L. intracellularis* isolates used for determining genetic relationships based on VNTR typing.

Isolate	Description	Source	Year	Origin	Profile	Disease?
PIG HOST*:*						
1-P	PHE00-MN1	Culture	2000	U.S.-MN	16-13-18-12	PHE
2-P	VPB 1-4	Culture	1991	U.S.-MN	15-11-16-9	PHE
3-P	15540D	Culture	1998	Denmark	10-10-17-8	PHE
4-P	963/93	Culture	1993	Scotland	16-12-16-9	PIA
5-1P	Pig herd I-1	Feces	2004	U.S.-MN	12-12-17-12	PIA
5-2P	Pig herd I-2	Feces	2004	U.S.-MN	12-12-17-12	Subclinical
5-3P	Pig herd I-3	Feces	2004	U.S.-MN	12-12-17-12	Subclinical
8-1P	Pig herd II-1	Feces	2005	U.S.-MN	12-13-21-11	PIA
8-2P	Pig herd II-2	Feces	2005	U.S.-MN	12-13-21-11	PIA
8-3P	Pig herd II-3	Feces	2005	U.S.-MN	12-13-21-11	PIA
11-1P	Pig herd III-1	Feces	2006	U.S.-MN	11-10-15-8	PIA
11-2P	Pig herd III-2	Feces	2006	U.S.-MN	11-10-15-8	Subclinical
11-3P	Pig herd III-3	Feces	2006	U.S.-MN	11-10-15-8	Subclinical
14-1P	Pig herd IV-1	Feces	2007	U.S-MN	13-10-18-8	PHE
14-2P	Pig herd IV-2	Feces	2007	U.S-MN	13-10-18-8	PHE
14-3P	Pig herd IV-3	Feces	2007	U.S-MN	13-10-18-8	PHE
18-P	Zoo pig	Intestine	2002	U.S.-MN	17-10-14-11	PHE
125-P	Gilt	Intestine	2004	U.S.-MN	13-11-12-15	PHE
128-P	Grower	Intestine	2004	U.S.-IA	11-13-14-12	PIA
141-P	7-1450	Intestine	2004	Denmark	10-10-17-7	na
142-1P	789	Intestine	2004	U.S.-MN	12-9-16-9	na
142-2P	790	Intestine	2004	U.S.-MN	12-9-16-9	na
148-P	992E	Intestine	2004	U.S.-MN	17-13-18-12	na
176-1P	310	Intestine	2005	U.S.-MN	13-10-15-9	PIA
176-2P	311	Intestine	2005	U.S.-MN	13-10-15-9	PIA
176-3P	312	Intestine	2005	U.S.-MN	13-10-15-9	PIA
215-P	MH	Intestine	2005	U.S.-MN	15-13-21-12	na
221-1P	Diarrhea	Intestine	2005	U.S.-IA	14-11-18-13	na
221-2P	Diarrhea	Intestine	2005	U.S.-IA	14-11-18-13	na
227-1P	Bloody diarrhea	Intestine	2006	U.S.-OK	13-10-11-14	PHE
227-2P	Bloody diarrhea	Feces	2006	U.S.-OK	14-10-11-14	PHE
227-3P	Bloody diarrhea	Feces	2006	U.S.-OK	14-10-12-14	PHE
236-1P	Diarrhea	Feces	2006	U.S.	16-12-12-14	PIA
236-2P	Diarrhea	Feces	2006	U.S.	16-12-12-14	PIA
243-P	Diarrhea	Feces	2006	U.S.	16-13-18-12	na
246-P	MH	Intestine	2006	U.S.	13-10-18-8	PHE
273-P		Culture	2006	U.S.-MN	17-13-18-11	PHE
274-P		Culture	2006	U.S.-MN	12-12-18-11	PHE
275-1P		Intestine	2006	U.S.-MN	14-11-15-12	PIA
275-2P		Intestine	2006	U.S.-MN	14-11-15-12	PIA
278-P		Culture	2006	U.S.-MN	17-13-18-9	na
279-P		Culture	2006	U.S.-MN	15-11-15-12	na
280-P		Paraffin	2006	U.S.-MN	11-12-12-13	na
282-P		Intestine	2006	U.S.-IA	15-11-11-11	na

284-P	Intestine	2006	U.S.-MN	13-10-13-8	na
289-P	Feces	2007	U.S.-IA	11-13-16-21	na
293-P	Paraffin	2007		11-13-16-20	na
309-1P MH	Intestine	2007	U.S.-MN	12-10-17-18	PHE
309-2P MH	Intestine	2007	U.S.-MN	12-10-17-18	PHE
311-1P MH	Intestine	2007	U.S.-MN	15-14-22-12	PHE
311-2P MH	Intestine	2007	U.S.-MN	15-14-22-12	PHE
319	Intestine	2007	U.S.	12-10-16-18	
320-1	Intestine	2007	U.S.	15-13-15-12	PIA
320-2	Intestine	2007	U.S.	15-13-15-12	PIA
350-1P	Feces	2006	U.S.	13-10-18-10	na
350-2P	Feces	2006	U.S.	13-10-18-10	na
350-3P	Feces	2006	U.S.	13-10-18-10	na
350-4P	Feces	2006	U.S.	13-10-18-10	na
350-5P	Feces	2006	U.S.	13-10-18-10	na
351-1P	Feces	2006	U.S.	14-11-18-12	na
351-2P	Feces	2006	U.S.	14-11-18-11	na
351-3P	Feces	2006	U.S.	14-11-18-11	na
352-1P	Feces	2006	U.S.-MN	16-11-12-14	PHE
352-2P	Feces	2006	U.S.-MN	16-11-12-14	PHE
352-3P	Feces	2006	U.S.-MN	16-11-12-14	na
355-P	Feces	2006	U.S.	12-11-19-8	na
357-P	Feces	2006	U.S.	15-10-17-10	na
358-1P	Feces	2007	U.S.	14-14-21-13	PIA
358-2P	Feces	2007	U.S.	15-14-22-12	PIA
358-3P	Feces	2007	U.S.	15-14-21-12	PIA
358-4P	Feces	2007	U.S.	14-14-22-12	PIA
358-5P	Feces	2007	U.S.	14-14-21-13	PIA
359-P	Feces	2007	U.S.	15-10-15-10	na
360-P	Feces	2006	U.S.	17-10-16-13	na
362-1P	Feces	2007	U.S.	12-12-13-9	na
362-2P	Feces	2007	U.S.	12-12-13-9	na
362-3P	Feces	2007	U.S.	11-12-16-15	na
363-1P	Feces	2006	U.S.	13-10-16-9	na
363-2P	Feces	2006	U.S.	13-10-16-9	na
363-3P	Feces	2006	U.S.	13-10-16-9	na
363-4P	Feces	2006	U.S.	13-11-16-9	na
363-5P	Feces	2006	U.S.	13-10-15-9	na
367-1P	Feces	2006	U.S.	15-11-13-11	na
367-2P	Feces	2006	U.S.	15-11-13-11	na
368-P	Feces	2007	U.S.	14-11-14-11	na
414-P	Feces	2007	U.S.	11-12-16-19	na
425-1P	Feces	2007	U.S.	13-10-15-9	na
425-2P	Feces	2007	U.S.	13-10-15-9	na
426-1P	Feces	2007	U.S.	12-11-16-15	na
426-1P	Feces	2007	U.S.	12-11-16-15	na
427-P	Feces	2007	U.S.	12-13-13-15	na
428-P	Feces	2007	U.S.	15-11-18-11	na
434-1	Feces	2007	England	13-8-11-9	na
434-2	Feces	2007	England	13-8-10-9	na
434-3	Feces	2007	England	13-8-10-9	na

491-1P		Feces	2008	U.S. – MN	10-11-19-13	na
491-2P		Feces	2008	U.S. – MN	10-11-19-13	na
491-3P		Feces	2008	U.S. – MN	10-11-19-13	na
491-4P		Feces	2008	U.S. – MN	10-11-19-13	na
491-5P		Feces	2008	U.S. – MN	10-11-19-12	na
496-1P		Feces	2008	U.S. – IN	14-10-20-10	na
496-2P		Feces	2008	U.S. – IN	14-10-19-10	na
496-3P		Feces	2008	U.S. – IN	14-10-20-10	na
518-P		Feces	2008	U.S.	13-10-14-12	na
521-P		Feces	2008	U.S.	15-10-16-9	na
522-P		Feces	2008	U.S.	13-11-22-9	na

NON-PIG, MISCELLANOUS ANIMAL HOST*:*

102-O	Ostrich	Intestine	2002	United States	15-5-15-8	PE
101-M	Spider monkey	Paraffin	1990	United States	13-5-18-13	PE
100-F	Ferret	Paraffin	1990	Scandinavia	16-5-19-8	PE
207-H	Hamster	Intestine		U.S.	16-5-16-18	PE
326-S	Skunk	Feces	2007	U.S.-CA	10-5-12-10	na
327-S	Skunk	Feces	2007	U.S.–CA	10-5-12-10	na
331-R	Rabbit	Feces	2007	U.S –CA	17-5-18-12	na

EQUINE HOST*:*

23-E	Foal/96	Culture	1996	U.S.-MO	17-5-16-12	PE
104-E	Mini pony	Intestine	2001	U.S.-IA	14-5-13-9	PE
217-E	Mare	Feces	2006	U.S.	11-5-15-11	PE
218-E		Feces	2006	U.S.	14-5-13-12	PE
223-E	Foal/217-E	Feces	2006	U.S.	11-5-14-11	PE
224-E		Feces	2006	U.S.	12-5-16-11	PE
225-E		Feces	2006	U.S.	15-5-16-11	PE
233-E	Icelandic	Feces	2006	U.S.	15-5-16-11	PE
248-E		Feces	2006	U.S.	11-5-12-9	PE
249-E		Feces	2006	U.S.	9-5-12-13	PE
250-E		Feces	2006	U.S.	9-5-13-12	PE
283-E		Feces	2006	U.S.	14-5-17-9	PE
410-E		Feces	2007	U.S.-MI	11-5-17-15	PE
411-E		Feces	2007	U.S.-	11-5-13-10	PE
412-E		Feces	2007	U.S.- OH	12-5-13-15	PE
413-E		Feces	2007	U.S.- OR	14-5-16-16	PE
417-E		Feces	2008	U.S.- MD	14-5-15-13	PE
418-E		Feces	2008	U.S.- NC	13-5-17-8	PE
419-E		Feces	2008	U.S.- KY	12-5-17-16	PE
421-E		Feces	2008	U.S.- OH	16-5-20-17	PE

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na=information not available; PE=proliferative enteropathy; PHE=proliferative hemorrhagic enteropathy; PIA=porcine intestinal adenomatosis; E=equine; P=porcine; O=ostrich; M=spider monkey; F=ferret; H=hamster; S=skunk; R=rabbit.

Figure 1. Dendogram showing VNTR relationships between *L. intracellularis* isolates from various pig herds and animal species. See Table 1 for strain epidemiologic data. E(green)=equine; P(red)=porcine; O=ostrich; M=spider monkey; F=ferret; H=hamster; S=skunk; R=rabbit.