Martina Ustulin

Stage on isolation, identification and characterization of *Brachyspira* spp. by University of Leon (Spain), Department of Animal Health.

The aim of the three week stage conducted at the University of Leon was the acquisition of the technical know-how regarding the isolation, identification and characterization of Brachyspira spp., including antibiotic susceptibility analysis.
Brachyspira hyodysenterie and *Brachyspira pilosicoli* are considered etiological agents of respectively swine dysentery and intestinal spirochaetosis but other Brachyspira spp. can be involved in the pathogenesis. Those are severe diarrheal diseases affecting growing and finishing pigs and causing heavy economic loss due to increased mortality, decreased rate of growth and cost of treatments.
The stage was developed in order to mimic the management of clinical sample from the isolation of *Brachispira* spp. from feces to the last phase of genetical characterization of the isolate.

The first step of the protocol used at Leon Univeristy for the diagnosis of Brachyspira spp. infection is the isolation of strains from fecal samples.
The media involved are the following:
CVS (colistin-vancomycin-spectinomycin): selective solid medium containing colistina, vancomicina and spectinomicina, used to reduce the growth of contaminant bacteria and to detect heamolysis of *Brachyspira* strains.
BA (blood agar): solid medium used to isolate strains, allows seeing haemolysis.
BHI (Brain hearth infusion): liquid medium used for Minimal Inhibitory Concentration (MIC) testing.
FBS (fetal bovine serum): supplement for BHI and TSB.
TSB (Trypton soy agar): liquid medium used for the storage at –80°C of pure strains.
FAA (Fastidious anaerobe agar): solid medium that allows *Brachyspira* spp. to grow on the surface of the plate.

The isolation protocol starts with direct plating of feces on CVS and incubation of plates at 41°C in anaerobic conditions. After 48 hours the plates are checked for the typical complete haemolysis of *Brachyspira* spp. If it is not present, plates are reincubated and checked every 24 hours for up to 7 days.
If the characteristic haemolysis caused by *Brachyspira* spp. is recognized, a small portion

of the agar should be plated on a BA or, if the original plate has an high amount of contaminating bacteria, on another plate of CVS and incubated 2 days at 41°C.
Brachyspira spp. does not form colonies on the surface of the plates but grows inside the agar on both CVS and BA. Its presence is suggested by a diffused haemolysis. In order to rapidly confirm the presence of typical helical *Brachyspira* spp., a small amount of agar can be placed on a slide with a drop of physiologic solution and examined under dark-field microscopy.
To obtain visible colonies the pure strain should be plated on FAA. After bacterial growth it's possible to proceed with MIC analyses or to freeze the strain for long time storage.

MIC analyses are performed using commercial plates (VetMIC Brachy, SVA, Sweden): a small amount of bacterial growth from FAA is suspended in 2 ml of BHI; then 500 ul of this suspension is added to 30 ml of a solution of BHI with 10% of FBS. 500 ul of the final solution are distributed in each well of the MIC plate. The plate is incubated for 4 days at 40°C under agitation.
At the end of the incubation the growth will be visible as turbidity in the liquid medium. The lowest antibiotic concentration where there is no growth represents the MIC.

The *Brachyspira* spp. strains are frozen in a liquid medium of 66,6% TSB and 33,3% FBS. The strains are kept at -20°C for a night and then stored at -80°C.
Previous experience indicates that media containing the cryo-conservant glycerol are not suitable for *Brachyspira* spp., although with the actual procedure many strains loose vitality in the freezing-thaw process.

The differentiation among Brachyspira species is performed by PCR. It's not possible to distinguish the species by morphology or by biochemical tests. The only biochemical test previously done, the indole spot, is not considered reliable because indole negative *B. hyodisenteriae* are reported. PCR targeting specie-specific region is the most accurate and fast way for differentiation.
According to the procedure of Leon University, the isolated strains are subjected to a duplex PCR for the most frequent pathogenic species: *B. hyodisenteriae* and *pilosicoli*. The target gene for *B. hyodisenteriae* is the tlyA, while for *B. pilosicoli* the target is located on the 16S gene. In case of negative result the strain is tested for the non pathogenic specie *B. murdochii*, which shows on blood agar haemolysis very similar to that caused by pathogenic species. The target for *B. murdochii* is located on the nox gene.

Otherwise the strain can be subjected to nox gene PCR and the amplified fragment can be sequenced and analyzed to identify the specie.
Sequencing of nox gene is the method used to identify the recently discovered *B. hampsoni*. There are still few isolations of *B. hampsoni* from pigs farm, but it is frequently found in wild boars.

Further investigation on strains of *B. hyodisenteriae* can be done by MLVA or MLST.
MLVA is a procedure based on PCR amplification of loci of minisatellites called “variable number of tandem repeats” (VNTR) consisting of DNA repeats present in all bacterial genome that can be used to clusterize bacterial strains.
In *B. hyodisenteriae* genome, 8 VNTR loci have been identified and can be used for this test.
Every locus should be amplified by a PCR, using primers whose targets are the conserved DNA regions external to the repeated locuses. The number of repeats is deduced from the expected size of the single minisatellite, the size of the external region, and the size of the obtained amplification product, according to the subsequent formula: (product size – external region size)/ repeat size.
The complete MLVA analyses can be a useful tool to compare different strains in order to understand the epidemiological traits of the infection.
The MLST test uses the same target regions as the MLVA but the amplification product is sequenced. This test is more accurate and gives supplemental information about mutation in the target sequence but it is rarely performed, being more expensive and time consuming.

Another possibility for *B. hyodisenteriae* diagnostics is to perform a quantitative real time PCR (qPCR). This method is specific for *B. hyodisenteriae* and does not require an isolate. On the other side it is a fast way to detect and quantify *B. hyodisenteriae* in a fecal or intestinal sample. The total DNA is extracted from stool with a commercial kit and subjected to a real time PCR at the same time of standard samples with a known amount of target DNA. The software calculates the number of copies present in the original sample through a comparison with the standard curve elaborated from the standards.
To obtain the standard curve, a reference strain of *B. hyodisenteriae* is cultured in liquid medium, BHI with 10% FBS, for three days. The number of bacteria after the incubation is evaluated using a modified Neubauer chamber and the medium is diluted until the required concentrations are reached. Five or six different standard concentrations, from 10^6 to

10^1 cell/ml, are needed for the curve. The standards are extracted with the same commercial kit used for feces, in order to purify the maximum amount of DNA.

My overall evaluation of the experience is positive. The diagnostic protocol applied at Leon University is a good combination of traditional microbiological techniques and more modern genetic analysis.
My first purpose for the next period will be to put into practice isolation procedure and identification PCR. Subsequently it will be possible to implement the MIC testing.
The development of the MLVA can be evaluated in a subsequent time, after collecting a sufficient number of strains.