猪消化道疾病国际研讨会
International Pig Digestive Diseases Symposium

yoyoung

ELISA antibody test for porcine ileitis Research and application of kits

猪回肠炎ELISA抗体检测试剂盒的研究与应用

李雪平　高级兽医师 senior veterinarian
18620758386

猪消化道疾病国际研讨会

GUANGZHOU YOYOUNG BIOTECH. CO.,LTD.
广州悦洋生物技术有限公司

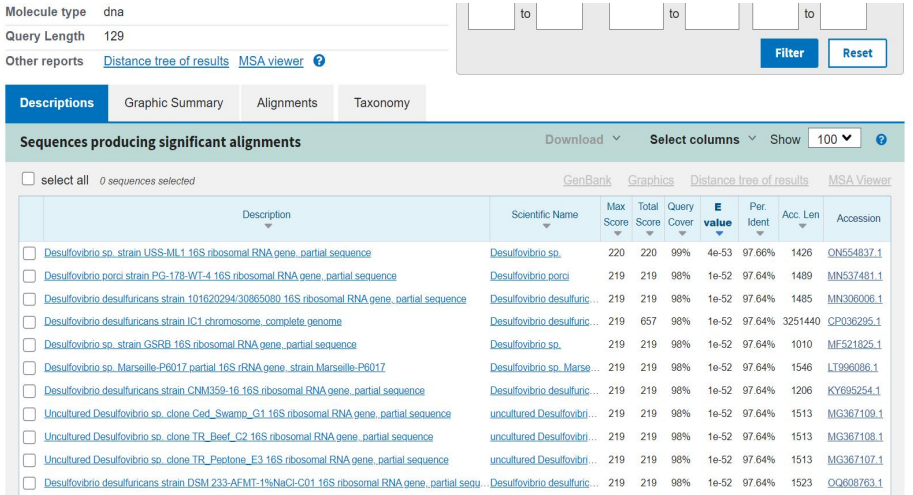
临床案例 Clinical cases

- 某集团公司场发生腹泻：后备母猪，180日龄左右，秋季10月发病，发病数量62头，拉土黄色稀粪。
> Diarrhea occurred in a group of farms: reserve sows, about 180 days old, onset of disease in October in the fall, the number ofonset of disease was 62, with soil-yellow scanty feces.>
- 检测：采集肛拭子A试剂阴性、某第三方检测公司阴性，YY试剂阳性。复核：YY检测了常见腹泻病原，部分样品*L. i*核酸强阳，测序与*L. i*同源性98~99%。最终确定为胞内劳森氏菌感染。
> Test: The anal swab collected was negative for reagent A, negative for a third-party testing company, and positive for reagent YY.Re check: YY detected the common diarrhea pathogen, and some samples were strongly positive for L. i nucleic acid. Sequencingshowed 98~99% homology with L. i. Finally, it was determined that the infection was caused by Lawsonia cytidis.
- ✓ 处理方案及结果：大环内酯类（泰乐菌素）+季铵盐消毒，5天后基本恢复平稳。
> Treatment plan and results: macrolides (tylosin)+ quaternary ammonium salt disinfection,5 days after the return to stable.

疑似回肠炎导致的腹泻 Diarrhea due to suspected ileitis

序号	样本信息 Sample	荧光PCR检测结果（CT）
1	28栏（E101103）	25.098
2	58栏(E400044)	38.051
3	2栏(E401178)	39.176
4	78	28.465
5	79	NOCT
6	阴性对照 N Control	NOCT
7	空白对照 B Control	NOCT
8	阳性对照 P Control	19.395

荧光PCR检测结果 PCR Result



扩增产物序列比对确认 Sequence Comparison

猪消化道疾病国际研讨会

胞内劳森氏菌感染面广、危害大

Widespread and dangerous infections with intracellular Lawsonella spp

育肥猪：大肠杆菌，胞内劳森氏菌，沙门，螺旋体，PED，TGE

育成猪：胞内劳森氏菌，沙门，PED，TGE

后备猪（限抗）阳性率：44.44%~66.67%

Finishing pigs: *Escherichia coli*, *Lawsonia intracellular*, Salmon, spirochete, PED, TGE

Growing pigs: *Lawsonia intracellular*, *Salmonella*, PED, TGE

Positive rate of gilts(Restricted antibiotics): 44.44%~66.67%

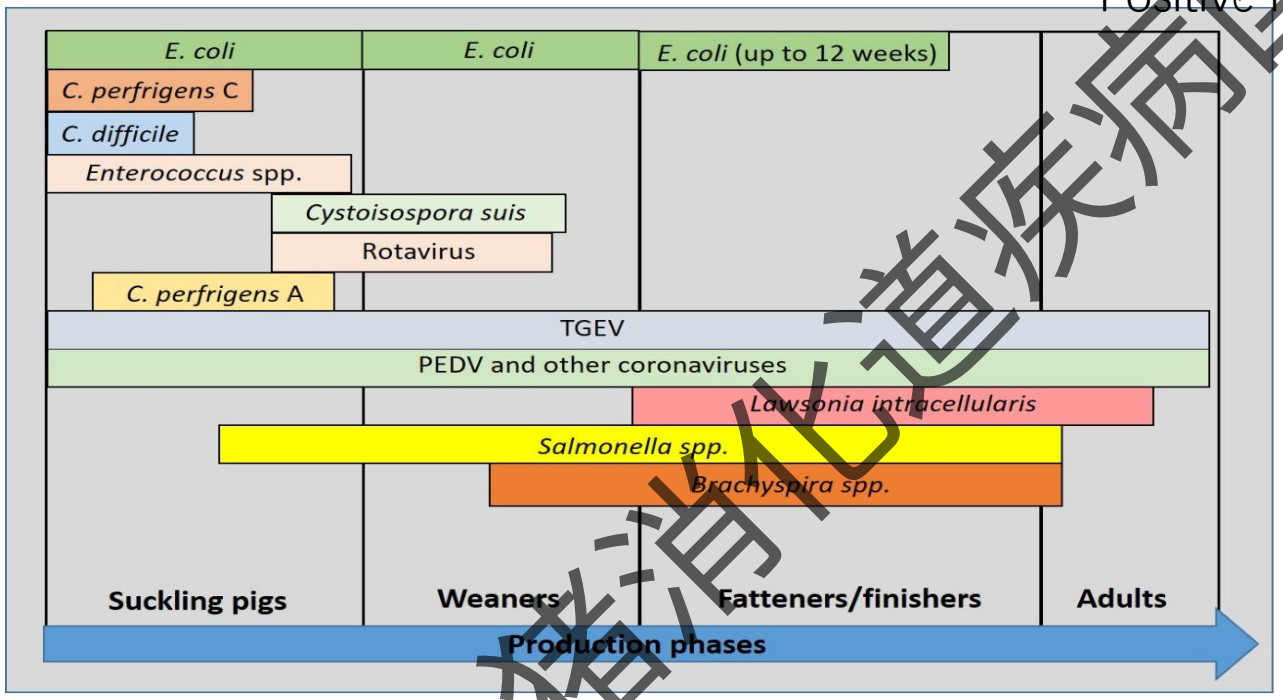


Figure 2. Incidence of pathogens in enteric disease in pigs related to age (modified from Ségales et al., 2013) [6].

表4 后备母猪胞LI感染的追踪调查

采样日龄(d)	样品数量	阳性数量	阳性率(%)
175	18	12	66.67
189	18	8	44.44
203	18	10	55.56

-支康发等，2023

猪消化道疾病国际研讨会

*L. i*感染后抗原抗体的消长规律

Growth and decline of antigen and antibody after iinfection

回肠炎发生后： Ileitis occurs

✓临床症状1.5~4.5周；
√ Clinical symptoms 1.5 to 4.5 weeks.

✓1周后可从粪便中检测到核酸（PCR）；
√ Nucleic acid (PCR) can be detectedin feces after I week;

✓2周内可检测到IFN-γ（ELISPOT）；
√ 'IFN can be detected within 2 weeks- Y(ELISPOT);

✓2周后可检测到抗体（ELISA），持续时间长
Antibody can be detected after 2weeks (ELISA), and the duration islong

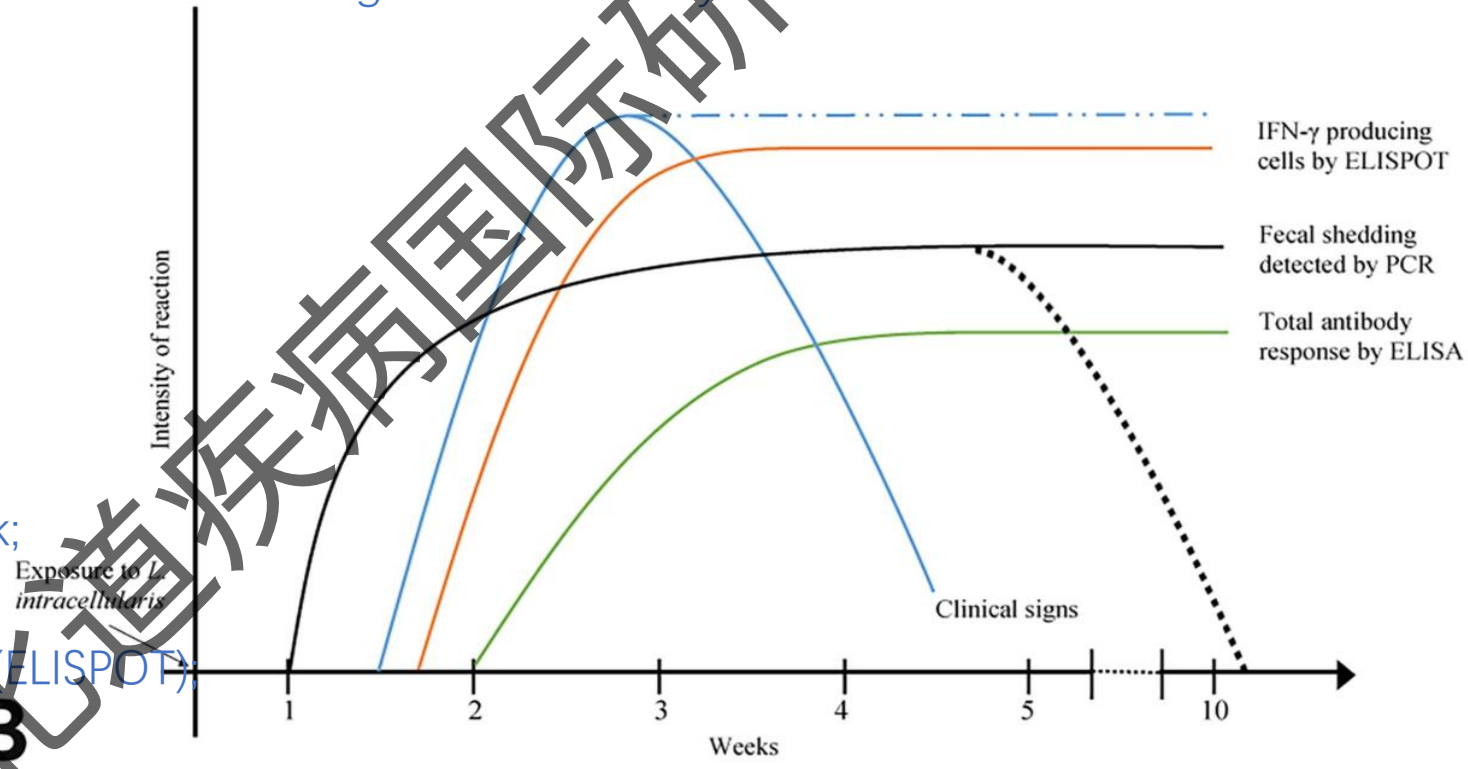


Figure 2. Detection of *Lawsonia intracellularis* infection. **A.** Time post-infection of detection of infection by each technique. Boldfaced techniques are those most widely used in veterinary diagnostic laboratories globally. dpi = days post-infection. **B.** Graphic representation of the course of *L. intracellularis* infection, clinical signs, immune reaction, and adaptive immune response. Solid blue line = clinical signs with interventions. Dashed blue line = clinical signs without interventions. Orange line = IFN-γ detection. Solid black line = fecal shedding detection without interventions. Dotted black line = fecal shedding detection with interventions. Green line = total antibody response.

-Campillo et al., 2021

猪消化道疾病国际研讨会

*L. i*感染后不同时期的检测技术

Detection techniques at different stages after infection

7 days after infection
Fecal samples/anal

7-10 days

21-28days

感染后7d 粪便样品/肛拭子	感染后7-10天 感染组织	感染后21-28天 血清
• 普通PCR • 荧光定量PCR • LAMP • RPA-LFD	• Warthin-Starry银染技术(WS) • 免疫组织化学(IHC) • 免疫荧光测定(IFA) • 荧光原位杂交技术(FISH)	• 酶联免疫吸附实验 ELISA • 间接免疫荧光抗体检测IFAT • 免疫过氧化物酶单层分析(IPMA)

- Common PCR
- Fluoresce quantitative PCR
- LAMP
- RPA-LFD

- Warthin Starry silver dyeing technology(WS)
- Immunohisto chemistry(IHC)
- Immunofluo rescenceassay (IFA)
- Fluorescence In Situ Hybridization(FISH)

- ELISA
- IFAT detection by indirect Immunofluorescence antibody
- Immunoperoxidase monolayer analysis(IPMA)

猪消化道疾病国际研讨会

回肠炎抗体检测试剂盒的立项背景

The background of the project for the ileitis antibody test kit

- 减抗禁抗的背景下，*L. i*对养猪业带来的经济损失将愈加严重。

Against the background of resistance reductionand resistance prohibition, the economic lossescaused by L. i to the pig industry will becomemore and more serious.

- 猪群感染后间歇性排菌，PCR不能完全准确检测猪群状态。

The pigs were discharged intermittently after infection, and PCR could not completely andaccurately detect the status of pigs.

- 目前国内外尚缺乏可靠、准确、适的用商品化ELISA 抗体诊断试剂盒。

At present, there is a lack of reliable, accurateand suitable commercial ELISA antibody diagnostickits at home and abroad.

- 旨在为回肠炎流行病学调查、疫苗免疫或药物效果评估、疾病净化效果评估提供有效工具

Aiming to provide an effective tool for theepidemiological investigation of ileitis, theassessment of the effectiveness of vaccineimmunization or drugs, and the assessment of theeffectiveness of disease decontamination

ELISA

Several ELISAs have been developed for the detection of *L. intracellularis* antibodies in serum.[16,44,54,61] Most are competitive ELISAs, the major advantage of which is their ability to selectively detect the antibody recognized by the plated antigen, even in crude or impure samples. To date, only one commercial kit is available (Svanovir *L. intracellularis*/*Ileitis*-Ab; Table 1).[44,109] This test is unable to quantify the response against *L. intracellularis*, and sensitivity may vary depending on the value of percent inhibition (PI) selected; at PI = 35, the test sensitivity is 72% and the specificity reaches 93%.[44] *Lawsonia*-specific serum antibody responses, IgG and IgM, can be detected in serum 2 wk after infection (Fig. 2B); they peak at 3–4 wk and remain detectable for up to 13 wk post-infection.[31,82]

--Jacobson，2011

猪消化道疾病国际研讨会

R&D process for animal diagnostic products (antibodies)

动物诊断制品（抗体）的研发流程

laboratory tests
实验室试验

- 病原体基础种子建立和鉴定；抗原性、特异性和纯净性、 保存条件
- 比较实验：与全菌体等、其他片段
- 生产工艺研究:抗原制备
- 诊断方法优化
- 组装和成品检验
- 试剂盒的试验以及成品检验项目和标准确定：性状、无菌、敏感性、特异性、重复性、消长规律
- 保存期
- Pathogen-based seed establishment and identification; antigenicity, specificity and purity, preservation conditions
- Comparative experiments: with whole bacteriophage, etc., other fragments that
- Production process studies: antigen preparation
- Optimization of diagnostic methods
- assembly and finished product inspection
- Tests of the kits as well as finished product testing programs and criteria fordetermining: traits, sterility,sensitivity, specificity,reproducibility,pattern of extinction
- Shelf life

Pilot
中试

- GMP生产车间
- 连续生产5～10批试剂盒
- 全面检验
- 必要时调整生产工艺适应大规模生产
- GMP workshop
- Continuous productionof 5 to 10 batches of kits
- Comprehensive testing
- Adaptation of production processesto mass production, if necessary

Clinical Trials
临床试验

- 一定数量的试剂盒中试产品
- 大量临床样品(阳性和阴性样品)
- 检测试验
- 确定试剂盒的实际应用效果
- a certain number of kit pilot products
- Large number of clinicalsamples (positive andnegative)
- detection tests
- Determining theeffectiveness of thepractical application ofthe kit

猪消化道疾病国际研讨会

回肠炎抗体ELISA核心研发路径

Core research and development path of ileitis antibody ELISA

一、检测靶标选择及表达：

二、体系优化：工艺研究

- 棋盘滴定法确定包被浓度和酶标二抗浓度
- 样本孵育时间的确定
- 酶标抗体反应时间的确定
- TMB底物显色时间的确定
- 封闭液配方的优化样本
- 样本稀释液配方的优化
- 酶标二抗稀释液配方的优化
- ELISA临界值cut off line的确定
- 临床符合率

三、中试和临床试验

I. Target selection andexpression:

II. System optimization: process studies

- Checkerboard titration was used to determine the concentration of the coating and the concentration of the enzyme-labeled secondary antibody
- The Determination of sample incubation time
- Determination of reaction times of enzyme-labeled antibodies
- The determination of the color development time of TMB substrates
- The optimized samples of sealant formulations,
- 2nd antibody diluent Determination of
- ELISA cut-off threshold
- Clinical compliance rate

III. Pilot and clinical trials

猪消化道疾病国际研讨会

胞内劳森菌检测靶标选取的部分文献参考

	文献		年份	单位	作者	包被物	蛋白名称
1	ILEITIS DIAGNOSTIC ASSAY	专利	2006	BOEHRINGER	KROLL, Jeremy, J	脂多糖	/
2	Evaluation of a novel enzyme-linked immunosorbent assay for serological diagnosis of porcine proliferative enteropathy	期刊 elsevier/Veterinary Microbiology	2005	Danish Institute for Food and Veterinary Research	Henriette Toft Boesen	全菌裂解物	/
3	猪增生性肠炎诊断方法的初步建立及胞内劳森氏菌LI0065基因的克隆与表达	学位论文	2017	湖南农业大学	李啟皓	AM180252.1/L00065 NC_008011.1/LI_RS00355	Outer membrane lipoprotein-sorting protein/outer membrane lipoprotein chaperone LolA
4	胞内劳森菌表面蛋白的克隆表达及ELISA抗体检测方法的建立	学位论文	2021	湖南农业大学	廖荣莉	NC_008011.1/LI_RS03115"	flagellar hook-basal body complex protein flgE
5	胞内劳森氏菌LI0902蛋白单克隆抗体制备及间接ELISA方法建立和初步应用	学位论文	2021	江西农业大学	于振兴	AM180252.1/LI0902 NC_008011.1/LI_RS04915	outer membrane protein/OmpA family protein
6	猪胞内劳森氏菌抗原候选蛋白的原核表达及间接ELISA检测方法的建立与初步应用	学位论文	2013	广西大学	刘磊	AM180252.1/LI0902 NC_008011.1/LI_RS04915	outer membrane protein/OmpA family protein
						AM180252.1/LI1022 NC_008011.1/LI_RS05590	Outer membrane protein/OmpH family outer membrane protein
						AM180252.1/LI1024 NC_008011.1/LI_RS05600	Outer membrane protein/protective antigen OMA87/outer membrane protein assembly factor BamA
						AM180252.1/LI0235 NC_008011.1/LI_RS01305	Surface lipoprotein/ VacJ family lipoprotein
8	胞内劳森菌LI0710和LI0649基因的原核表达及ELISA检测方法的建立	学位论文	2022	佛山科学技术学院	李复坤	AM180252.1/LI0649 NC_008011.1/LI_RS03555	NA/autotransporter outer membrane beta-barrel domain-containing protein
						AM180252.1/LI0710 NC_008011.1/LI_RS03890	fliC/flagellin
9	Antigenic and functional profiles of a Lawsonia intracellularis protein that shows a flagellin-like trait and its immuno-stimulatory assessment	期刊Vet Res	2018	College of Veterinary Medicine, Chonbuk National University	Gayeon Won and John Hwa Lee	AM180252.1/LI0570 NC_008011.1/LI_RS03135	Flagellin and related hook-associated proteins/flagellin

猪消化道疾病国际研讨会
International Pig Digestive Diseases Symposium
猪消化道疾病国际研讨会

检测靶标选择 Detecting targetselection

1、包被胞内劳森菌、脂多糖

优点：覆盖面广，更灵敏

缺点：细菌为专性胞内菌。体外难以培养。不安全。

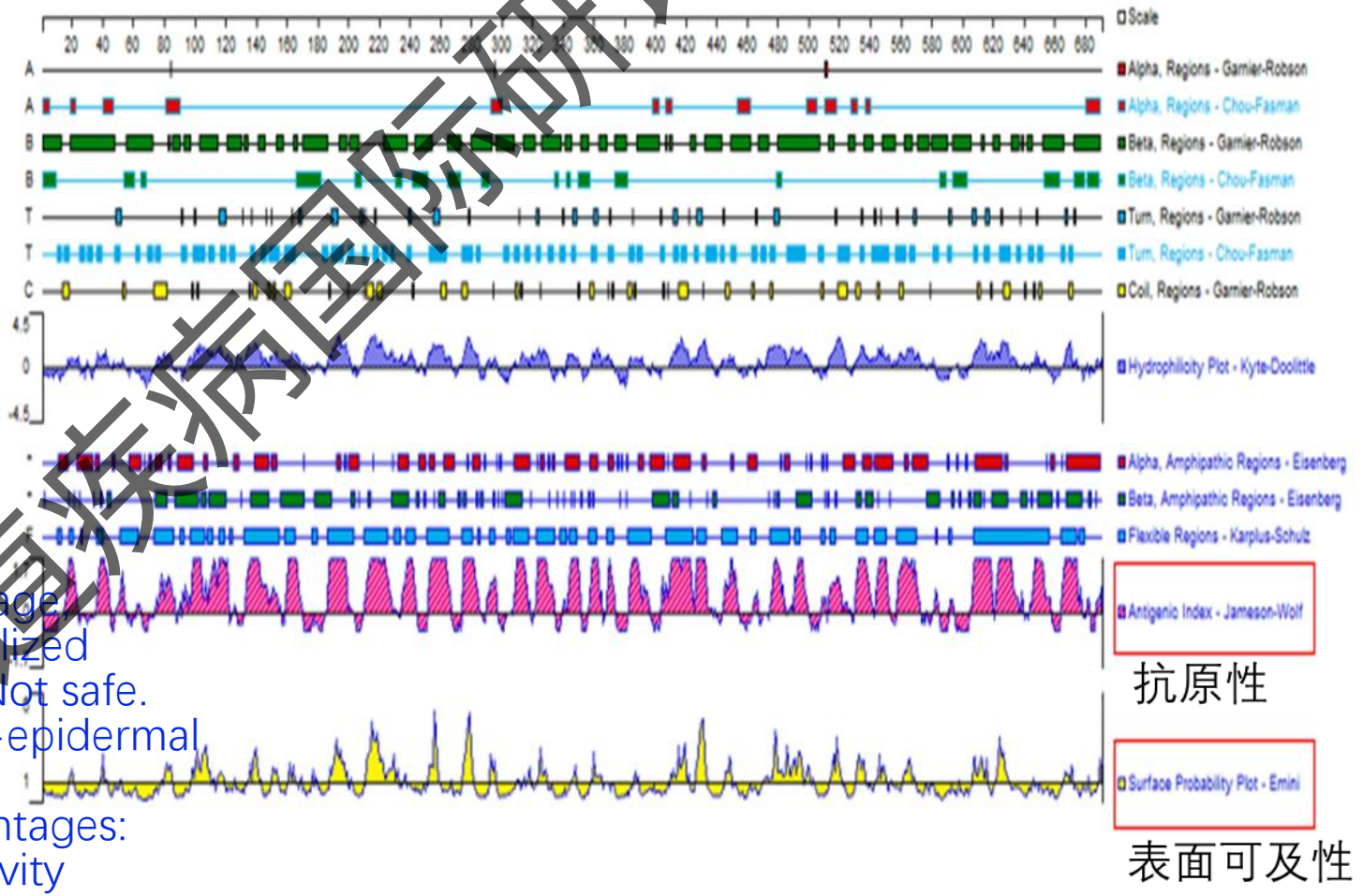
2、包被胞内劳森菌表外膜蛋白、鞭毛膜蛋白

优点：能体外高效表达

缺点：单个蛋白覆盖面不广，灵敏度差

3、选择flgE蛋白（鞭毛钩-基体复合蛋白），根据序列特点有选择性进行优化蛋白表达。

1. Encapsulation of intracellular Lawsonia, lipopolysaccharide that Advantages: Wide coverage, moresensitive. Disadvantages: Bacteria arespecialized intracellular bacteria. Difficult to culture invitro. Not safe.
2. Encapsulation of intracellular Lawsonian extra-epidermal membraneproteins, flagellar membrane proteins Advantages: Efficient in vitroexpression. Disadvantages: poor coverage ofindividual proteins, poor sensitivity
3. Select flgE protein (flagellar hook matrix complex protein) and selectively optimize protein expression based on sequence characteristics.



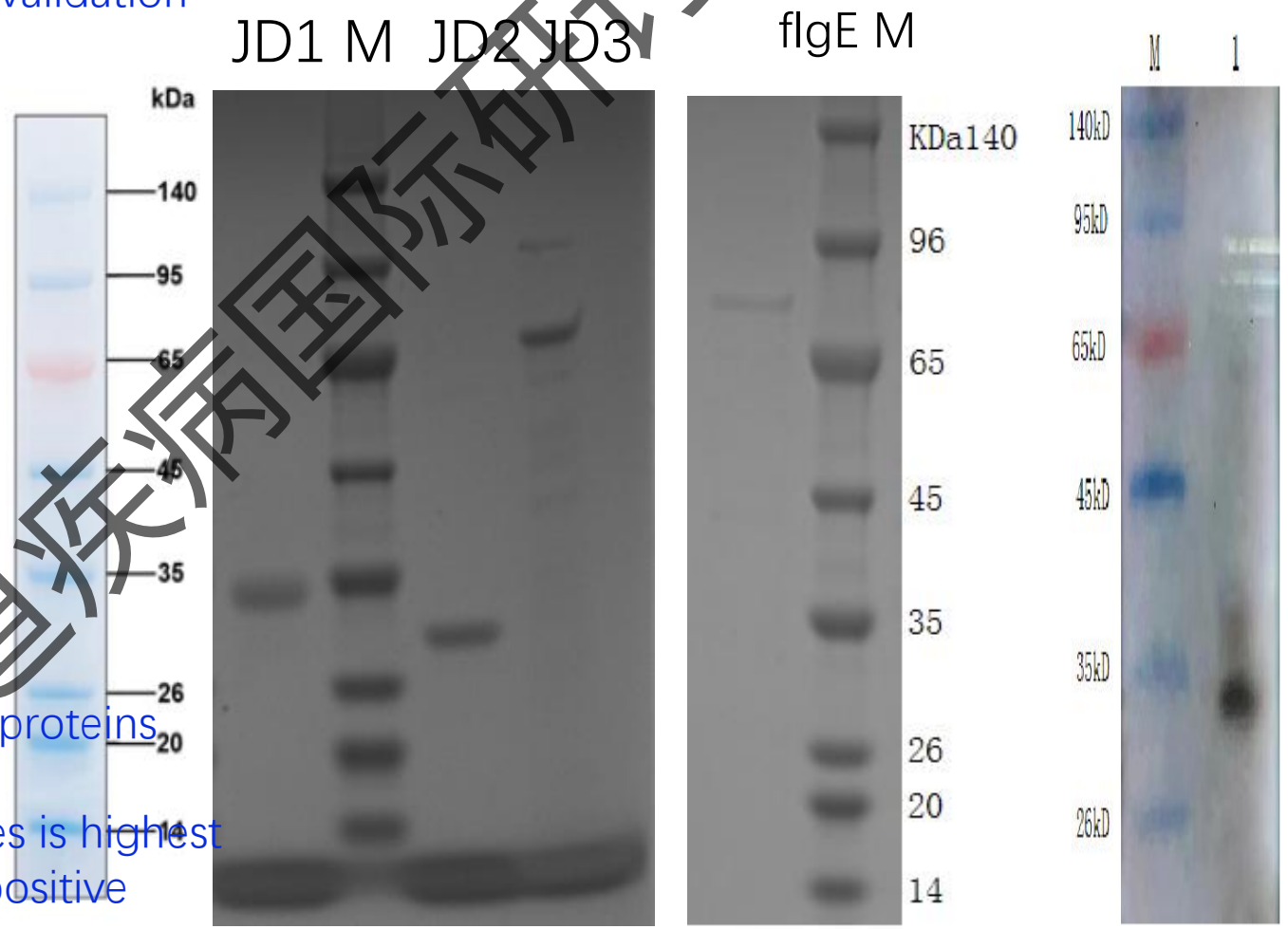
flgE蛋白（鞭毛钩-基体复合蛋白）部分抗原性预测

猪消化道疾病国际研讨会

蛋白表达及验证 Protein expression and validation

- 全长和三种截短蛋白均能有效表达。
- 胞内劳森菌阳性血清的杂交中，JD1蛋白与抗体的结合能力最高。
- 仍需进一步分析，来筛选最适的靶标抗原。

- Both full-length and three types of truncated proteins can be effectively expressed.
- The binding ability of JD1 protein to antibodies is highest in the hybridization of intracellular Lawsonia positive serum.
- Further analysis is still needed to screen for the most suitable target antigen



3种不同的截短可溶性表达及全长flgE蛋白表达及鉴定

猪消化道疾病国际研讨会

标准样品盘建立-IFAT方法确认

Establishment of standard sample tray- IFAT method confirmation

标准样品盘背景		
Attacking a poisoned pig	攻毒猪	5
naturally infected pigs	自然感染猪	
10 weeks after weaning	断奶后10周	26
20 weeks after weaning	断奶后20周	28
Total	总计	59
SPF pig	SPF猪	39
Nucleic acid-negative pigs	核酸阴性猪	25
Total	总计	123

猪消化道疾病国际研讨会

System Optimization 1

体系优化1

Determination of coating concentration and enzyme-linked secondary antibody concentration using chessboard titration method

棋盘滴定法确定包被浓度和酶标二抗浓度

	JD1蛋白				
	001号封闭液封闭，37度干燥1小时，PBS稀释。				
	4μg/ml	2μg/ml	1μg/ml	0.5μg/ml	0.25μg/ml
二抗1w	4.83	5.02	5.87	5.57	2.87
二抗2w	4.66	4.87	5.86	5.89	2.67
二抗4w	4.78	4.67	5.40	4.97	1.39

Determination of sample incubation time

样本孵育时间的确定

IFAT背景		样本孵育时间对比		
		30min	60min	90min
1：400	强阳	1.435	1.721	1.821
1：200	中阳	0.967	1.246	1.526
1：150	弱阳1	0.675	0.897	1.265
1：100	弱阳2	0.786	0.987	1.345
/	阴性1	0.145	0.443	0.431
/	阴性2	0.243	0.453	0.723
P AVE		0.966	1.213	1.489
N AVE		0.194	0.448	0.577
P/N		4.978	2.707	2.581

猪消化道疾病国际研讨会

System Optimization 2

体系优化2

Determination of reaction time for enzyme-linked antibody

酶标抗体反应时间的确定

IFA背景		酶标抗体孵育时间对比		
		30min	60min	90min
1：400	强阳	1.675	1.876	2.021
1：200	中阳	0.854	1.052	1.287
1：150	弱阳1	0.579	0.897	1.065
1：100	弱阳2	0.632	0.864	1.234
/	阴性1	0.189	0.435	0.564
/	阴性2	0.243	0.348	0.429
P AVE		0.935	1.172	1.402
N AVE		0.216	0.392	0.497
P/N		**4.329**	2.994	2.823

TMB底物显色时间的确定

Determination of TMB substrate color development time

IFA背景		显色时间对比		
		10min	15min	20min
1：400	强阳	1.387	1.535	1.876
1：200	中阳	0.967	1.282	1.354
1：150	弱阳1	0.675	0.642	1.231
1：100	弱阳2	0.786	0.786	1.143
/	阴性1	0.145	0.134	0.375
/	阴性2	0.243	0.256	0.567
P AVE		0.954	1.061	1.401
N AVE		0.194	0.195	0.471
P/N		4.916	**5.442**	2.975

猪消化道疾病国际研讨会

Optimization sample of sealing liquid formula
封闭液配方的优化样本

IFA背景		封闭液种类				
		方案1	方案2	方案3	方案4	方案5
1：400	强阳	1.904	1.124	1.653	2.388	1.052
1：200	中阳	1.217	1.109	1.189	1.589	0.779
1：150	弱阳1	0.623	0.535	0.794	0.976	0.446
1：100	弱阳2	0.907	0.572	0.639	0.940	0.484
/	阴性1	0.325	0.149	0.189	0.233	0.165
/	阴性2	0.473	0.226	0.208	0.254	0.252
P AVE		1.163	0.835	1.069	1.168	0.570
N AVE		0.399	0.188	0.199	0.244	0.209
P/N		2.914	4.453	5.384	4.798	2.732

Optimization of Sample Dilution Formula
样本稀释液配方的优化

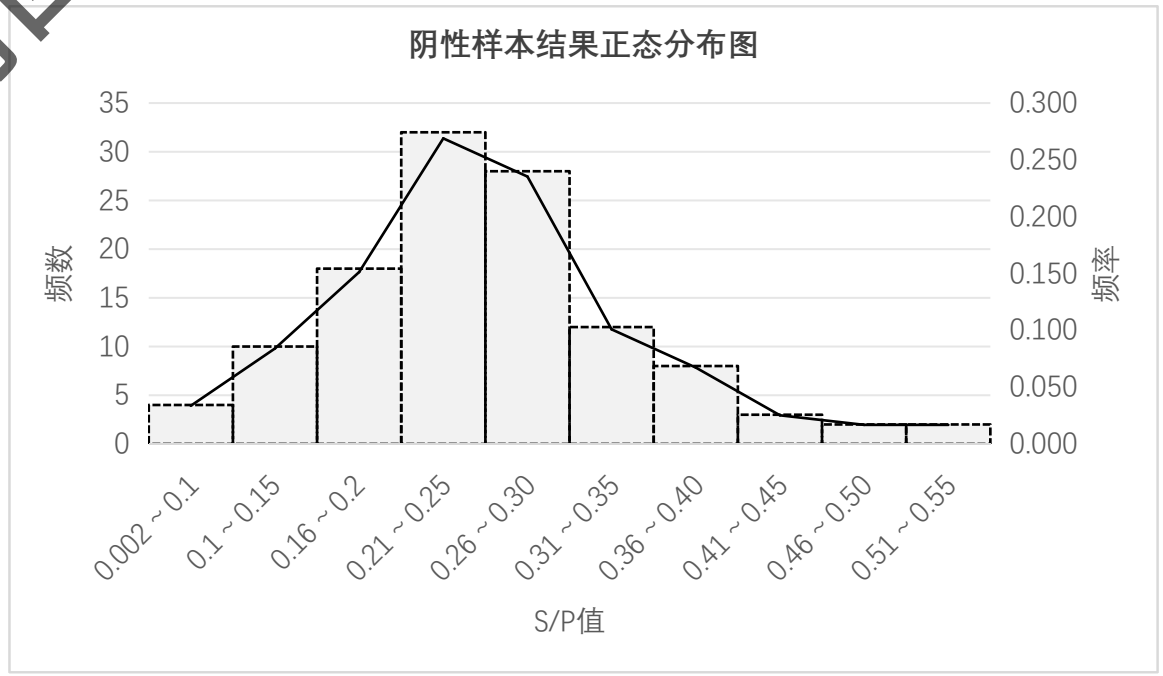
IFA背景		样本稀释液种类优化				
		方案1	方案2	方案3	方案4	方案5
1：400	强阳	1.789	0.692	1.112	1.527	1.387
1：200	中阳	1.265	0.578	0.690	0.931	1.003
1：150	弱阳1	0.865	0.346	0.523	0.725	0.776
1：100	弱阳2	0.754	0.385	0.710	0.715	0.851
/	阴性1	0.221	0.125	0.243	0.228	0.297
/	阴性2	0.189	0.168	0.154	0.124	0.143
P AVE		1.168	0.500	0.759	0.975	1.004
N AVE		0.205	0.147	0.199	0.176	0.220
P/N		5.699	3.415	3.822	5.537	4.565

猪消化道疾病国际研讨会
International Pig Digestive Diseases Symposium
猪消化道疾病国际研讨会

ELISA临界值cut off line的确定 Determination of ELISA cut-off threshold

117份正常猪血清样品（IFA验证为阴性的样本）稀释，在筛选的最佳条件下进行间接ELISA测定，设置阴阳性对照孔，对检测结果进行统计学分析，得到OD450nm，计算S/P值平均值（X）和标准差（SD），将S/P值≥X+3SD（即S/P值0.504）时，判为阳性；S/P值＜X+2SD 即（S/P值0.415）时，判为阴性，介于两者之间判为可疑。

117 normal pig serum samples (samples verified as negative by IFA) were diluted and subjected to indirect ELISA testing under the best screening conditions. A negative and positive control well was set up, and the test results were statistically analyzed to obtain OD450nm. The average S/P value (X) and standard deviation (SD) were calculated. When the S/P value ≥ X+3SD (i.e. S/P value 0.504), it was judged as positive; When the S/P value is less than X+2SD (S/P value 0.415), it is considered negative, and if it falls between the two, it is considered suspicious.



猪消化道疾病国际研讨会

ELISA诊断敏感性和诊断特异性 ELISA diagnostic sensitivity and specificity

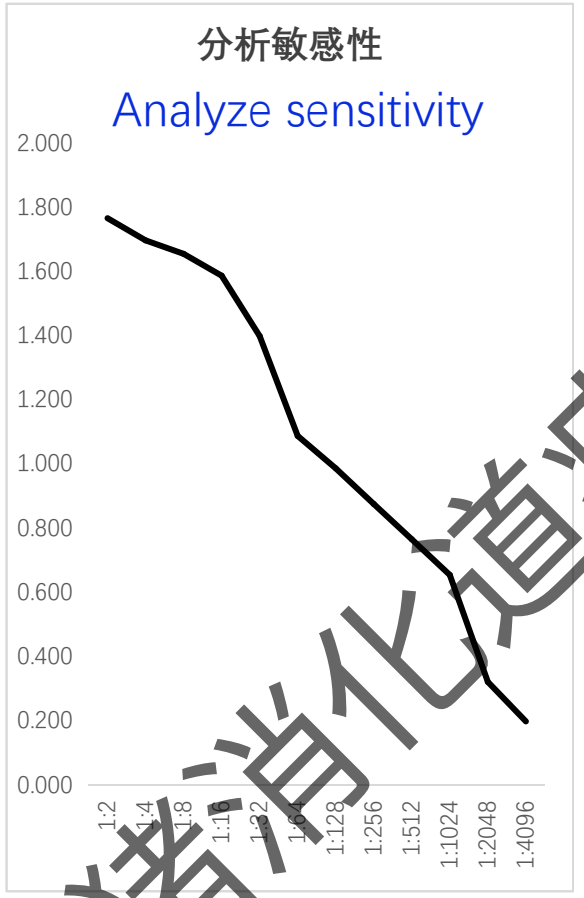
ELISA sensitivity and diagnostic specificity　　ELISA敏感性和特异性

	Positive quantity/tested quantity	阳性数量/检测数量	敏感性(%)
Attacking a poisoned pig	攻毒猪	22/23	95.70%
naturally infected pigs	自然感染猪		
10 weeks after weaning	断奶后10周	25/26	96.20%
20 weeks after weaning	断奶后20周	27/28	96.40%
Total	总计	74/77	96.10%
		阴性数量/检测数量	特异性（%）
SPF pig	SPF猪	39/39	100%
Nucleic acid-negative pigs in clinically negative herds	临床阴性猪群核酸阴性猪	99/99	100%
Total	总计	138/138	100%

猪消化道疾病国际研讨会

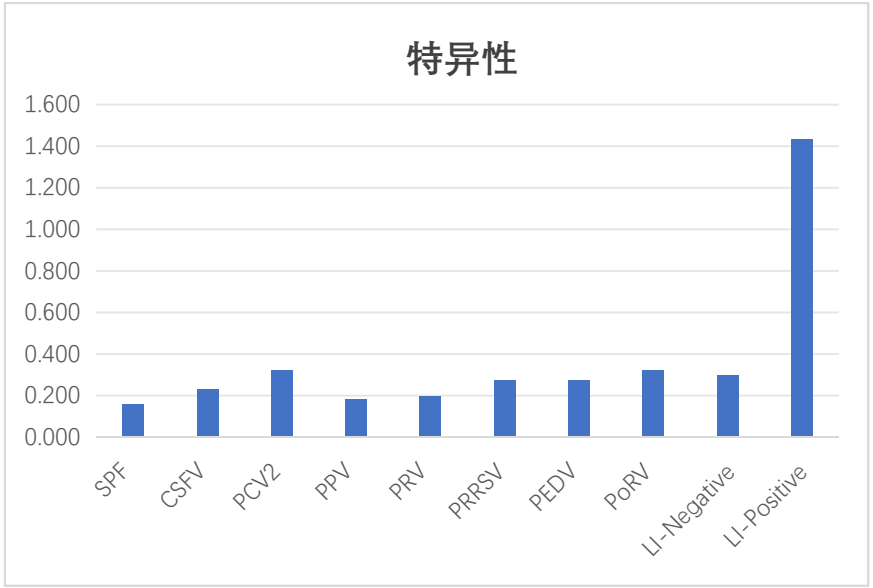
分析敏感性和特异性 Analyze sensitivity and specificity

分析敏感性	
IFA 1:400 稀释倍数	S/P
1:2	1.765
1:4	1.696
1:8	1.654
1:16	1.586
1:32	1.398
1:64	1.087
1:128	0.986
1:256	0.875
1:512	0.765
1:1024	0.654
1:2048	0.321
1:4096	0.198



特异性样本	S/P
SPF	0.160
CSFV	0.231
PCV2	0.323
PPV	0.180
PRV	0.198
PRRSV	0.276
PEDV	0.276
PoRV	0.321
LI-Negative	0.298
LI-Positive	1.432

Specificity



猪消化道疾病国际研讨会

临床符合率 Clinical compliance rate

悦洋公司试剂盒		IFAT		
		阳性	阴性	合计
JD1	阳性	144	5	149
	阴性	10	62	72
	合计	154	67	221
阳性符合率（敏感性）		93.51%		
阴性符合率（特异性）			92.54%	
总符合率				93.21%

悦洋公司试剂盒		IFAT		
		阳性	阴性	合计
JD3	阳性	139	8	147
	阴性	15	59	74
	合计	154	67	221
阳性符合率（敏感性）		90.26%		
阴性符合率（特异性）			88.06%	
总符合率				89.59%

悦洋公司试剂盒		IFAT		
		阳性	阴性	合计
flgE（全基因）	阳性	138	12	150
	阴性	16	55	71
	合计	154	67	221
阳性符合率（敏感性）		89.61%		
阴性符合率（特异性）			82.09%	
总符合率				87.33%

悦洋公司试剂盒		IFAT		
		阳性	阴性	合计
JD2	阳性	140	7	147
	阴性	14	60	74
	合计	154	67	221
阳性符合率（敏感性）		90.91%		
阴性符合率（特异性）			89.55%	
总符合率				90.50%

猪消化道疾病国际研讨会

重复性 Repeatability

批内重复性 Within batch repeatability						
样品名称	20231201-1	20231201-2	20231201-3	平均值	标准差	变异系数(%)
阳性血清	1.275	1.23	1.16	1.222	0.058	4.74%
弱阳性血清	0.837	0.887	0.876	0.867	0.026	3.03%
阴性血清	0.159	0.163	0.149	0.157	0.007	4.59%

批间重复性 Repeatability between batches						
样品名称	批次			平均值	标准差	变异系数(%)
	20231201	20231202	20231203			
阳性血清	1.121	1.165	1.151	1.146	0.022	1.96%
弱阳性血清	0.865	0.885	0.979	0.910	0.061	6.69%
阴性血清	0.134	0.128	0.14	0.134	0.006	4.48%

猪消化道疾病国际研讨会

总结:回肠炎防控手段

Summary: Prevention and control measures for ileitis

- ✓ 定期监测、及时隔离淘汰阳性动物

Regular monitoring, timely isolation and elimination of positive animals

- ✓ 适当提高疫苗接种率

Appropriately increase the vaccination rate

- ✓ 药物预防

Drug prevention

核酸检出率高：

- 采样污染：新鲜粪便
- 环境污染：猪舍环境
- 建议采集肛拭子
- 采样时间（夏秋多发）
- 对象：断奶仔猪和育肥猪

High nucleic acid detection rate:

- Sampling contamination: fresh feces
- Environmental pollution: pigsty environment
- Suggest collecting an anal swab
- Sampling time (frequent in summer and autumn)
- Target: Weaned piglets and fattening pigs

抗体检测的三个应用场景：

- 流行病学摸底：是否需要用药和疫苗
- 用药用苗效果评估
- 回肠炎净化效果评估

Three application scenarios for antibody detection:

- Epidemiological investigation: whether medication and vaccines are needed
- Evaluation of the effectiveness of drug use vaccines
- Evaluation of purification effect on ileitis

猪消化道疾病国际研讨会

广东省专精特新中小企业

国家高新技术企业

广州市科技创新小巨人企业

兽医诊断制品(A类)GMP生产线企业

猪消化道疾病国际研讨会

感谢聆听！

李雪平
18620758386