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Immunoproteomic analysis of *Lawsonia intracellularis* identifies candidate neutralizing antibody targets for use in subunit vaccine development

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ABSTRACT

Lawsonia intracellularis is an obligate intracellular microorganism and the causative agent of porcine proliferative enteropathy. Due to its obligate intracellular nature, characterization of antigens and proteins involved in host-pathogen interaction and immune recognition have been difficult to achieve using conventional microbiological techniques. In this work, we used 2-dimensional gel electrophoresis coupled with Western-immunoblotting, mass spectrometry and bioinformatics to identify bacterial proteins that interact *in vitro* with pig intestinal cells (IPEC-1), have immunogenic properties and the potential to be used as subunit vaccine antigens. We detected eleven immunogenic bacterial proteins from which fliC (LI0710), LI1153 (annotated by NCBI as Putative protein N), and LI0649 (annotated as autotransporter) were predicted to be expressed on the outer membrane while LI0169 (oppA; annotated as ABC dipeptide transport system) was predicted to be periplasmic with a transmembrane domain forming a central pore through the plasma membrane. Genes coding for these four proteins were cloned and expressed in *Escherichia coli* and the corresponding recombinant proteins were purified using affinity chromatography. Porcine hyperimmune serum against whole *Lawsonia* lysate established that all four recombinant proteins were immunogenic. Further, rabbit hyperimmune sera generated against the vaccine strain of *L. intracellularis* and rabbit serum specific for each recombinant protein showed an inhibitory effect on the attachment and penetration of live, avirulent *L. intracellularis*, thus indicating that each protein is a potential neutralizing antibody target and a candidate for subunit vaccine formulation.

1. Introduction

Lawsonia intracellularis is an obligate intracellular Gram negative bacteria with fastidious microaerophilic growth requirements. It is the causative agent of proliferative enteropathy (PE) which is an economically important disease in pigs and is commonly found in other mammals such as non-human primates, horses and rodents or even birds emus and ostriches (Kroll et al., 2005b). *L. intracellularis* infects enterocytes in distal ileum and jejunum and cannot replicate outside eukaryotic cells. Attachment of bacteria to enterocytes is an important step in bacterial infection but the mechanism by which these bacteria interact with the host cells has not yet been determined (Vannucci and Gebhart, 2014). Proteins which comprise the Type III secretion system (T3SS), a common secretion system found in many enteroinvasive pathogens that plays the role in invasion and suppression of innate defences, have been detected in three *Lawsonia* isolates (Pilar Alberdi et al., 2009).

These T3SS proteins and other uncharacterized bacterial proteins that facilitate contact with enterocytes are potentially important immunogens as they are expressed on the surface and therefore accessible to the host immune system. Establishing that these proteins are important for attachment has been hampered by the obligate intracellular growth requirement of *L. intracellularis* and by difficulties of removing eukaryotic host cell proteins from sample preparation. Modern proteomic analysis coupled with a fully sequenced and partially annotated *L. intracellularis* genome offers the means to identify potential antigens. For example, *L. intracellularis* autotransporter protein (LatA) was detected by Watson et al. (2011) using mass spectrometry (MS) and bioinformatics (Watson et al., 2011). The same group applied shotgun proteomic analysis to identify 19 unique proteins during *in vitro*

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infection of which two proteins, LI0841 and LI0902, were shown to have antigenic properties (Watson et al., 2014). The success and efficacy of proteomic analysis to detect yet uncharacterized *L. intracellularis* proteins prompted us to perform 2-dimensional gel electrophoresis (2DE) coupled with Western blot analysis to identify antigens that could be detected by hyperimmune serum and be used towards formulation of neutralizing subunit vaccine.

2DE is an efficient analytical tool for separation of complex protein mixtures from tissue, mammalian and bacterial cells, and secretions (Magdeldin et al., 2014) and, 2DE coupled with Western blot (WB) and Mass Spectrometry (MS), can detect antigens from bacteria (Lahner et al., 2011) (Havlasová et al., 2005), cancer cells (Kellner et al., 2002) and fungi (Pitarch et al., 1999) that were recognized by the human immune system. Bacterial cells are good candidates for 2DE analysis due to their lower complexity compared to mammalian cells, which is an essential requirement for successful protein separation (Rabilloud et al., 2010).

To detect *L. intracellularis* antigenic proteins that could be used as recombinant proteins to formulate subunit vaccines, we combined the separation power of 2DE with WB and MS. The resulting target proteins were cloned, expressed and then evaluated for their immunoreactivity and capacity to interfere with bacterial-host interactions under competitive conditions. Our analysis revealed eleven unique *L. intracellularis* proteins and further bioinformatics analysis and flow cytometry assay indicated that 4 proteins were predicted to be possible vaccine antigens.

2. Materials and methods

2.1. Animals and generation of immune serum

All animal experimental procedures were performed in accordance with Procedures for Ethics Review of Animal Use Protocols and approved by University Committee on Animal Care and Supply (UCACS), University of Saskatchewan.

Rabbit serum against whole cell *L. intracellularis* was acquired as reported in Obradovic et al. (2016). Briefly, to obtain hyperimmune serum specific for each recombinant protein of interest, four female New Zealand White rabbits (2–3 kg weight) were kept in isolation units in the Animal Care Unit of VIDO-InterVac. Rabbits were sourced from *L. intracellularis*-free stock and, before immunization, we established that their sera did not recognize *L. intracellularis* proteins from whole cell or recombinant proteins in WB (Supplementary Fig. 1B, lane 2, and Supp. Fig. 2). Rabbits were injected *via* the subcutaneous route with the inoculum consisting of 100 µg of recombinant protein for the first immunization and 50 µg of the same recombinant protein for two booster injections. For all injections, recombinant proteins were suspended in 500 µl sterile PBS and mixed with 500 µl sterile Incomplete Freund's adjuvant (Sigma-Aldrich) to 1 ml final volume and the vaccines were administered subcutaneously at 4 injection spots with 250 µl of inoculum per site. Each rabbit received just one of the four recombinant proteins on day 0, 20, and 40. Rabbit immune sera were collected *via* exsanguination following euthanasia of the rabbits (Euthanyl, Bimeda-MTC Animal Health INC., Cambridge ON, Canada) 60 days after the first vaccination. Pig sera was obtained from field animal that were naturally exposed to infectious *L. intracellularis* and showed clinical signs of PHE. All blood samples were collected in SST tubes, centrifuged (2500 × *g*) then sera were stored at −20 °C until use.

2.2. Removal of antibodies against LPS from rabbit immune serum

To preclear any LPS-specific antibodies from sera, we incubated, 10,000 EU/ml LPS from *E. coli* 055:B5 (Sigma-Aldrich), per ml of each rabbit serum for one hour at room temperature to allow serum anti-LPS antibodies to bind. After one hour of incubation, endotoxin removing gel (Pierce High-Capacity Endotoxin removing gel, Thermo scientific) was used according to manufacturer's protocol, to remove LPS and LPS-bound antibodies from rabbit sera. Flow through fractions before elution of LPS and after elution of LPS were collected and used in WB analysis to test the efficacy of the LPS-antibody clearing procedure. WB was performed on LPS (as a control), whole cell *L. intracellularis*, and all 4 recombinant proteins and detection was performed using LPS cleared rabbit serum in 1:500 dilution as primary antibody and anti-rabbit IR800 antibody (1 µg/ml; Li-COR) as secondary antibody (Supplementary Fig. 1B, C). As expected, endotoxin removal and preclearing sera for LPS-specific antibodies did not remove antibodies specific for *L. intracellularis* proteins.

2.3. Cell culture conditions

Undifferentiated porcine intestinal epithelial cell lines (IPEC-1), which were derived from the jejunum and ileum of unsuckled one day old piglets (Cano et al., 2013), were cultured and maintained in DMEM/F-12 (SH30271.01; HyClone, Thermo Fisher Scientific, San Jose, CA, USA) with 5% Fetal bovine serum (FBS) (Sigma-Aldrich, Oakville, ON, Canada), penicillin/streptomycin (Gibco 5000 units/ml Penicillin, 5000 µg/ml Streptomycin), insulin (10 µg/ml), transferrin (5.5 µg/ml), selenium (5 ng/ml) (ITS; (Sigma-Aldrich)) and 5 ng/ml of epidermal growth factor (Sigma-Aldrich). Cells were kept in humidified incubator in an atmosphere of 5% CO_2 and 95% air at 37 °C and passaged two times per week at 1:5 ratio in Corning 75 cm^2 cell culture flasks. IPEC-1 cells used for *L. intracellularis* infection and neutralization assays were grown as indicated above but in the absence of antibiotics.

2.4. L. intracellularis protein sample preparation

L. intracellularis pellets were prepared from Enterisol® (Boehringer Ingelheim), an avirulent, vaccine strain of *L.intracellularis* that was grown for 2 passages in McCoy cells. To prepare for lysis, McCoy cells were resuspended in RIPA buffer (0.05 M Tris pH 8 (Bio Basic Canada INC, Markham. ON, Canada); 0.15 M Sodium Chloride (Bio Basic Canada INC.); 0.10% SDS, (Bio Basic Canada INC.); 1% Deoxycholic acid, (VWR-Amresco, Dublin, Ireland); 1% Nonidet P40 substitute (Sigma-Aldrich); distilled water) complete with 0.1 mM PMSF (Sigma-Aldrich) in isopropanol (Sigma-Aldrich). Lysis of McCoy cells to extract the bacteria was performed as published in (Lawson et al., 1993). The supernatant was carefully discarded and the pellet dried before suspending with $NaHCO_3$ buffer and quantifying by BCA analysis (following the manufacturer's instructions (Pierce, Thermo Fisher Scientific)).

L. intracellularis proteins were labelled with Cy5 dye (GE Healthcare Life Sciences-Amersham Biosciences, Mississauga, ON, Canada) in dye/protein molar ratio of 8:1, following the manufacturer's recommended protocols. The mixture was incubated for 4 h at room temperature in the dark. Unbound dye was removed by size filtration using 3000 MWCO 15 ml volume filters (Millipore, Etobicoke, ON, Canada) with 4 additional washes. The final concentration of Cy5-labelled L. *intracellularis* proteins was determined by BCA assay (Pierce) prior to 2DE.

2.5. Binding of Cy5-labeled L. intracellularis proteins to IPEC cells

IPEC-1 cells were grown to confluence in T-75 flasks trypsinized and washed three times with antibiotic- and FBS-free IPEC medium. Next, 1×10^6 IPEC cells were incubated with 700 µg of Cy5 labelled bacterial proteins for 3 h with gentle nutation at 4 °C. Cells were centrifuged as indicated above and the unbound *L. intracellularis* proteins were removed with the supernatant. The IPEC-1 cells and bound *L. intracellularis* proteins were then suspended in RIPA buffer with PMSF (Sigma-Aldrich) and subjected to repeated freeze/thaws as indicated above. IPEC-1 proteins and Cy5-labeled adherent *L. intracellularis* proteins were then subjected to 2DE.

2.6. Two dimensional gel electrophoresis

Proteins from lysed IPEC-1 cells and bound Cy-5 labeled *L. intracellularis* (250 µg analytical gels 600 µg for prep gels) were suspended in rehydration buffer overnight (9 M urea, 2% CHAPS (Fisher BioReagents), 1% DTT (Promega, Medison, Wi, USA), 2% pharmalyte 5–8 (GE Healthcare), 0.002% bromophenol blue (BioRad, Hercules, CA, USA) and were loaded onto an IPG strip (Immobiline^{tm} DryStrip, pH 4–7, 13 cm, GE Healthcare). The strips were individually subjected to isoelectric focusing (IEF) using IPGphor device (GE Healthcare-Amersham Biosciences) using a stepwise protocol (150 V step and hold for 3 h, 300 V step and hold 1200 Vh, 1000 V gradient for 3900 Vh, 8000 V gradient for 13,500 Vh and 8000 V step and hold for 25,000 Vh). After IEF, both IPG strips were stored at −80 °C. IPG strips with isoelectric focused proteins were thawed at room temperature and equilibrated with SDS equilibration buffer with 1% DTT (6 M urea, 75 mM Tris-HCl pH 8.8, 29.3% glycerol, 2% SDS and 0.002% bromophenol blue) for 15 min at room temperature followed by washing with SDS equilibration buffer with 2.5% Iodoacetamide (GE Healthcare) for 15 min. After equilibration, strips were placed over SDS gels and covered with sealing solution (0.5% agarose in 1x SDS running buffer). Second dimension electrophoresis was performed using BIO-RAD protean II xi Cell Apparatus and two medium size, 10% SDS PAGE gels. Electrophoresis was performed using 90 V constant voltage for 16 h with constant water cooling of apparatus (Bio-Rad Power pack 200).

2.7. Western blot analysis and silver staining

Proteins on the analytical gel were transferred with semi-dry transfer to a nitrocellulose membrane (BIO-RAD, 162-0094) using Bio-Rad Trans-Blot SD Semi-Dry transfer cell (15 V for 60 min) and then Western blot analysis was performed using rabbit hyperimmune serum (1:500; obtained from rabbits prior to immunization (negative sera) and from rabbits immunized with whole bacteria) as primary antibodies. Anti-rabbit IR 800 antibody (1 µg/ml; Li-COR, Lincoln, NE, USA) was used as secondary antibody. The membrane was scanned with Odyssey scanner (Li-COR) in the IR 700 and IR 800 channels. IR800-stained proteins indicate bacterial proteins with affinity for IPEC-1 cells and bound by rabbit serum against whole bacteria (See Fig. 1A (cropped image) and Supplementary Fig. 3A).

For the preparative gel, *L. intracellularis* proteins were stained with Silver stain kit, (PROTSIL-1-KT, Sigma Aldrich) according to manufacturer's protocol and this gel was reserved for excising gel spots for Mass Spectrometry analysis (See Fig. 1B (cropped image) and Supplementary Fig. 3B).

2.8. Preparing samples for mass spectrometry

Silver-stained proteins on the preparative gel which correspond to IR-800 labelled proteins detected by WB analysis were excised from the gel using a sterile biopsy punch (3 mm diameter) to avoid contamination of gel samples with environmental proteins. Gel plugs were collected, and stored in ultrapure water at −20 °C. Gel plug samples (annotated as 1.4, 2.3, 3.1, 3.2 and 4 (Fig. 1B)) were sent to Plateforme Protéomique Centre de Recherche du CHU de Québec CHUL, Québec, Canada for Mass Spectrometry analysis.

2.9. Mass spectrometry

Protein digestion and MS analyses were performed by the Proteomics Platform of the CHU de Québec Research Center (Quebec, Canada). Bands of interest were reduced and alkylated then digested with trypsin at 37C for 18 h. Peptides were extracted using 1% formic acid, 2% acetonitrile followed by 1% formic acid, 50% acetonitrile then dried. Peptides were then injected on a Dionex Ultimate 300 nanoRSLC chromatography system (Thermo Fisher Scientific / Dionex Softron GmbH, Germering, Germany) connected to an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific, San Jose, CA,USA) on a 50 cm 75 u reversed phase column made of ReproSil-Pur *C18-AQ* 3-µm resin (Dr. Maisch HPLC GmbH, Ammerbuch-Entringen, Germany) for 60 min. Full scan mass spectra (350–1800 m/z) were acquired in the orbitrap followed by fragmentation of the most intense ions in the HCD cell with detection in the linear trap.

2.10. Database searching

All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.5.1). Mascot was set up to search the TAX_Desulfovibrio_CI_194924_20160714 database (unknown version, 104,802 entries) assuming digestion with trypsin. Mascot was searched

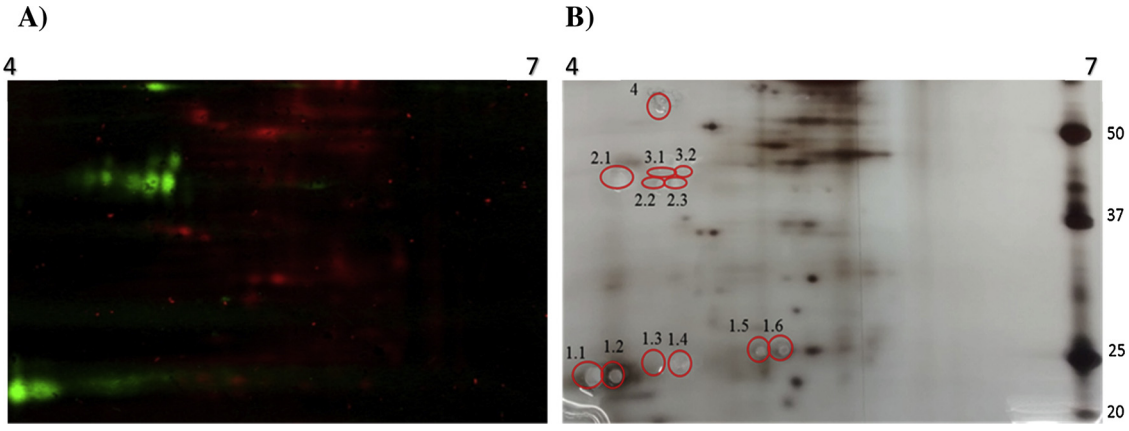

Fig. 1. Protein separation by 2-Dimensional electrophoresis and selection of spots for mass spectrometry, cropped image. Cy5-labeled Lawsonia intracellularis proteins that bound to IPEC1 cells were subjected to isoelectric focusing using IPG strip 4–7 (horizontal plane) followed by SDS-PAGE using 10% SDS-PAGE gel (vertical plane). Molecular weight markers are indicated (kD). A) Proteins were transferred to a nitrocellulose membrane and incubated with rabbit hyperimmune serum as primary antibody (1:500) and anti-rabbit IR800 secondary antibody (1:10,000). Proteins visible in IR700 channel are red and indicate all Cy5 labelled bacterial proteins. The proteins visible in IR800 channel are green and indicate proteins bound by rabbit antibodies from rabbits immunized with whole-cell L. intracellularis. Note, all green proteins are also red and therefore should appear yellow in colour but they are overwhelmed by the green fluorescence. B) A replicate gel was stained with PROTSil-1 silver stain kit. Position and numbering of gel spots are indicated by red circles. Gel plug samples 1.4, 2.3, 3.1, 3.2 and 4 were sent to Plateforme proteomique Centre de recherché du CHU de Québec CHUL, Québec, Canada for Mass Spectrometry analysis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

with a fragment ion mass tolerance of 0.60 Da and a parent ion tolerance of 10.0 PPM. Carbamidomethyl of cysteine was specified in Mascot as a fixed modification. Deamidated asparagine and glutamine and oxidation of methionine were specified in Mascot as variable modifications. Two missed cleavages were allowed.

2.11. Criteria for protein identification

Scaffold (version Scaffold_4.7.5, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Peptide Prophet algorithm (Keller et al., 2002) with Scaffold delta-mass correction. Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al., 2003). Proteins that contain similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

2.12. Bioinformatics analysis of proteins

Amino acid sequences from peptides identified by mass spectrometry were submitted to BLAST algorithm (Altschul et al., 1997) to identify corresponding proteins. Prediction of functional domains and motifs was performed using UniProt (https://www.uniprot.org) and Pfam (http://pfam.xfam.org). To compute physical and chemical parameters of MS/MS detected proteins, protein sequences were submitted to ExPasy ProtParam tool (https://web.expasy.org/protparam) and proteins are listed in Table 1 (Gasteiger et al., 2005). To predict presence of B-cell epitopes, sequences were submitted to BepiPred-2.0, a web server for sequence-based B-cell epitope prediction (http://tools.iedb.org/bcell/) (Jespersen et al., 2017)

2.13. Molecular cloning

Bacterial genomic DNA from avirulent *L. intracellularis* N343 was isolated using GenElute^{tm} Bacterial Genomic DNA Kit, (Sigma-Aldrich) following manufacturer's protocol. PCR amplification of open reading frames were performed using Phusion^{R} High-Fidelity PCR kit, (NEB, Ispwitch, MA, USA). Primers with cleavage sites for in-frame cloning with the N-terminal his-tag contained within the expression vector pET30a are listed in Table 2; these were based on the genomic sequence of *L. intracellularis* (PHE/MN1-00). DNA was gel purified cut with the appropriate restriction enzymes (either BamHI/XhoI or NcoI/XhoI) and ligated into pET30a using T4 DNA Ligase (NEB). The resulting constructs were transformed into competent Dh5α *E. coli* using standard procedures (Sambrook and Russell, 2001). Stocks of the plasmid DNAs were isolated from the bacteria using Presto Mini plasmid kit (Genaid, New Taipei City, Taiwan). Finally the cloned sequences and vector insertion were validated by DNA sequencing and restriction digests (data not shown).

2.14. Expression and purification of recombinant proteins

Recombinant proteins were expressed in LOBSTR-BL21 (DE3) pRosetta2 *E. coli* after transformation with plasmid. *E. coli* grown to mid-exponential phase (OD = 0.6) in 2xYT medium and induced by the addition of IPTG to 0.5 mM. Incubation was continued for16 h at 16 °C with shaking at 200 rpm. Bacteria were harvested by centrifugation and resuspended in urea lysis buffer (8 M urea, 50 mM $NaHPO_4$, 300 mM NaCl) followed by sonication to lyse bacterial cells. Lysate was centrifuged at 20,000 x g for 15 min to remove insoluble material. Recombinant His-tagged protein was purified from the supernatant using His60 Superflow Resin (Clontech, Takara Bio USA, Inc., Mountain View, CA, USA) equilibrated with urea lysis buffer. Proteins were purified in accordance with the manufacturer's protocol and eluted protein was dialyzed in PBS. Recombinant proteins were subjected to SDS-PAGE (rLI1153 10% SDS gel, rFlagellin LI0710 12% SDS gel, rLI0649 8% SDS gel, and rLI0169 10% SDS gel), and analysed by Western blot using pigs sera from animals with clinical symptoms of PHE.

2.15. Neutralization assay

To determine the effect of recombinant *L. intracellularis* protein-specific sera on penetration of bacteria into IPEC cells, we performed neutralization assay using CFSE stained bacteria, as previously described (Obradovic et al., 2016). Briefly, CFSE was used to stain avirulent *L. intracellularis* (propagated from Enterisol® as indicated above) and stained bacteria were incubated with low (500 µg/ml), medium (1000 µg/ml) and high (2000 µg/ml) complement-inactivated, LPS-antibody precleared rabbit hyperimmune serum for 1 h at room temperature. Bacteria bound with serum antibodies were used to infect 10^5 IPEC-1 cells in 24 well plate (Corning) incubated in a tri-gas environment (10% hydrogen, 10% carbon dioxide and 80% nitrogen gas (Praxair Canada Inc., Mississauga, ON, Canada)) in zip lock bags at 37 °C. (Vannucci et al., 2012). After 4 h incubation, cells were trypsinized then centrifuged at 500 x g for 5 min to remove medium and unbound bacteria. The cells were then re-suspended in PBS (Gibco Life Technologies) with 2% FBS (Gibco Life Technologies) and analyzed by flow cytometer. This assay was repeated 4 times independently to obtain biological replicates. Flow cytometric analysis was performed using a BD FACS Calibur™ flow cytometer (BD Biosciences, Mississauga, ON, Canada). CFSE fluorescence was detected in the FL1 channel with gating selected based on uninfected IPEC-1 cells (negative control) and IPEC-1 cells infected with CFSE labelled bacteria (positive control). Thirty thousand events were acquired per sample and flow cytometer results were analyzed in Kaluza software (Beckman-Coulter, Indianapolis, Indiana, USA). The percent inhibition was calculated using

Table 1
Lawsonia intracellularis proteins detected by mass spectrometry.

PHE/MN1-00 Locus tag	NCBI PHE/MN1-00 annotation	Spot	Sequence coverage %	# Peptides identified	Probability %	MW (kDa)/PI (ExPasy)
LI0710	Flagellin, FliC	2.3	36	9	100	31/5.97
LI0649	Autotransporter	4	4	4	100	91.86/4.81
LI0169	ABC type dipeptide transport system	4	4	2	100	63.615/6.52
LI1153	Putative outer protein N	3.1	7	2	100	44/4.62
LI0786	DNA polymerase III subunit beta	3.2	13	6	100	43.622/4.70
LI1171	5'nucliotidase/2'3' cyclic phosphodiesterase	4	11	5	100	62.294/5.71
LI0608	Cysteine-tRNA ligase	4	5	2	100	55.492/5.55
LIO726	S-adenosylmethionine synthase	2.3	6	2	100	44.352/5.41
LI0823	Xaa-Pro aminopeptidase	2.3	5	2	100	40.953/5.6
LI0625	60 kDa chaperon, groL	3.2	5	2	100	58.641/5.63
LI0794	ATP-dependant Clp protease proteolytic subunit	1.4	21	4	100	23.465/4.73

Table 2
Bacterial isolates, plasmids and primers used in this study.

Isolate	Description	Origin
Avirulent *L. intracellularis*	Vaccine strain, B3903 isolate	Live vaccine, Enterisol®
Dh5α	*E. coli* cloning strain	
LOBSTR-BL21(DE3) pRosetta2	*E. coli* expression strain	Kerafast
Plasmid		
pET30a	IPTG inducible, T7 expression vector, C-terminal 6xHis tag	Novagen
Primers	Sequence 5' to 3'	Restriction site
Flag-F	GACGGATCCTCTCTTGTCATTAATAACAACCTGATGG	BamHI
Flag-R	GAGCTCGAGTTAGCCAATAAGTTGCTGAGCC	XhoI
LI1153-F	GAGGGATCCGCTAATGTTAGTGGAATCCCTGC	BamHI
LI1153-R	GAGCTCGAGTTATTGTATATTATTTTCATCTGGTTGTAGTG	XhoI
LI0649-F	TCCCATGGCTGAGGCTGTTGAACACTTTG	NcoI
LI0649-R	GGCTCGAGTTAGAATCTATAAGTAGCTCCTACC	XhoI
LI0169-F	CGCCATGGACAGTGATGAGGACCTTAGTACAG	NcoI
LI0169-R	AGCTCGAGTAGGAATCCACCACTGATCAAG	XhoI

the following formula: Percent inhibition = (1- % of fluorescence of CFSE bacteria incubated with serum / % of fluorescence of CFSE bacteria (control)) x 100.

2.16. Statistical analysis

The Shapiro-Wilk normality test was used to determine whether data follows a Gaussian distribution. One way ordinary ANOVA test was used to compare means of values of percentage of inhibition of each serum. All statistical analyses and graphing were performed using GraphPad Prism 5 software (GraphPad Software, San Diego, CA). Differences were considered significant if $p < 0.05$.

3. Results and discussion

3.1. Identification of bacterial proteins that interact with IPEC cells as putative antigens

In the present work, we utilized 2DE coupled with Western blot analysis and MS/MS to identify *L. intracellularis* proteins that interact with IPEC-1 cell's surface proteins and are recognized by rabbit hyperimmune serum. Prior to 2DE, we established that rabbit negative sera (*i.e.* sera from rabbits obtained prior to immunization) failed to bind *L. intracellularis* proteins indicating that the rabbits had not been naturally exposed to *L. intracellularis* (Supplementary Fig. 1A). The rabbit sera did bind LPS so LPS antibodies were precleared from the negative sera and from all sera from immunized rabbits. After being precleared of LPS-antibodies, they failed to bind LPS and *L. intracellularis* proteins (Suppl Fig. 1B).

On the WB, Cy-labeled *L. intracellularis* proteins appear as red spots (Fig. 1A; full image in Supplementary Fig. 3) whereas any protein also bound by the rabbit antibodies will also be green as the green fluorescence is overwhelming the red fluorescence (note the unmerged images confirm that they are both red and green spots (data not shown)). Characteristic accumulation of the abundant albumin protein was observed in the region from 75 kDa to 100 kDa, which was also confirmed by MS/MS analysis (data not shown). Despite three washes with serum-free medium, contaminating albumin was consistently present and attempts to preclear samples of albumin failed (see arrows in Supplementary Fig. 3). However, despite the presence of albumin, low MW proteins were well separated and we were able to visualize both red and green spots indicating Cy-labeled *L. intracellularis* proteins alone and bound by antibodies. Using the WB (Fig. 1A) as a template, we were able to isolate the corresponding spots in the silver-stained preparative gel (indicated by a red circles in Fig. 1B). These proteins/spots were excised from the gel and subjected to MS/MS analysis.

Bioinformatics analysis of MS/MS detected proteins by UNIPROT and PFAM revealed 11 unique bacterial proteins identified by mass spectrometry (Table 2). Four of the indicated proteins were predicted to be expressed in the outer membrane of bacteria and these were selected for further analysis. These proteins were identified as Flagellin (fliC, LI0710), Putative outer protein N (LI1153), ABC dipeptide transport system, OppA (LI0169) and previously described autotransporter (LI0649), LatA (Watson et al., 2011). Selected proteins were analyzed with BepiPred-2.0, a web server analysis tool for sequence-based B-cell epitope prediction (Jespersen et al., 2017). All four protein sequences were predicted to have a significant presence of B-cell epitopes thus suggesting their potential immunogenicity *in vivo* (Supplementary Fig. 4). Among the eleven proteins detected in our study, proteins (LI0625) and (LI1171) were also reported previously using a shot-gun proteomic approach which increased our confidence in our approach (Watson et al., 2014).

Flagellin (LI0710, fliC) is a subunit protein that polymerizes to form flagella that plays an important role in bacteria locomotion and chemotaxis. Flagellum was observed as a bacterial cell structure of *L. intracellularis* by Lawson and Gebhart (Lawson and Gebhart, 2000) and a *L. intracellularis* recombinant protein similar to Flagellin (LI0710; identified as flagellar associated protein, FliC) which was recently shown to be immunogenic and shown to induce expression of IL-8 in HEK-Blue™-hTLR5 cells in a TLR5-specific manner (Won and Lee, 2018). Flagellin LI0710 (NCBI-proteinID: CAJ54764; UNIPROT: Q1MQG3) and putative Flagellin-like protein LI0570 (NCBI-proteinID: CAJ54624; UNIPROT: Q1MQV3) share 79.8% amino acid sequence similarity with highly conserved domains although they are coded by discrete regions of the bacterial genome; LI0710 resides between 894,199 and 895,080 nt and LI0570 resides between 701,958 and 702,842 nt of the bacterial chromosome. Flagellin LI0710 has 293 amino acids and consists of an N-terminal helical region (PF00669) and a C-terminal helical region (PF00700) domain.

Flagellin is a TLR5 agonist which plays an important role in the process of immune recognition of Gram negative bacteria. Immunization of mice by the systemic route with *Yersinia pseudotuberculosis*-derived Flagellin triggered protection in mice orally infected with *Y. pseudotuberculosis,* likely through the activation of TLR5 receptors on intestinal epithelial cells and CD103+ dendritic cells in the *lamina propria* (Porte et al., 2016). These mice showed increased secretion of Flagellin-specific IgA antibodies in the intestinal mucosa (Flores-Langarica et al., 2012) which likely played a primary role in protection. Due to its dual antigen and adjuvant nature, we predict that *L. intracellularis* Flagellin LI0710 may be an optimal subunit vaccine candidate antigen.

LI1153, annotated as Putative outer protein N is a part of the T3SS system. LI1153 consists of two prominent domains with important functions during invasion into eukaryotic cells: HrpJ-like domain

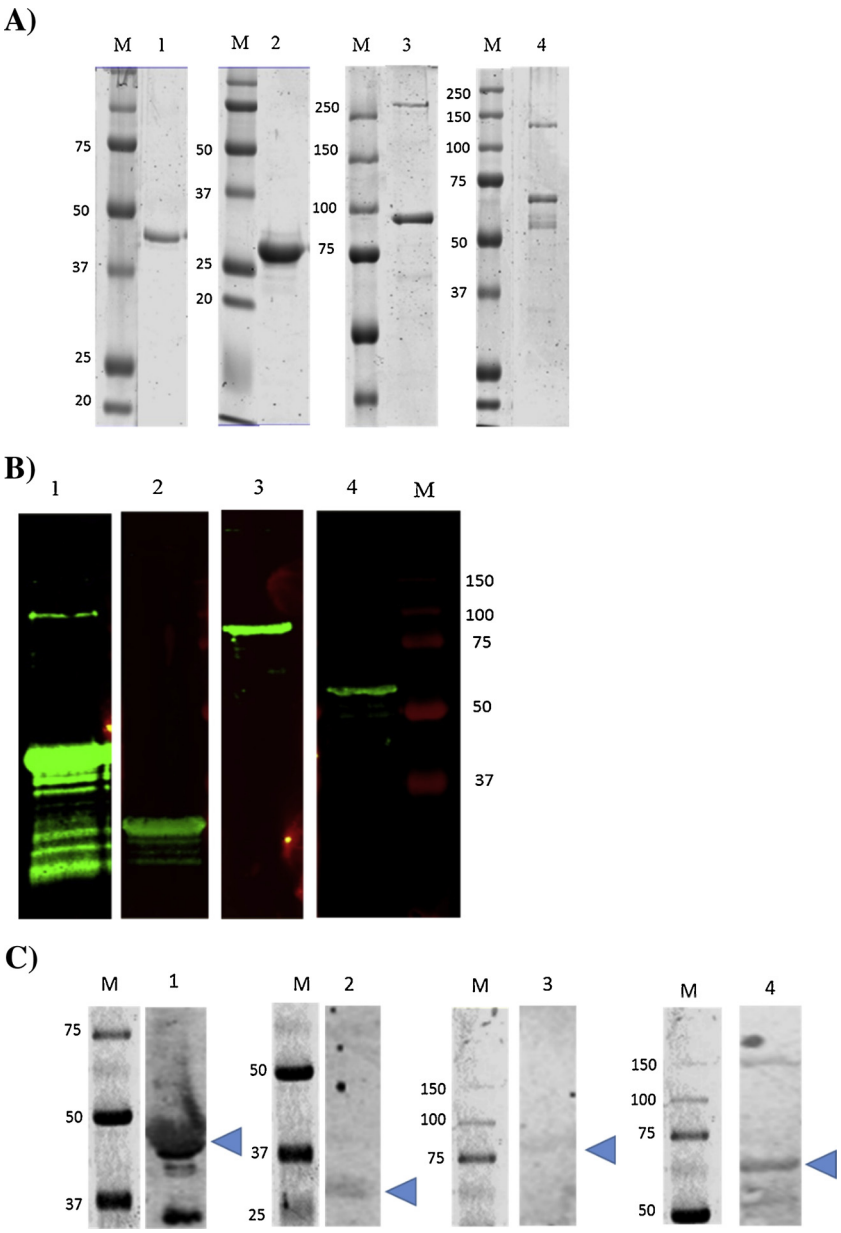

Fig. 2. Coomassie stained gels loaded with Nickel-purified recombinant proteins and Western blot analysis to confirm that the recombinant proteins remained immunogenic. Marker lanes are indicated by M and sizes in kd are indicated. A) Lane 1: rLI1153 44k Da (10% SDS gel), Lane 2: rLI0710 fliC 32 kDa (12% SDS gel), Lane 3: rLI0649 92 kDa (8% SDS gel), Lane 4: rLI0169 64 kDa (10% SDS gel. B) Western Blot analysis of recombinant proteins detected with sera from rabbits immunized with one of each recombinant protein. For all blots, recombinant proteins were recognized by antigen-specific IgG purified form rabbits immunized with recombinant proteins (1:500) followed by the secondary anti-rabbit IR800 antibody (1:10,000). Rabbit sera was generated against recombinant protein LI1153 44 kDa (Lane 1), recombinant protein LI0710 fliC 32 kDa (Lane 2), recombinant protein LI0649 92 kDa (Lane 3) and recombinant protein LIi0169 64k Da (Lane 4).). C) Recognition of purified recombinant proteins by sera pooled from pigs naturally infected and showing clinical symptoms of PE (1:500). Anti-pig IR800 (1:10,000) was used as the secondary antibody. Arrows indicate predicted MW of recombinant proteins. Lane 1: rLI1153 44 kDa, Lane 2: rLI0710 31 kDa, Lane 3: LI0649 92 kDa, Lane 4:LI0169 64 kDa.

(PF07201) and (TyeA; (PF09059) domain. HrpJ-like domain is predicted to be part of the T3SS and related proteins include SsaL and InvE invasion protein from *Salmonella typhimurium* which are involved in host pathogen interaction (Hensel et al., 1997) and invasion (Ginocchio et al., 1992), respectively. A related *E. coli* protein, SepL, plays a crucial role in the infection of enterohemorrhagic *E. coli* and has a potential role in secretion of EspA, EspD, and EspB (Kresse et al., 2000). Domain TyeA was identified in *Yersinia spp*. and it plays an essential role in controlling the secretion of effector proteins, YopE and Yop H (but not YopM, YopO and YopP) into eukaryotic cells (Iriarte et al., 1998). TyeA is localized at the bacterial surface and together with YopD and YopN contributes to a translocation-control apparatus within the T3SS (Iriarte et al., 1998). These secretion regulator proteins have been described as "gate-keepers" in major Gram negative bacterial species and their deletion leads to decreased secretion of translocon proteins or increased secretion of effector proteins (Burkinshaw and Strynadka, 2014). Alberdi et al., 2009 identified the gene that encodes three components (*YscN, YscO* and *YscQ*) of the T3SS system in three *L. intracellularis* isolates (Pilar Alberdi et al., 2009). Expression of two components, *YscQ and YscN,* were also identified using comparative transcriptional analysis of homologous pathogenic and non-pathogenic *L. intracellularis* isolates with *YscQ* being expressed in both isolates, while *YscN* expression was identified exclusively in the non-pathogenic isolate (Vannucci et al., 2012). Further, this study found expression of five out of a total of fifteen *L. intracellularis* genes that code T3SS components (Vannucci et al., 2012). Thus, our results indicate that Putative Outer protein N belongs to the *L. intracellularis* T3SS apparatus and has a *in silico* predicted role in controlling secretion of effector proteins into host cells analogous to InvE (*Salmonella*), YopN/TyeA (*Yersinia*), MxiC (*Shigella*) and SepD/SepL (*EPEC*) (Burkinshaw and Strynadka, 2014). Further structural and functional studies are needed to elucidate the interactions between LI1153 with T3SS effector proteins of *L. intracellularis* during invasion and attachment of the bacteria to small intestine enterocytes.

LI0169, OppA is coded by gene *oppA* and predicted to be expressed as part of the ATP-binding cassette (ABC) transporter complex. In bacteria, the ABC transporter complex plays a central role in the uptake of sugars, amino acids, metals, growth factors, ions and other solutes across the cell membrane (Singh and Röhm, 2008). Beyond transporting oligopeptides, Opp transporters are known to have important

roles in pathogenesis of different bacterial species (Garai et al., 2016). They are important factors in survival inside the host in *Campylobacter jejuni* infections (Garai et al., 2016), adhesion to the host cell in *Corynebacterium pseudotuberculosis* infections (Moraes et al., 2014), survival of *Listeria monocytogenes* inside macrophages (Borezee et al., 2000) and they are expressed during bacteraemia in mouse model of *Staphylococcus aureus* infection (Mei et al., 1997). LI0169 consists of transmembrane helical domain (12–34 aa), a periplasmic domain (PF00496) and an ATP-binding coiled domain (476–496 aa) at the intracellular face of the membrane that together form a central pore. It transports di- and tripeptides in an ATP-dependent manner (Higgins, 2001).

Protein LI0649 has been previously identified as autotransporter protein LatA (Watson et al., 2011). Using an approach similar to ours involving WB analysis coupled with MS, Watson et al., 2011 determined that LatA was recognized by pig hyperimmune serum thus representing a potential target for use in immunodiagnostics. The re-occurrence of this protein in our results further validates the 2DE data which indicates that LatA is immunogenic as it is bound by rabbit anti-*L. intracellularis* hyperimmune serum and that it plays a role in bacterial-host interactions. LatA had a predicted molecular mass (ExPasy) of 91.2 kDa but the corresponding protein was 60 kDa in our 2DE SDS-PAGE gel, indicating that some cleavage may have occurred. This protein is immunogenic and represents a good candidate to be a recombinant protein in a subunit vaccine formulation.

3.2. In vitro evaluation of recombinant protein antigenic properties

Because the bioinformatics analysis of these four proteins predicted their localization to the bacterial surface and therefore potential accessibility to host antibodies, we selected these proteins for expression in *E. coli* and further analysis. Recombinant proteins were expressed into LOBSTR-BL21 (DE3) pRosetta2 *E. coli*. SDS-PAGE analysis and Coomassie staining confirmed that rLI1153 (44 kD; Fig. 2A, Lane 1), rLI710 Flagellin (32 kD; Lane 2), rLI0649 (92 kD; Lane 3), rLI0169 (64 kD; Lane 4) were expressed at their predicted molecular weights.

To test the immunogenic potential of each of the four recombinant proteins, we vaccinated rabbits with one of the four recombinant proteins to generate hyperimmune serum specific for each target. Hyperimmune serum was then used in WB analysis and visualized with anti-rabbit secondary IR800. Fig. 2B, Lane 1 shows recombinant LI1153 bound by rabbit hyperimmune sera (from a rabbit vaccinated against recombinant LI1153). The band at approximately 100 kD and the smaller bands at 35 kd are *E. coli* proteins present after Ni-affinity column purification.

Lane 2 shows recombinant LI0710 Flagellin (32 kD) bound by rabbit hyperimmune sera (from a rabbit vaccinated against recombinant Flagellin). Lane 3 shows recombinant LI0649 (92 kD) bound by rabbit hyperimmune sera (from a rabbit vaccinated against recombinant LI0649). Finally, Lane 4 shows recombinant LI0169 (64 kD) bound by rabbit hyperimmune sera (from a rabbit vaccinated against recombinant LI0169). Importantly, sera from unimmunized rabbits did not recognize any of our four recombinant proteins (Supplementary Fig. 2). Our results indicate that the recombinant proteins were recognized by rabbit hyperimmune sera thus indicating their immunogenic properties.

Next, to confirm that these recombinant proteins were immunogenic in pigs, we pooled serum from pigs diagnosed with PE from *L. intracellularis* endemic farm and used the sera in WB analysis. All four recombinant proteins were recognized by sera from PE-infected pigs (Fig. 2C; rLI1153 (44 kD; Lane 1), rLI0710 FliC (32 kD; Lane 2), rLI0649 (92 kD; Lane 3), rLI0169 (64 kD; Lane 4)), which indicates that these recombinant proteins remained immunogenic in both species and, critically, despite being selected based on rabbits immunized with the avirulent strain, these proteins were present in the virulent strain and generated a humoral response during infection in pigs with clinical disease. Interestingly, rLI0169 (OppA) was recognized by the pig sera from field cases of PE, indicating that although it has a periplasmic region, this protein is expressed during *L. intracellularis* infection and it is targeted by specific antibodies. OppA is conserved among many bacteria species and these results correspond to reported antigenicity and surface exposure of OppA in *Streptococcus suis* serotype 2 (Zheng et al., 2018). rLI0649 is weakly recognized by sera from PE-infected pigs in our WB possibly due to the fact that we had pooled porcine sera from only 5 animals infected from one farm. Previously it was also shown that porcine sera against *L. intracellularis* from different animals have variable intensity of binding to recombinant proteins, rLI0649 (Watson et al., 2011) or rLscQ (Alberdi et al., 2009), possibly due to the difference in immune response against bacteria or infectious dose of bacteria each animal was exposed to.

3.3. In vitro evaluation of proteins antigenic properties using flow cytometry

We performed a neutralization assay (methodology optimized in (Obradovic et al., 2016) to quantify the level of inhibition that antigen-specific antibodies had on preventing penetration of CFSE-labelled avirulent *L. intracellularis* into eukaryotic cells. Because others have shown that negative mouse serum showed 48% to 59% inhibition of *L. intracellularis* invasion, likely due to the presence of anti-LPS antibodies present prior to generation of hyperimmune serum (McOrist et al., 1997), we tested whether negative rabbit serum bound to LPS. We observed that rabbit negative serum did bind to one band with molecular weight between 20 and 25 kDa (Supplementary Fig. 1A) which corresponds to the molecular weight of LPS (Kroll et al., 2005a). Therefore, we precleared anti-LPS antibodies from all sera prior to performing the neutralization assays. WB analysis of sera pre- and post-incubation with LPS to remove LPS binding antibodies is shown in Supplementary Fig. 1A–C The following sera were tested at low 500 μg/ml, medium 1000 μg/ml and high 2000 μg/ml concentrations: rabbit sera before immunization, rabbit sera against whole avirulent bacteria, rabbit hyperimmune serum specific for rLI0169, rLI0649, rLI0710 FliC, or rLI1153. MOI of 0.1 of CFSE stained *L. intracellularis* remained constant.

The gate in flow cytometry analysis was based on percentages of fluorescence detected in FL-1 channel (Gate B from representative biological replicate shown in Fig. 3) for IPEC-1 cells alone (Fig. 3A) and IPEC-1 cells infected with CFSE *L. intracellularis* (Fig. 3B). As expected, IPEC-1 cells alone had negligible positive fluorescence events (mean value of 0.20% fluorescence in FL1 channel; Fig. 3A) and IPEC-1 cells infected with CFSE-labelled *L. intracellularis* showed mean value of 8.86% fluorescence in FL1 channel, 4 h post-infection (Fig. 3B). The percentage of positive events in FL1 channel when CFSE-labelled *L. intracellularis* was incubated with 2000 μg/ml of serum from rabbit immunized with whole bacteria was reduced to 2.29% (Fig. 3C).

Fig. 4 represents the percent inhibition that serum antibodies blocked *L. intracellularis* invasion of IPEC-1 cells. As our negative control sera, we pooled the sera from rabbits prior to immunization for hyperimmune serum generation. We observed that pre-incubation of CFSE-*L. intracellularis* with low (500 μg/ml), medium (1000 μg/ml) and high (2000 μg/ml) concentrations of negative control sera showed 46.7% ± 7.9, 58.9% ± 8, 65.7% ± 5.7 percent inhibition (hatched bars). This inhibition by negative control serum is not unexpected as others have shown that rabbit polyclonal sera prepared against *E. coli* and other negative control serum obtained prior to generation of *L. intracellularis* hyperimmune sera inhibited *L. intracellularis* penetration of cultured rat enterocytes (IEC-18) (McOrist et al., 1997).

We used rabbit hyperimmune generated against whole *L. intracellularis* as our positive control serum and results indicated CFSE-labeled *L. intracellularis* incubated with low (500 μg/ml), medium (1000 μg/ml) and high (2000 μg/ml) serum resulted in 64.8% ± 5.7, 73.4% ± 4.7 and 79.88% ± 5.9 percent inhibition of infection (Fig. 4A–C, second bars in each groups). Relative to the negative control sera, positive control sera inhibited significantly more cellular

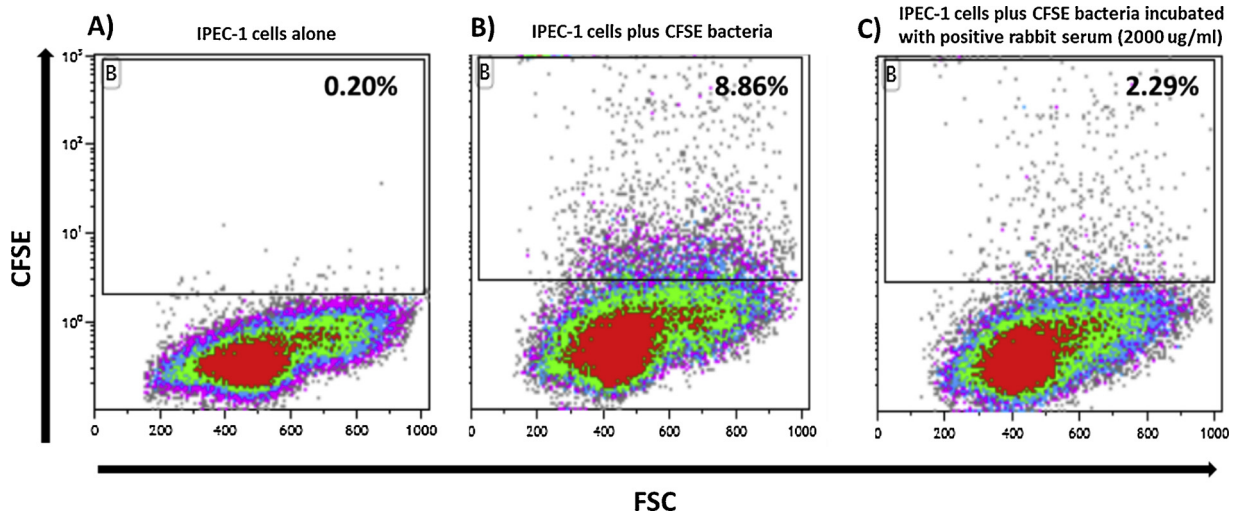

Fig. 3. Representative dot blots and gating for flow cytometry analysis: A) Gate was drawn on live IPEC-1 cell forward scatter/CFSE plot, 0.20% of fluorescent events, B) IPEC-1 cells infected with CFSE- labeled L. intracellularis MOI 0.1, 8.86% of fluorescent events, C) IPEC-1 cells infected with CFSE- labeled L. intracellularis MOI 0.1 incubated with 2000 µg/ml of positive serum from rabbits vaccinated against whole bacteria, 2.29% of florescent events (C). Plots represent one of four biological replicates for all sera precleared for LPS antibodies to obtain mean values and to calculate the level of inhibition. Thirty thousand events were acquired for each sample.

adhesion/ penetration for low ($p < 0.05$; Fig. 4A), medium ($p < 0.01$; Fig. 4B) and high ($p < 0.001$; Fig. 4C) sera concentrations indicating that anti-*L. intracellularis* antibodies were neutralizing. To discern whether antibodies specific for rLI0169, rLI0649, rLI0710 and rLI153 blocked bacterial adherence/penetration into IPEC-1 cells, we pre-incubated the CFSE-*L. intracellularis* with 500 µg/ml (Fig. 4A), 1000 µg/ml (Fig. 4B) and 2000 µg/ml (Fig. 4C) hyperimmune sera specific for each recombinant protein. At the lowest concertation of hyperimmune sera (Fig. 4A), anti-rLI0169 showed 68.7% ± 5.9 percent inhibition, anti- rLI0649 showed 64% ± 9.0 percent inhibition, anti-rLI0710/FliC showed 69.5% ± 5.2 percent inhibition and anti-rLI1153 showed 60.4% ± 11.8 percent inhibition. With the exception of anti-rLI1153, all 3 hyperimmune sera showed significantly higher percent inhibition relative to the negative control serum ($p < 0.01$, $p < 0.05$, $p < 0.01$, respectively). When the medium and high concentration of each antisera was used, statistically significant reduction of bacteria entrance into IPEC-1 cells was achieved relative to the corresponding dose of control serum: anti-rLI0169 ($p < 0.01$ Fig. 4B, $p < 0,001$ Fig. 4C), anti-rLI0649 ($p < 0.01$ Fig. 4B, $p < 0.001$ Fig. 4C), anti-rLI0710/FliC ($p < 0.01$ Fig. 4B; $p < 0.001$ Fig. 4C), and anti-rLI1153 ($p < 0.01$ Fig. 4B; $p < 0.01$ Fig. 4C). We can conclude that sera antibodies specific for each recombinant protein showed comparable inhibitory effect to that observed with the positive rabbit serum against whole bacteria and that the inhibitory effect of all sera was higher with increased serum concentration. The results from recombinant serum neutralization assay suggest that use of recombinant proteins may generate neutralizing antibodies capable of inhibiting *L. intracellularis* penetration and infection when used as antigens in subunit vaccine formulation.

Because *L. intracellularis* is obligate intracellular bacterium, the cellular immune response may be predicted to play the major role in protection against virulent bacteria (Cordes et al., 2012; Guedes and

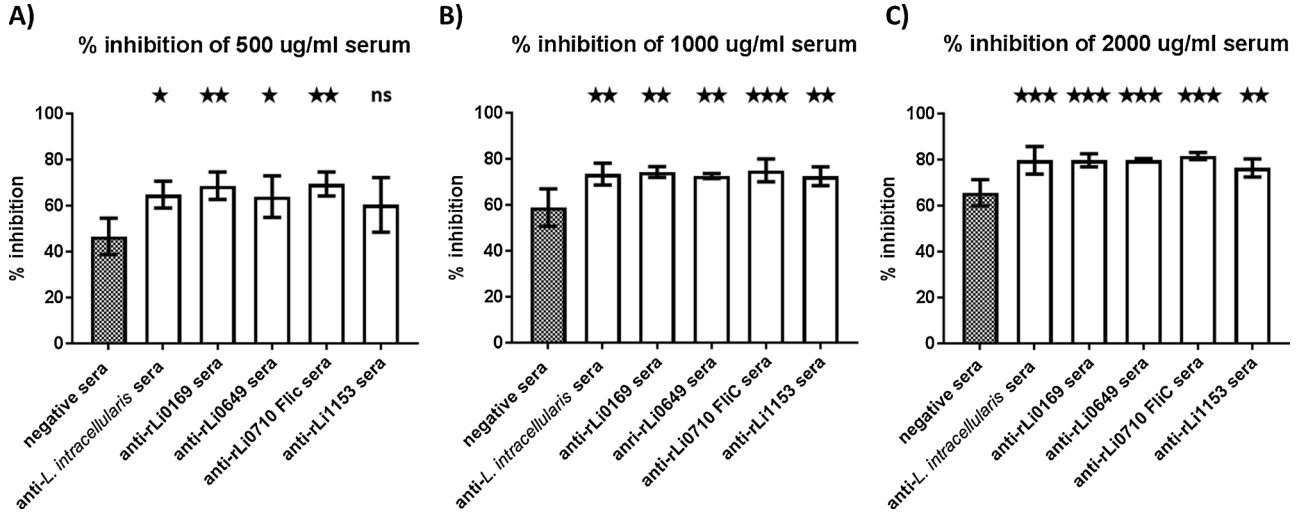

Fig. 4. Inhibitory effect of rabbit sera on CFSE- labelled avirulent L. intracellularis penetration in IPEC-1 cells: negative control sera (sera obtained prior to immunization, pooled and precleared of anti-LPS antibodies), anti-L. intracellularis sera (serum from rabbits immunized with whole avirulent bacteria), sera from rabbits immunized with recombinant proteins: anti-rLI0169, anti-rLI0649, anti-rLI0710 FliC, anti-rLI1153; serum concentrations used in assay 500 µg/ml (A); 1000 µg/ml (B) and 2000 µg/ml (C). All sera were cleared from antibodies against LPS. Percent inhibition = (1- % of fluorescence of CFSE bacteria incubated with serum / % of fluorescence of CFSE bacteria (control)) x 100. Data presented for 4 biological replicates. The error bar shows standard deviation of mean value of 4 biological replicates. ((***) $p < 0.001$, (**) $p < 0.01$ and (*) $p < 0.05$, (ns) not significant.

Gebhart, 2010), however, the humoral immune response may also play an important role in protecting against *L. intracellularis* infection. IgG antibodies against intracellular bacteria could bridge humoral and cellular immunity by targeting of intracellular pathogens to lysosomes through Ab-FcR-mediated stimulation of the host cells (Armstrong and Hart, 1975), protection against intracellular bacteria by Fc receptor-mediated lysosomal targeting (Joller et al., 2010) and modulation of cytokine secretion (Levy Polat et al., 1993). Also, IgA antibodies play an important role in protection against enteric pathogens. Accumulation of IgA bound to *L. intracellularis* inside enterocytes and *lamina propria* was reported previously (McOrist et al., 1992) and *L. intracellularis* specific IgA were detected in intestinal lavage of pigs 3 weeks after experimental infection (Guedes and Gebhart, 2010). Results from a vaccine trial where animals were vaccinated orally and challenged with virulent *L. intracellularis* suggested that protection was associated with mucosal cytokine and specific IgG and IgA responses and that systemic antibody responses were boosted following challenge (Nogueira et al., 2013). In this study we demonstrate that sera generated against recombinant proteins inhibited *L. intracellularis* invasion of IPEC-1 cells *in vitro*. Although this study was designed to identify proteins capable of generating a humoral response, it does not preclude the capacity of these proteins to elicit a cellular immune response and thus further investigation in this area is warranted.

A subunit vaccine comprised of Flagellin alone or coupled with these 3 other antigens may (or may not) induce specific protective immune response against *L. intracellularis* in intestinal mucosa of pigs and should be investigate further.

4. Conclusions

In conclusion, we used functional 2DE and WB analysis coupled with MS to identify eleven *L. intracellularis* proteins from which four showed potential to be suitable recombinant antigens for formulation of *L. intracellularis* subunit vaccine in pigs. *In vitro* neutralization assay based on flow cytometry indicated that rabbit hyperimmune sera generated against the vaccine strain of *L. intracellularis* and rabbit sera specific for each recombinant protein showed an inhibitory effect on the attachment and penetration of live, avirulent *L. intracellularis* to eukaryotic cells. Results from this *in vitro* study demonstrated a means to efficiently and economically select potential vaccine antigens prior *in vivo* studies thus enabling us to narrow down the number of antigens tested in future live vaccine trails. Extensive *in vivo* studies will need to be performed to ensure that such a quadrivalent subunit vaccine is effective in generating protective long term immunity. However, if effective such a vaccine against *L. intracellularis* may present the industry with an effective and economically attractive vaccine to use in high security pig barns.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.07.014.

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