Pathogen recognition proteins in rainbow trout (*O. mykiss*) plasma

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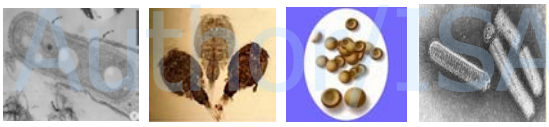
Functional approach - Direct binding assays

Protocol *(Brooks et al. 2003; Lillie et al., 2006)*

Rainbow trout plasma / TBSCa^{2+}

▼

Formalin-killed bacteria / chitin / *Artemia* / *Virus*



▼

Wash away unbound plasma proteins

▼

Elute with sugar cocktail / EDTA

▼

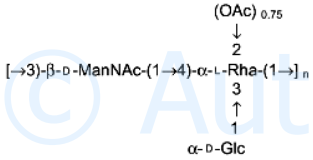
1-Dimension SDS PAGE

Identify soluble proteins in rainbow trout plasma which bind relevant targets

Bacteria ◂ | **Pathogen associated molecular patterns** | ▸ **Parasite**

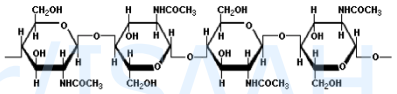
Aeromonas salmonicida

O- polysaccharrides – trisaccharide repeating units



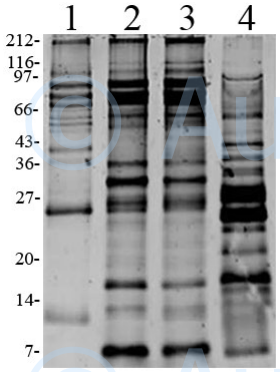
Lepeophtheirus salmonis **(salmon louse)**

Chitin = Exoskeleton



Soluble proteins in rainbow trout plasma which bind relevant targets

1-D Reducing SDS PAGE



37 kDa

34 kDa

29 kDa

16-18 kDa

Lane 1. R. trout plasma

Lane 2. Purified chitin eluate

Lane 3. *Artemia* cysts eluate

Lane 4. *A. salmonicida* eluate

Similar sized proteins identified

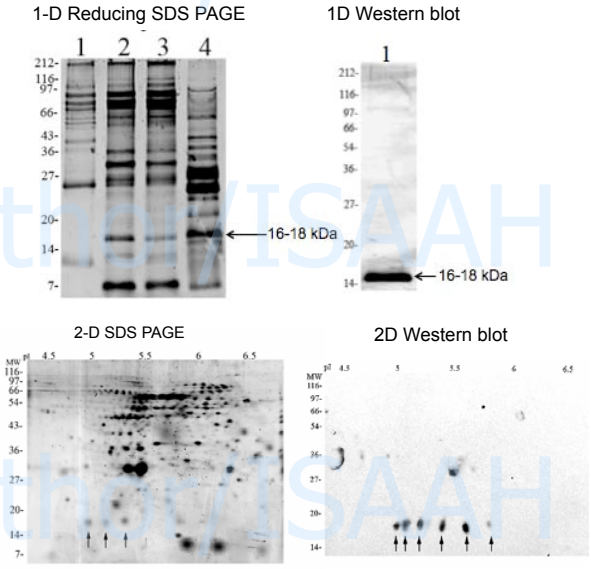
Functional binding studies

Bacterial isolates	Proteins identified Reduced kDa
Aeromonas salmonicida (Non virulent ATCC strain)	p16 + p37+ p29
Aeromonas salmonicida (Virulent Ontario strain)	p16+ p37, p25, p34
Aeromonas hydrophila (Virulent Ontario strain)	p16+ p37, p25, p34
Flavobacterium psychrophilum	p16+ p37
Psuedomonas species	p16+ p37
Chitin	p16+ p37+ p29
Artemia- brine shrimp eggs	p16+ p37+ p29

Similar proteins identified from relevant targets ‣ p16 + p37

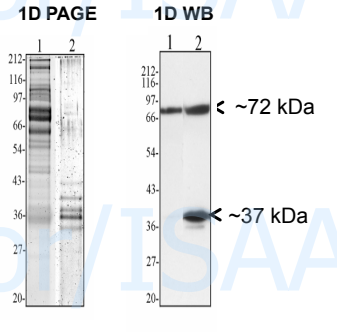
16 kDa = Rainbow trout ladderlectin (RTLL)

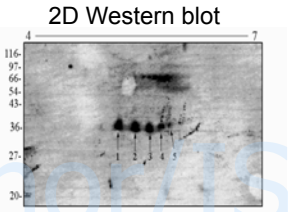
1. Mannose specific VII C-type lectin – similar to Atlantic salmon serum mannan-binding lectin
2. Structural heterogeneity of isoforms were observed using 2D SDS PAGE



37 kDa = Rainbow trout Intelectin

1. 975 bp cDNA and 325 AA sequence - intelectin protein
2. Structural heterogeneity of isoforms were observed using 2D SDS PAGE





> 5 isoforms identified by antiserum

Lectins as Pattern Recognition Receptors

Definition :
- non enzymatic carbohydrate binding proteins that are not immunoglobulins.
- Pattern recognition molecules (PRRs) that bind pathogen associated molecular patterns (PAMPs)

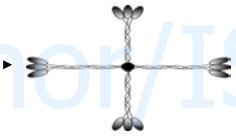
Classification :
1. Carbohydrate ligand
2. Biological process
3. Cellular localization
4. +/- divalent cation dependence

Carbohydrate recognition domain (CRD) - pattern of invariant and highly conserved amino acid residues at a characteristic spacing.

Mannose binding lectin
C1q
Ficolin (Ca^{2+} independent)

Opsonization
Complement
Activation

Conglutinin
SP-D

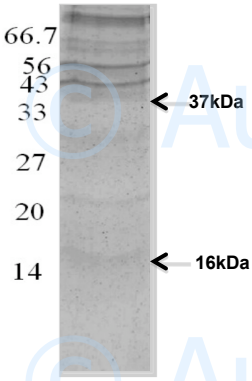


Agglutination

(Ichijo et al., 1993)

Viral Hemorrhagic Septicemia Virus direct binding assay

1D SDS PAGE of EDTA elution

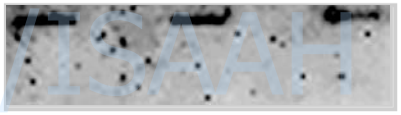


SLOT BLOTS

Rabbit α Intelectin

Whole plasma

5 ml EDTA



Rabbit α Ladderlectin

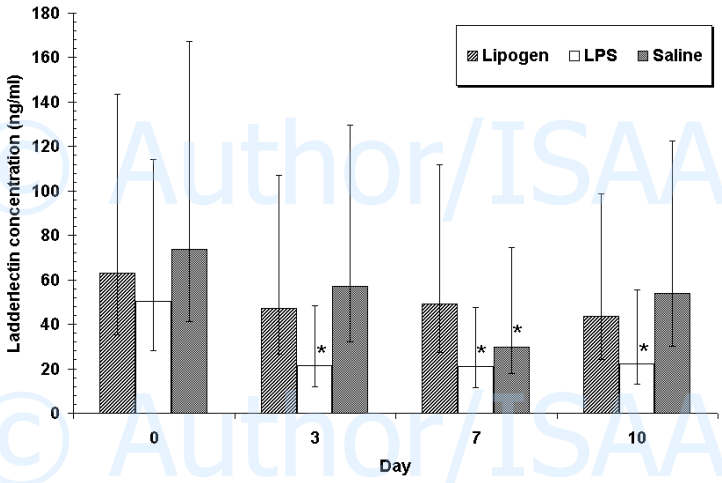
Whole plasma

5 ml EDTA

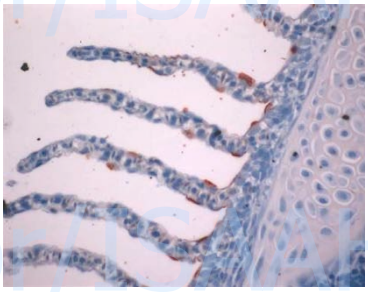
Ladder lectin binds VHSV but Intelectin does <u>not</u>

Are these lectins acute phase proteins?

ELISA for Rainbow trout ladderlectin (RTLL)

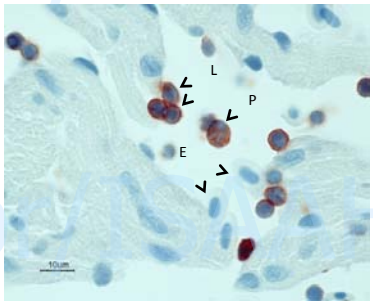


IHC healthy tissues – Ladderlectin p16



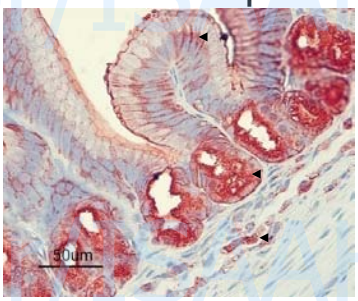
Cytoplasmic staining

- epithelial cells of gills and intestinal villi
- leukocytes epidermis, dermis and sub-mucosa
- cells within hepatic sinusoids
- renal hematopoietic interstitium



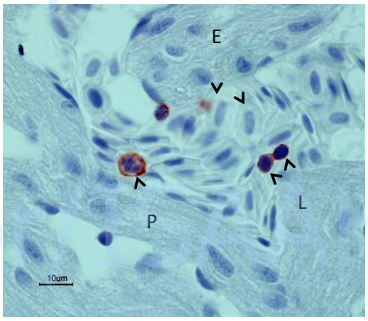
1° Ab - 1:4000
2° Ab - 1:10,000

IHC healthy tissues – Intelectin p37



Cytoplasmic staining

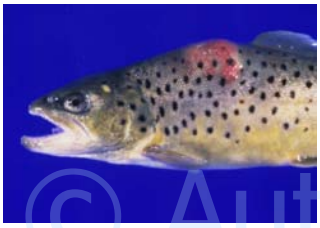
- epithelial cells of gills and intestinal villi
- leukocytes epidermis, dermis and sub-mucosa
- cells within hepatic sinusoids
- renal hematopoietic interstitium
- epithelial cells of swim bladder



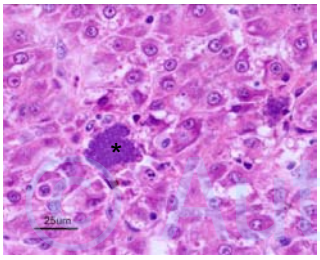
1° Ab - 1:4000
2° Ab - 1:10,000

A.salmonicida - Furunculosis

Gross pathology

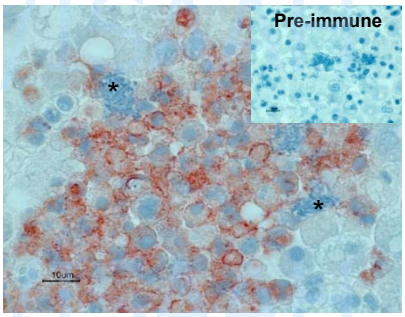


Histopathology

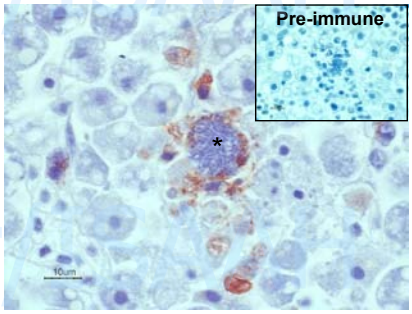


IHC

Ladderlectin



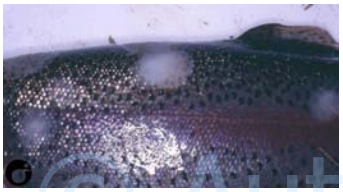
Intelectin



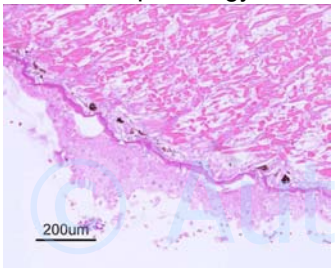
1° Ab - 1:4000 2° Ab - 1:10,000

Mycotic dermatitis

Gross pathology

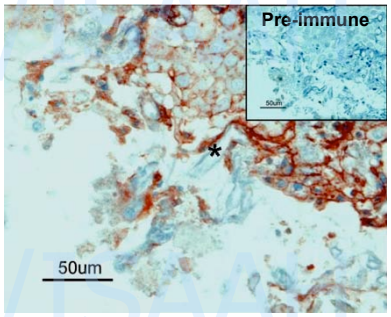


Histopathology

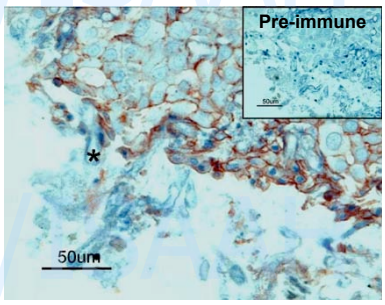


IHC

Ladderlectin



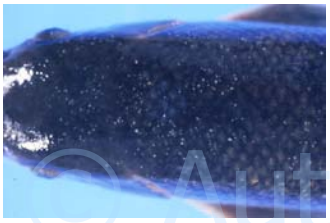
Intelectin



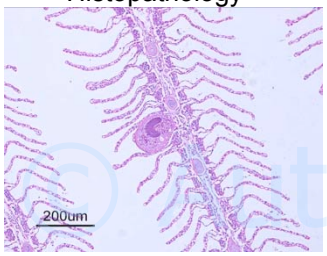
1° Ab - 1:4000 2° Ab - 1:10,000

***I. multifilis* – White spot disease**

Gross pathology

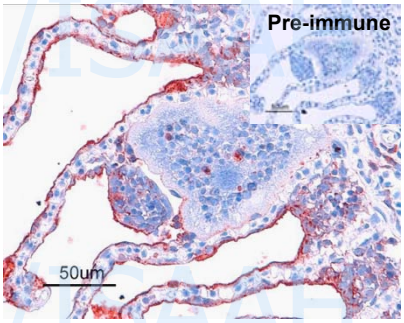


Histopathology

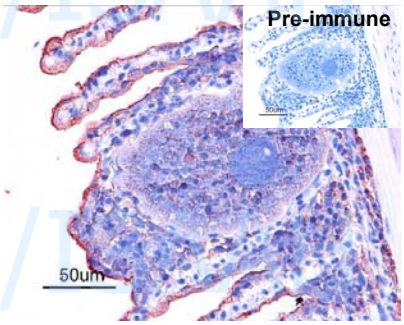


IHC

Ladderlectin



Intelectin



1° Ab - 1:4000 2° Ab - 1:10,000

Conclusions

- Both lectins acting as Pattern Recognition Receptors
  - Ladder lectin binds chitin, Gram -ve bacteria and VHSV
  - Intelectin binds chitin and Gram -ve bacteria and **not** VHSV
  - multiple electrophoretic isoforms
- Both lectins do not act as acute phase reactants
- IHC of both RTLL and RTInt from healthy trout localized to
  - individual cells at mucosal surfaces
  - leukocytes
- IHC of RTLL and RTInt from clinically infected fish
  - extra-cellular spaces around bacteria, fungi and protozoa
  - leukocytes - inflammation

Future work

- Purification or expression production systems
  - functional assays – opsonization / agglutination / neutralization
  - differential binding of protein isoforms
  - ability to activate complement
- Alternative binding targets – Gram positive bacteria, virus etc
- Mechanisms producing multiple protein isoforms and tissue specific production
- Characterize other bacterial binding proteins identified

The end

Thank you - Acknowledgements

- Fish Path Lab - Karrie Young, Paul Huber, Mackenzie Smith and Shohreh Hesami, Arman Y.
- Beinzle lab - Paula Katavolos, Mary Ellen Clarke, Sherry Wen and Dr. Beinzle
- Hayes / Turner / Brooks lab - Brandon Lillie, Betty Anne Quinn, Jutta Hammermueller and Dr. Brooks
- Funding – NSERC PhD scholarship, OGS, NSERC Discovery, OMAFRA, CFIA

Fish intelectins

Channel catfish

- Intelectin gene sequences strongly (> 50 times) induced following i.p. of *Edwardsiella ictaluri* *(Peatman et al., 2007)*

Grass Carp

- Intelectin gene sequences induced in multiple tissues following LPS stimulation *(Chang and Nie 2007)*

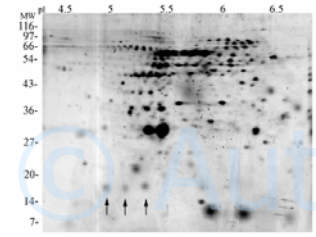
rainbow trout

- Partial intelectin gene sequence induced following i.p. injection of emulsified, killed *Listonella (Vibrio) anguillarum* *(Bayne et al., 2001; Gerwick et al., 2007)*

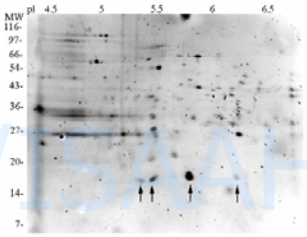
Ladderlectin isoforms

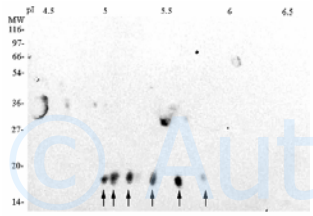
RT plasma

A. salmonicida eluate

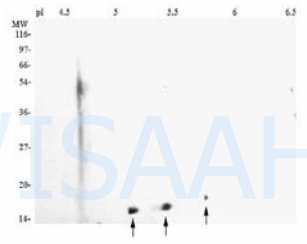


2-D SDS PAGE





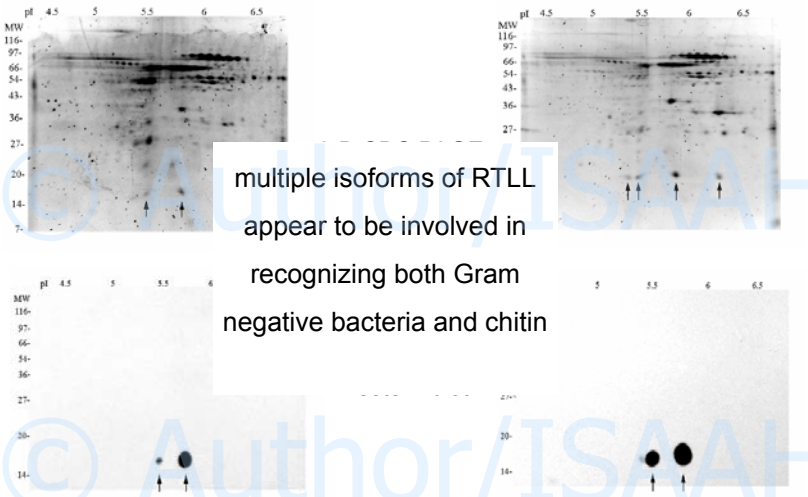
Western blot



Ladderlectin isoforms

Purified chitin eluate

Artemia cysts eluate

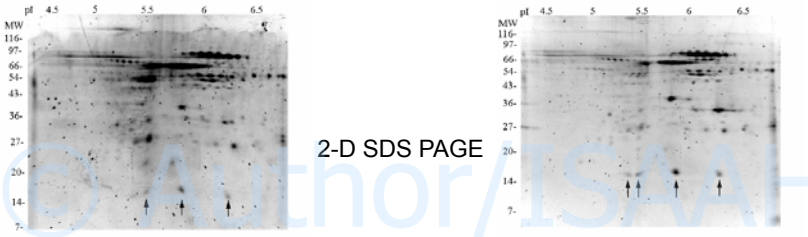


multiple isoforms of RTLL appear to be involved in recognizing both Gram negative bacteria and chitin

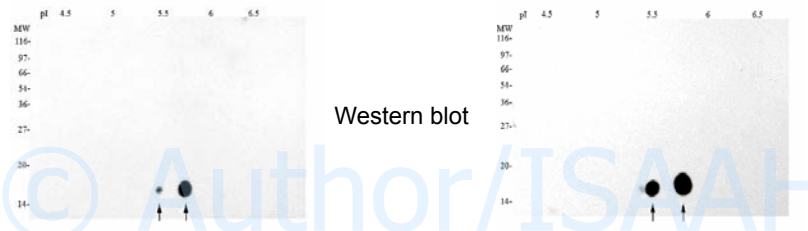
Ladderlectin isoforms

Purified chitin eluate

Artemia cysts eluate

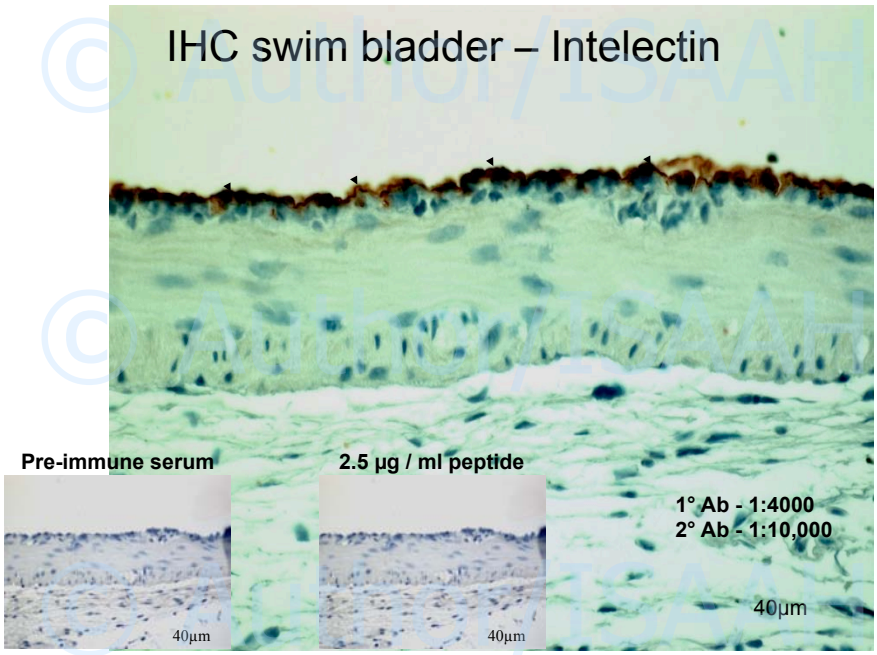


2-D SDS PAGE



Western blot

IHC swim bladder – Intelectin



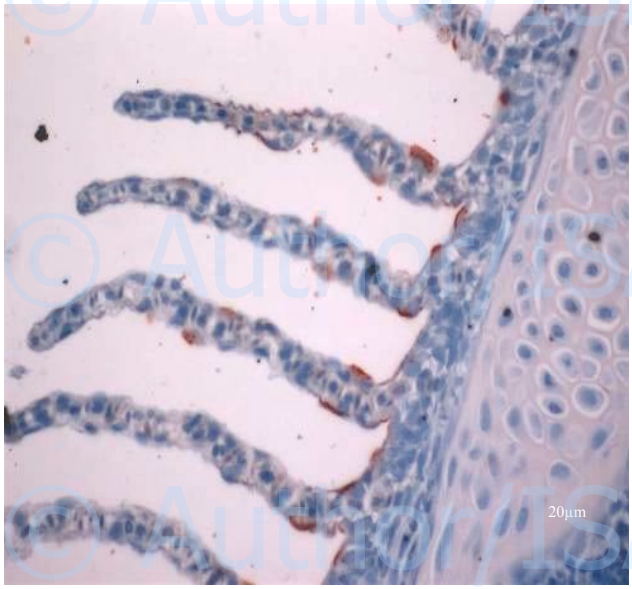
IHC localization of RTLL and RTInt in tissues from infected trout

- Infected rainbow trout – Clinical cases
  - 4 fish – *Aeromonas salmonicida subsp. salmonicida*
  - 4 fish – *Flavobacterium psychrophilum*
  - 2 fish – Mycotic dermatitis
  - 4 fish – *Ichthyophthirius multifilis*
  - 2 fish – *Loma salmonae*

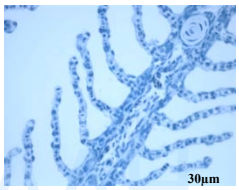
Acute phase response – rainbow trout

		Pre stimulus plasma			Post stimulus plasma					Total
	Days of experiment	-9	-5	0	1	3	7	10	14	fish
Trial 1	Saline (n=5)	◄	———	►	◄	———	———	►	-	5
	Vaccine (n=5)	◄	———	►	◄	———	———	►	-	5
	LPS/FIA (n=5)	◄	———	►	◄	———	———	►	-	5
Trial 2	Non injected (n=4)	-	-	n=4	-	-	-	-	-	4
	Saline injected	-	-	-	-	n=4	n=4	n=4	n=4	16
	Vaccine (n=16)	-	-	-	-	n=4	n=4	n=4	n=4	16

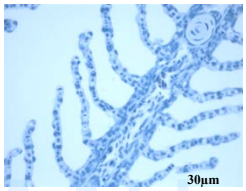
IHC gill – RTLL



Pre-immune serum



2.5 µg / ml peptide

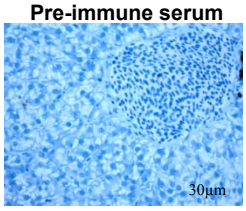


1° Ab - 1:4000
2° Ab - 1:10,000

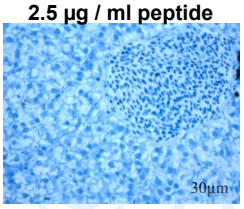
IHC Liver – RTLL

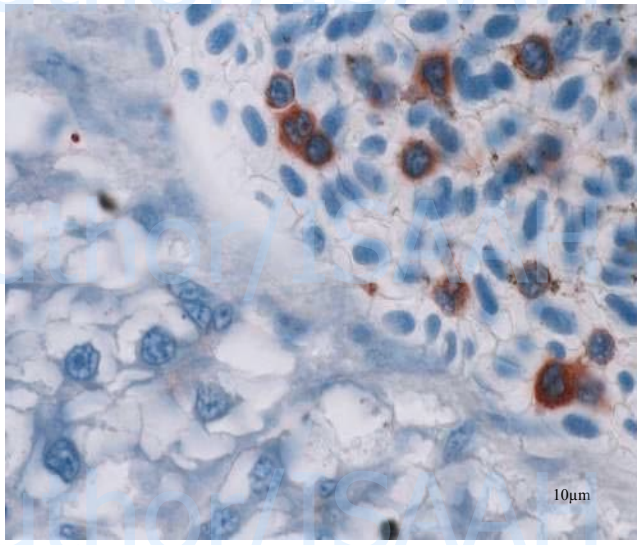
1° Ab - 1:4000
2° Ab - 1:10,000

Pre-immune serum



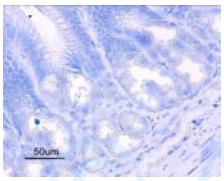
2.5 µg / ml peptide



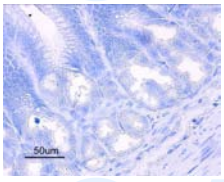


IHC intestine – Intelectin

Pre-immune serum



2.5 µg / ml peptide



1° Ab - 1:4000
2° Ab - 1:10,000