3. A primary cell line from allogynogenetic silver "crucian" carp, *Carrassius auratus gibelio* brain (**CrCB**) (Duan Hongan) and progress in CyHV-2 research

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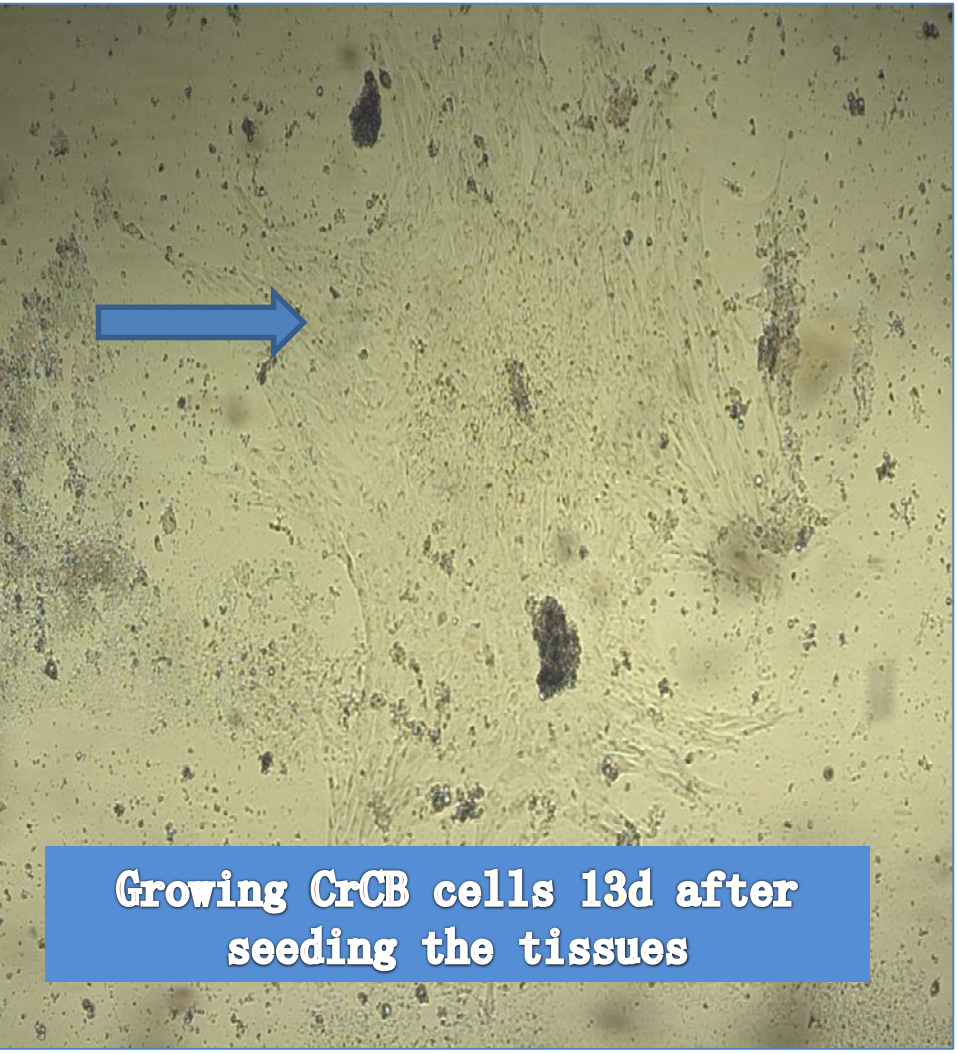
Main contents

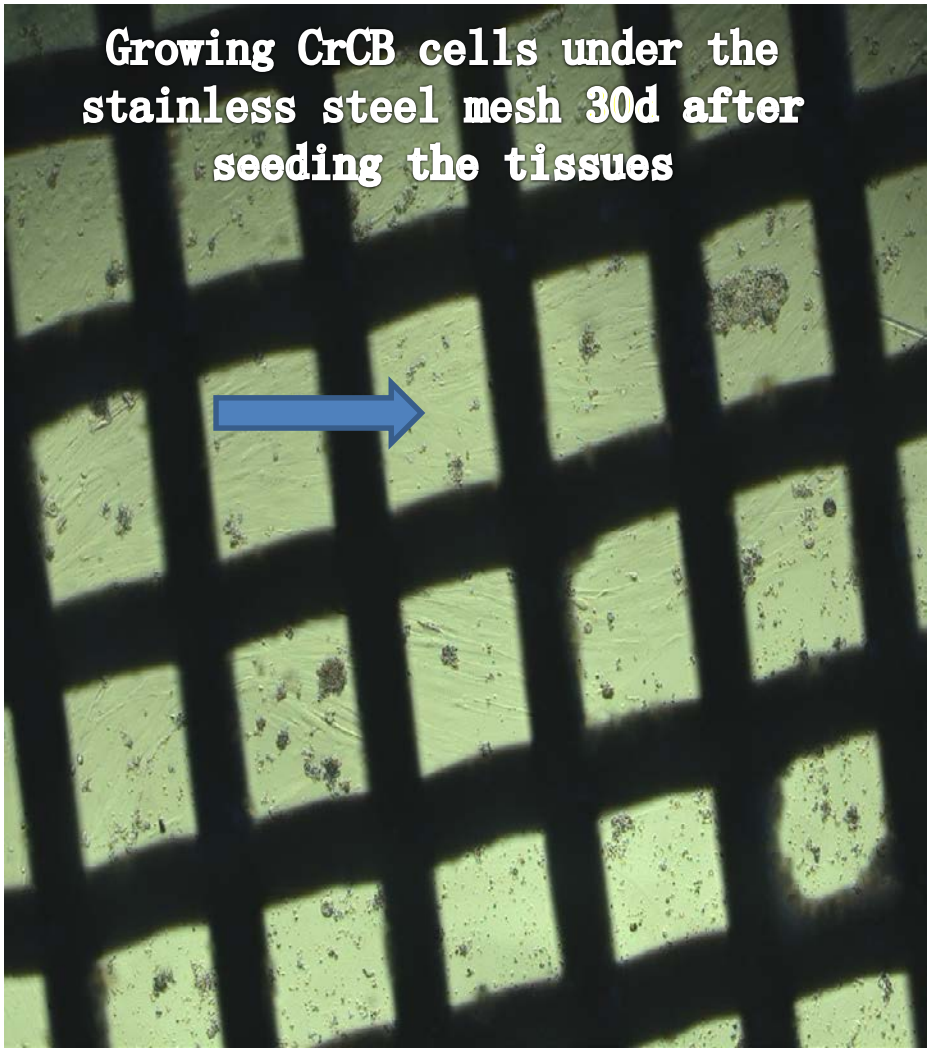
- Establishment of primary cell line from allogynogenetic *Car. auratus gibelio* brain
- Experimental infection of goldfish and PCR testing for CyHV-2
- Isolation of CyHV-2 strains from goldfish, and PCR
- Research in the future
- Acknowledgements

SOP Establishment of primary cell line from allogynogenetic *Car. auratus gibelio* brain

- Aseptically take brain from healthy allogynogenetic silver crucian carp (*Carassius auratus gibelio* of 8-10cm long, rinse 2x in 75% ethanol, 10-15 sec each, rinse 3x in sterile MEM or M199 medium
- Cut brain tissue into about $1mm^3$, transfer into 15ml sterile centrifuge tube, add 2 times the volume of trypsin solution, incubate about 5 min at 25 ℃
- Centrifuge for 2 min at 2000 rpm, discard the supernatant
- Suspend the pellet in appropriate amount of MEM medium with 10% FBS, centrifuge for 2 min at 2000 rpm, discard the supernatant
- Transfer the pellet into the cell culture flask with a sterile inoculation ring or other equipment, plate the tissue to a single layer, cover the tissue block with a sterile stainless steel mesh with wire diameter 0.20 mm and aperture 0.308 mm, incubate flask at 25℃ for 6-24h. Add M199 medium containing 20% FBS, penicillin 100 U/ml, streptomycin 0.1mg/ml, FGF (fibroblast growth factor) 10µg/ml and/or EGF (epidermal growth factor) 10µg/ml. Replace the fresh medium every 3-5 days
- When ~80% confluence: subculture the cells, then passage every 10 days
- From passage # 10 replace medium with 10% FBS, but no antibiotics, FGF and EGF.
- Passage the cells ~every 5 days.

Primary cell line from *C.aur.gibelio* brain





Further tests on these cells

- 16S and 18S goldfish mtDNA fragment primers were used to differentiate the cell lines. The amplified products were sequenced and analysed by Blast analysis.
- Giemsa staining, and the cell chromosome number and karyotype analysis were done.

Establishment of primary cell line from allogynogenetic *Car. auratus gibelio* brain

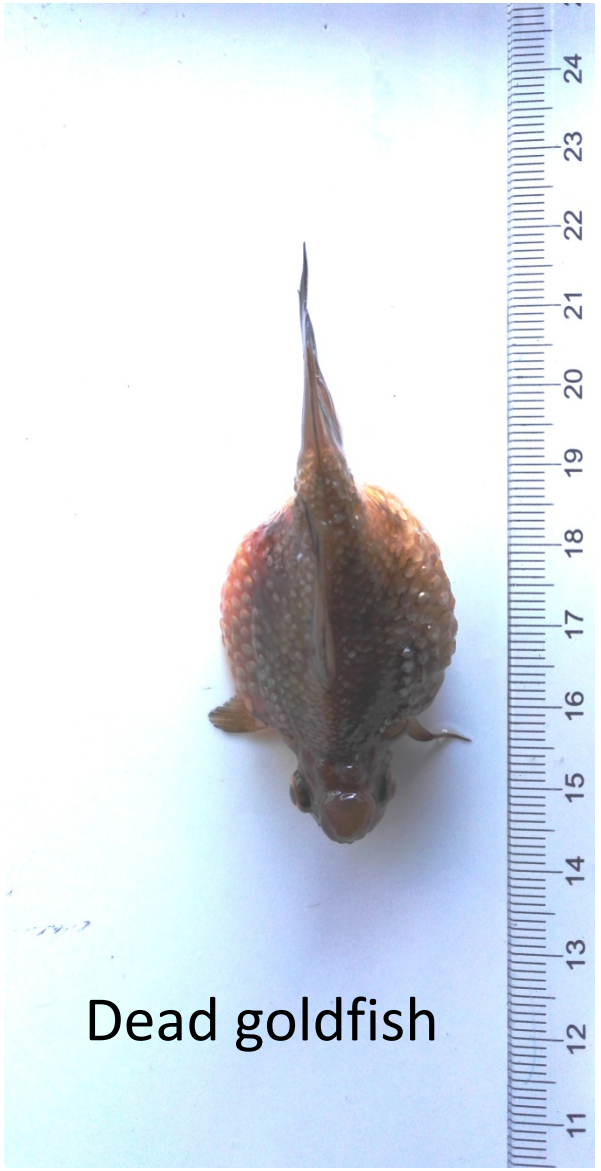
Results

- The first generations of CrCB cells adhered difficult to the flask and grew slowly into a confluent monolayer
- It took ~ 30 days for the CrCB cells to grow to ~80% confluence, from then cell growth gradually accelerated. From the 10^{th} passage it took 24-48h for the cells to grow into confluent monolayers
- CrCB has a fibrous cell morphology
- PCR showed target fragments (123bp and 234bp) that were amplified by 16S and 18S primers and confirmed by sequencing and blast analysis
- Chromosome analysis and further characterization is in progress

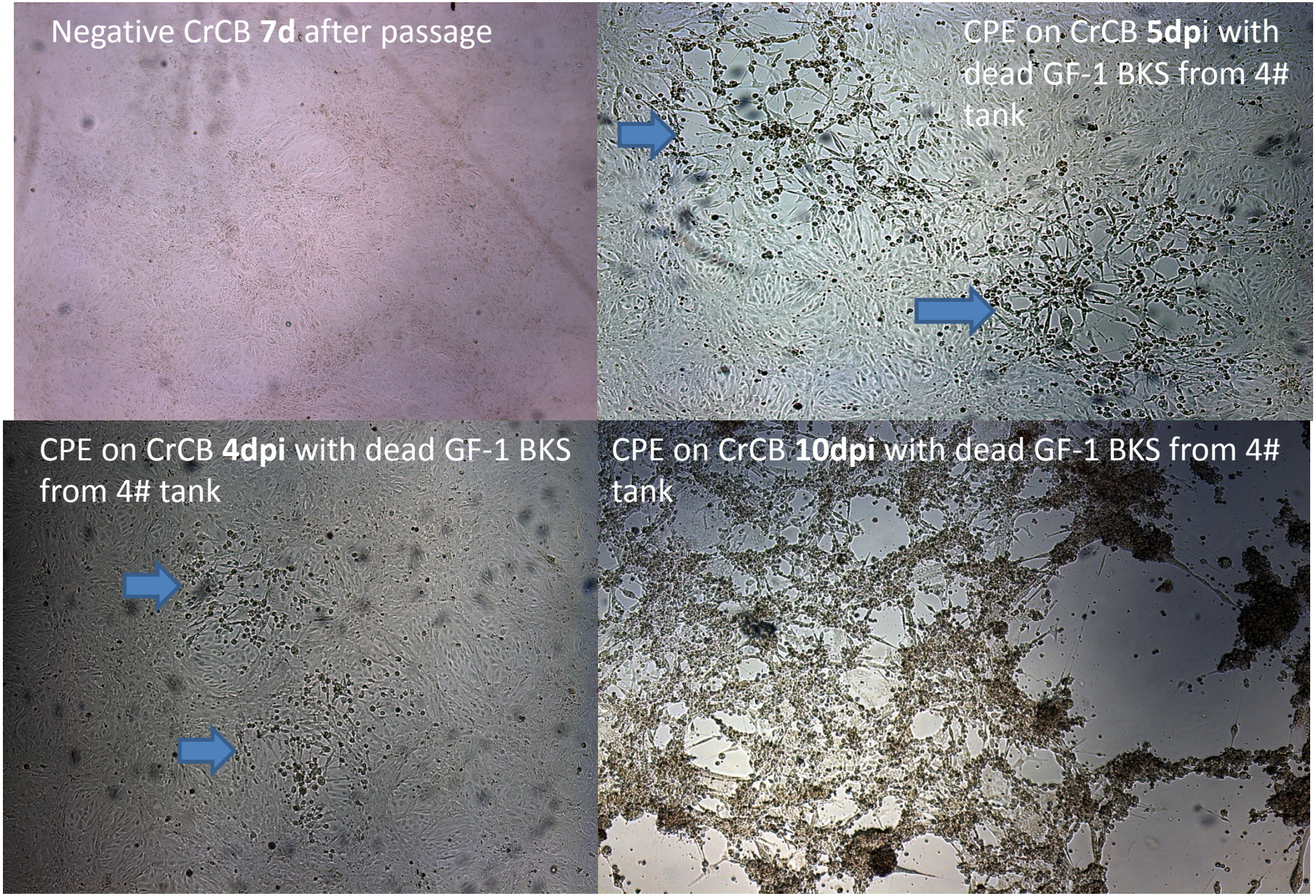
Experimental infection with and isolation of CyHV-2 from goldfish

- Feb 2017 goldfish kept at 3-6℃ were taken to the lab and put in the tank, and the water was heated to about 25 ℃
- Brain kidney and spleen (**BKS**) and intestine of sick and dead fish ➡ PCR test
- If PCR + for CyHV-2 ➡ homogenized and filtered with 0.45µm filter and inoculated onto the CrCB
- CPE positive CrCB tested by PCR for CyHV-2 and sequencing

Experimental infection of and Isolation of CyHV-2 from goldfish



Experimental infection of and Isolation of CyHV-2 from goldfish



Experimental infection of and Isolation of CyHV-2 from goldfish

Results

- Fish began to die from day 6 p.i. (PCR+ for CyHV-2)
- Tissues from sick or dead goldfish: PCR + for CyHV-2
- CrCB cells inoculated with tissue homogenates from sick/dead goldfish showed CPE at 3-5d post-inoculation.
- CPE+ CrCB suspensions were PCR positive for CyHV-2

Research in the future

- Further characterization of CrCB
- Further characterization of CyHV-2 strains
- Production of reagents for detection of CyHV-2
- Production of inactivated CyHV-2 vaccine and trial

Acknowledgements

- Thank you for your attention!
- Thanks for the organizers to give the opportunity for the inclusion the presentation in this conference
- Thanks for Dr Olga Haenen to edit and present this PPT to the meeting
- Welcome to research cooperation on diagnosis and prevention of this disease
- CrCB and CyHV-2 strains can be sent to you on request

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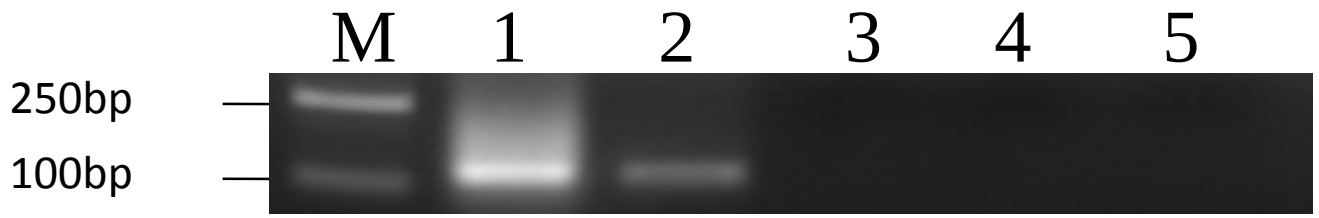
Characterization of primary cell line CrCB

reference： Establishment, characterization, and viral susceptibility of two cell lines derived from goldfish *Carassius auratus* muscle and swim bladder (Rougée et al., 2007）

16S fragment (123 bp) :

16S-F 5’-GCGACCACGGAGGAAAAA-3’
16S-R 5’-CGTTGATCGGCTTGTATTAG-3’



M: DL2000; 1: CrCB; 2: goldfish brain cell; 3-5: negative control

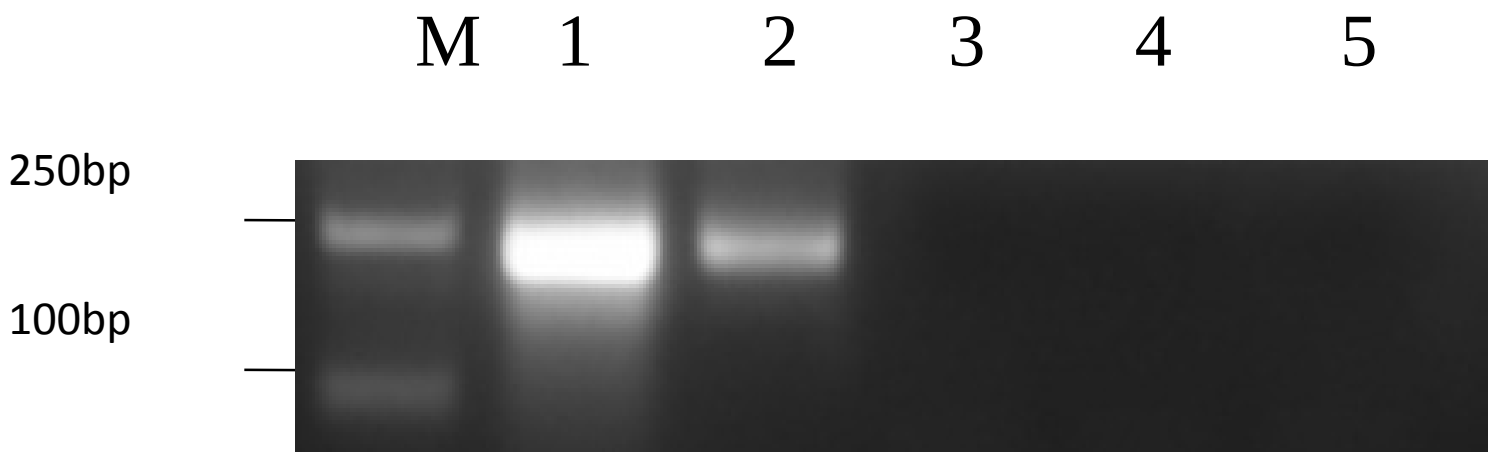
Fig1. **16S PCR results**

Characterization of primary cell line CrCB

reference： Establishment, characterization, and viral susceptibility of two cell lines derived from goldfish Carassius auratus muscle and swim bladder（Dis Aquat Org 77: 127–135, 2007）

18S fragment (234 bp) :

(18S-F)5’-GCGAGACGAGCCACCACCTATC-3’ (18S-R) 5’-CCCCCGGCCGTCCCTCTTA-3’.



M: DL2000; 1: CrCB; 2: goldfish brain cell; 3-5: negative control

fig2. **18S PCR results**

Characterization of primary cell line CrCB

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

16s F alignment result

Description	Max score	Total score	Query cover	E value	Ident	Accession
Carassius gibelio isolate 2 mitochondrion, complete genome	159	159	100%	6e-36	100%	KU896992.1
Carassius gibelio isolate 1 mitochondrion, complete genome	159	159	100%	6e-36	100%	KU896991.1
Carassius gibelio isolate CQFJ5 mitochondrion, complete genome	159	159	100%	6e-36	100%	KU668580.1
Carassius gibelio isolate CQFJ3 mitochondrion, complete genome	159	159	100%	6e-36	100%	KU668578.1
Carassius gibelio isolate CQFJ2 mitochondrion, complete genome	159	159	100%	6e-36	100%	KU668577.1
Carassius gibelio isolate CQBP2 mitochondrion, complete genome	159	159	100%	6e-36	100%	KU668573.1
Carassius gibelio isolate CQBA4 mitochondrion, complete genome	159	159	100%	6e-36	100%	KU668571.1
Carassius gibelio isolate CQBA2 mitochondrion, complete genome	159	159	100%	6e-36	100%	KU668569.1
Carassius gibelio isolate CQBA1 mitochondrion, complete genome	159	159	100%	6e-36	100%	KU668568.1
Carassius auratus grandoculis mitochondrial gene for 16S ribosomal RNA, partial sequence, isolate: TG53	159	159	100%	6e-36	100%	LC097884.1

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

16s R alignment result

Description	Max score	Total score	Query cover	E value	Ident	Accession
Carassius gibelio isolate 2 mitochondrion, complete genome	158	158	98%	2e-35	100%	KU896992.1
Carassius gibelio isolate 1 mitochondrion, complete genome	158	158	98%	2e-35	100%	KU896991.1
Carassius gibelio isolate CQBA2 mitochondrion, complete genome	158	158	98%	2e-35	100%	KU668569.1
Carassius gibelio isolate CQBA1 mitochondrion, complete genome	158	158	98%	2e-35	100%	KU668568.1
Carassius auratus 'high back crucian carp' mitochondrion, complete genome	158	158	98%	2e-35	100%	KT756205.1
Carassius carassius isolate Carcar1 16S ribosomal RNA gene, partial sequence; mitochondrial	158	158	98%	2e-35	100%	KR476838.1
Carassius auratus mitochondrion, complete genome	158	158	98%	2e-35	100%	KJ874431.1
Carassius auratus mitochondrion, complete genome	158	158	98%	2e-35	100%	KJ874429.1
Carassius auratus mitochondrion, complete genome	158	158	98%	2e-35	100%	KM659025.1
Carassius auratus isolate Ca-Mugla 16S ribosomal RNA gene, partial sequence; mitochondrial	158	158	98%	2e-35	100%	KM657136.1
Carassius auratus mitochondrion, complete genome	158	158	98%	2e-35	100%	KJ476998.1

Characterization of primary cell line CrCB

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accessio
Carassius gibelio 18S ribosomal RNA gene, partial sequence	285	285	99%	3e-73	94%	KT002365.1
Carassius carassius 18S ribosomal RNA gene, partial sequence	285	285	99%	3e-73	94%	HQ615531.1
Carassius auratus 18S ribosomal RNA gene, partial sequence	285	285	99%	3e-73	94%	FJ710819.1
Carassius auratus gibelio 18S ribosomal RNA gene, partial sequence	285	285	99%	3e-73	94%	EF189737.1
Cyprinus carpio 18S ribosomal RNA gene, partial sequence	279	279	99%	1e-71	93%	KT002364.1
Cyprinus carpio genome assembly common carp genome, scaffold 000028946	279	1098	99%	1e-71	93%	LN591197.1
Onychostoma macrolepis 18S ribosomal RNA gene, partial sequence	279	279	99%	1e-71	93%	JQ283288.1
Cyprinus carpio intergenic spacer, partial sequence; 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer	279	279	99%	1e-71	93%	JN628435.1
Cyprinus carpio 18S ribosomal RNA gene, partial sequence	279	279	99%	1e-71	93%	FJ710826.1
Cyprinus carpio intergenic spacer region, partial sequence; 5' external transcribed spacer, 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal	279	279	99%	1e-71	93%	AF133089.2

18s F alignment result

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accessio
Carassius gibelio 18S ribosomal RNA gene, partial sequence	241	241	98%	6e-60	90%	KT002365.1
Carassius carassius 18S ribosomal RNA gene, partial sequence	241	241	98%	6e-60	90%	HQ615531.1
Carassius auratus 18S ribosomal RNA gene, partial sequence	241	241	98%	6e-60	90%	FJ710819.1
Carassius auratus gibelio 18S ribosomal RNA gene, partial sequence	241	241	98%	6e-60	90%	EF189737.1
Cyprinus carpio 18S ribosomal RNA gene, partial sequence	235	235	98%	3e-58	89%	KT002364.1
Cyprinus carpio genome assembly common carp genome, scaffold 000028946	235	706	98%	3e-58	89%	LN591197.1
Onychostoma macrolepis 18S ribosomal RNA gene, partial sequence	235	235	98%	3e-58	89%	JQ283288.1
Cyprinus carpio intergenic spacer, partial sequence; 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer	235	235	98%	3e-58	89%	JN628435.1
Cyprinus carpio 18S ribosomal RNA gene, partial sequence	235	235	98%	3e-58	89%	FJ710826.1
Cyprinus carpio intergenic spacer region, partial sequence; 5' external transcribed spacer, 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal	235	235	98%	3e-58	89%	AF133089.2

18s R alignment result

Experiment infection of and Isolation of CyHV-2 from goldfish

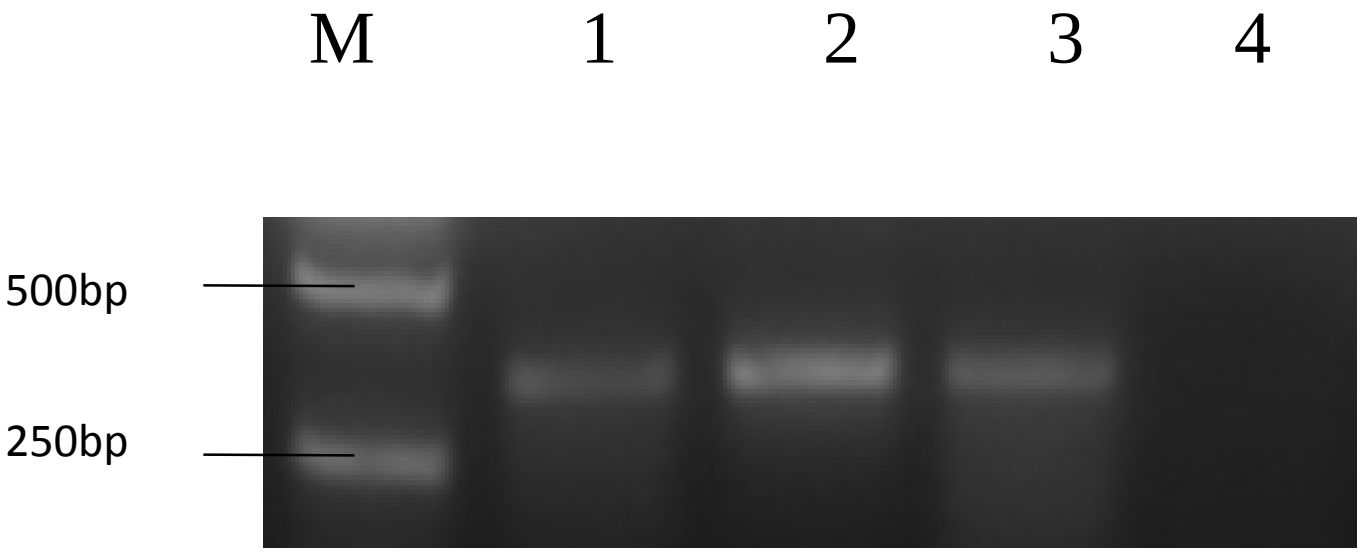
3# tank of gold fish			4# tank of gold fish			Tank of PCR- goldfish		
Total 13			Total 12			Total 13(divided after Feb24)		
Date	Death	PCR	date	death	PCR	4 in 3# tank	4 in 4# tank	5 control
Feb15	2	+	Feb18	1	+	1 death on Feb2 PCR$^-$	no death	no death
Feb 17	1 morbid	+	Feb21	1	+			
Feb18	1	+	Feb22	2	+	1 death on Mar14 PCR$^-$		
Mar8	1	+	Feb25	1	+			
Mar24	1	+	Feb26	1	+	1 death on May4 PCR$^-$		
			Mar13	1	+			
total	7		total	7				

CyHV-2 PCR

Primers: CyHV pol-FOR（CCCAG CAACATGTGCGACGG）

CyHVpol-REV（CCGTARTGAGAGTTGGCGCA）

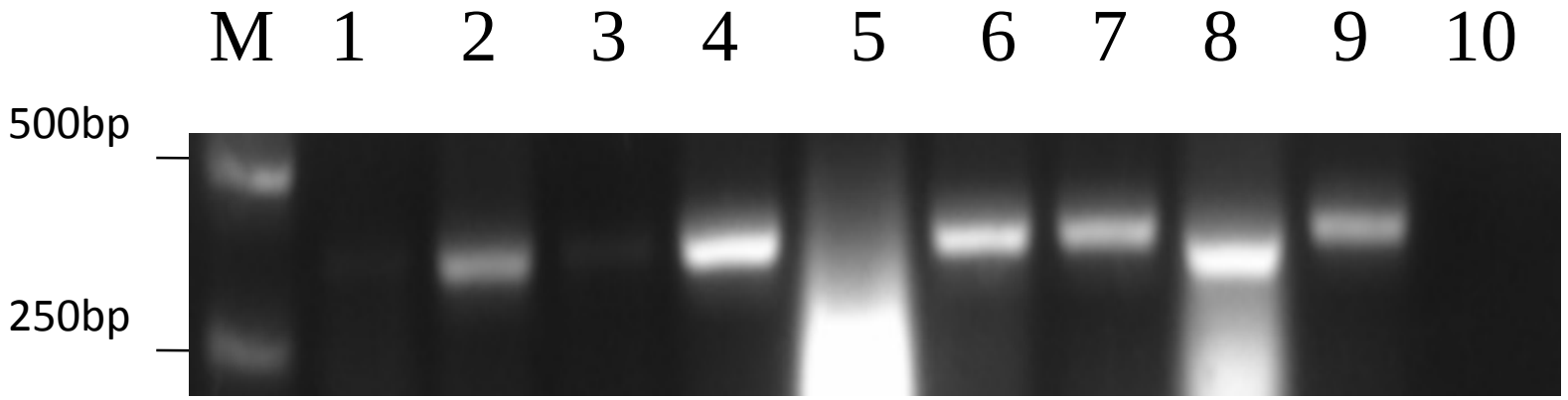
Fragment size： 362 bp



M: DL2000 ladder; lane1: intestine; lane 2: brain kidney and spleen mixture ; lane 3: positive control; lane 4 blank control

fig3. PCR results for dead goldfish on Feb 15,2017

PCR results

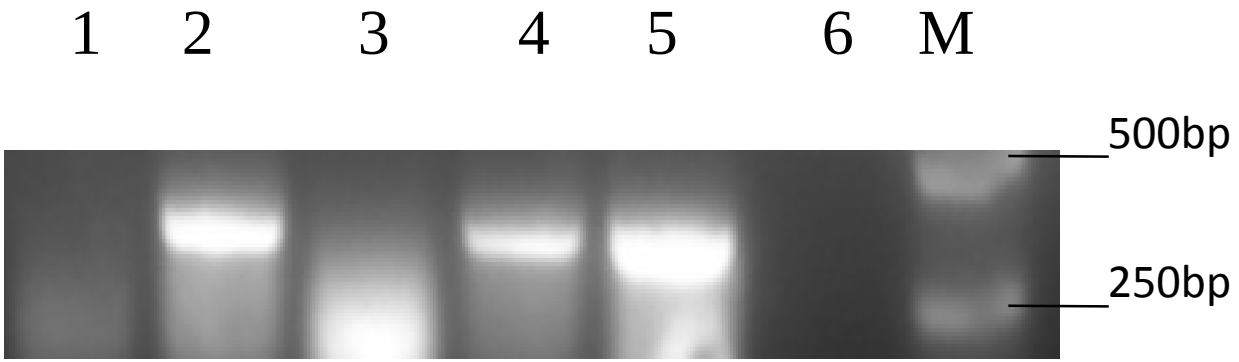


M: DL2000 marker ; lane1:zzxz21(XUZHOU GF); lane2-6: BKS, intestine of GF died on Feb17 in 3# tank; intestine,BKS,brain of GF died on Feb18 in 3# tank; Lane7-8: intestine ,BKS of GF died on Feb18 in 4# tank; lane 9: positive control(PC); lane10: blank control(BC);

Note：GF-goldfish，DGF-dead goldfish，BKS –brain kidney and spleen

Fig4. PCR results from cell suspension inoculated with infected tissue homogenate

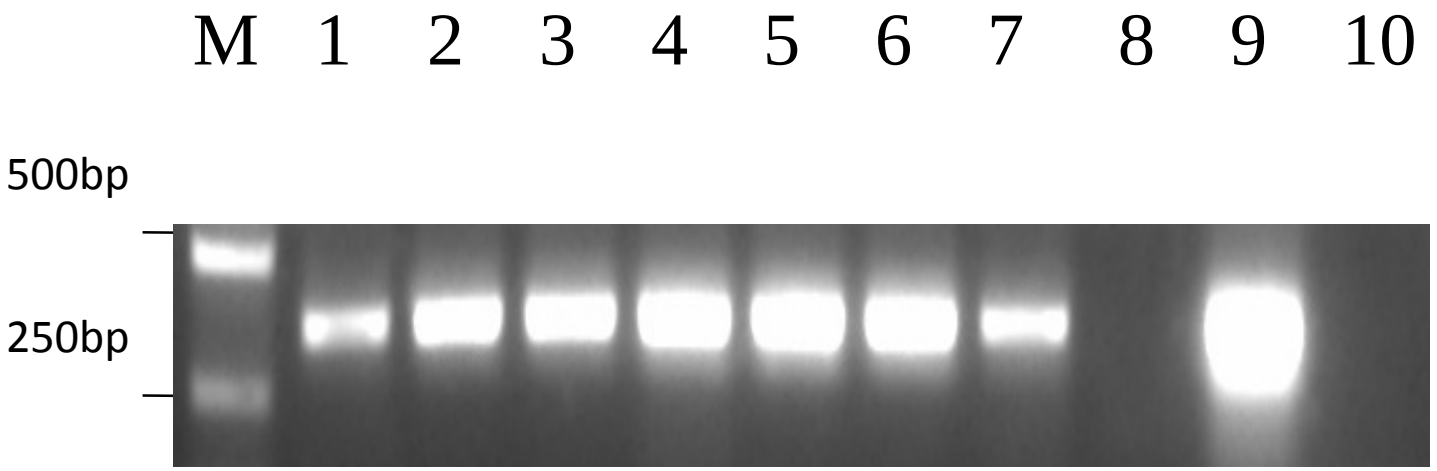
PCR results for 2 dead goldfish from 4#tank



Lane 1,2: intestine, brain kidney and spleen from dead goldfish No.1; lane3,4: intestine, brain kidney and spleen from dead goldfish No.2; lane5:positive control; lane6 : blank control; M: DL2000

Fig6. PCR results for 2 dead goldfish from 4#tank

20170303 PCR results for CrCB suspension infected by BKS of DGF



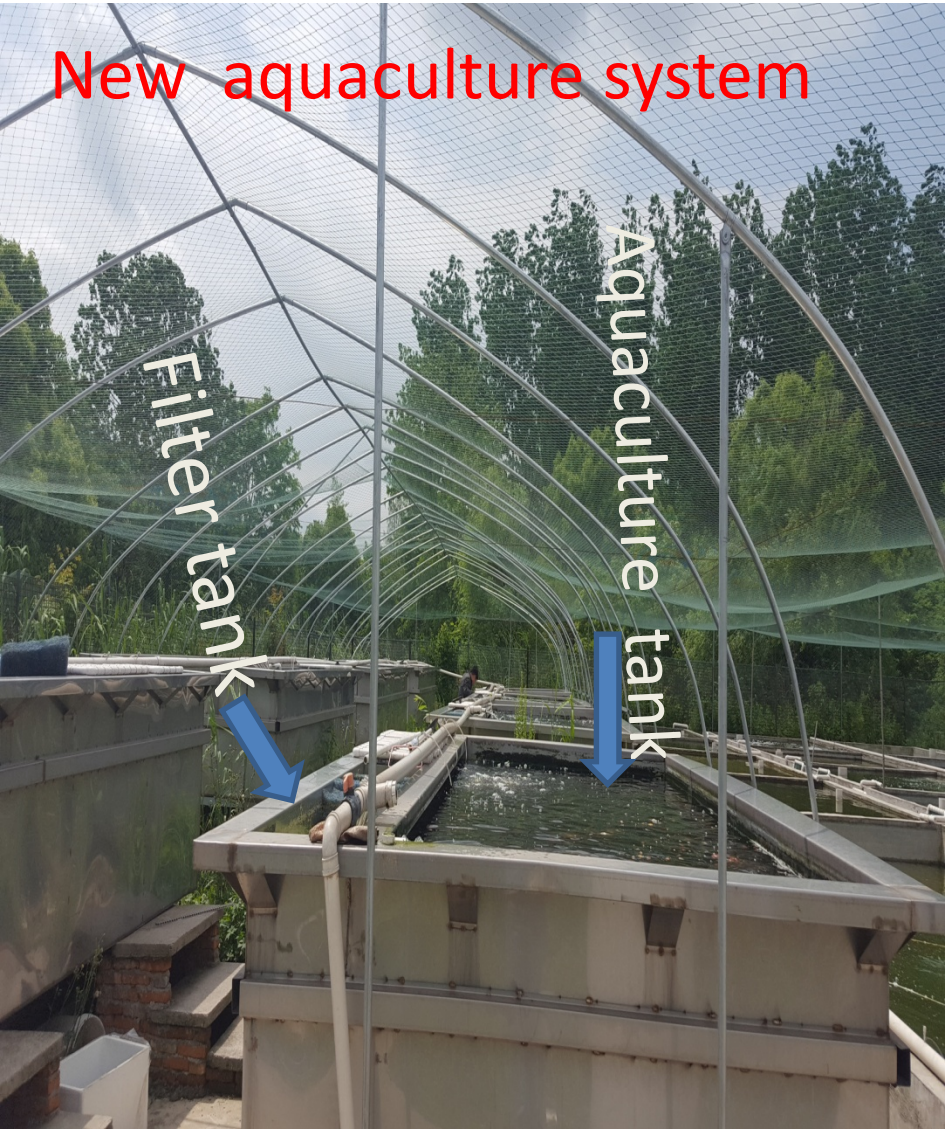
M: DL2000; lane1-8: CrCB suspension infected by BKS of DGF from 4# tank died and inoculated on the CrCB on Feb21(1,2) ,Feb22（3，4），Feb25（5，6）,Feb26（only brain, 7，8）; lane9: positive control; lane10: Blank control; BKS: brain kidney and spleen mix; DGF: dead goldfish

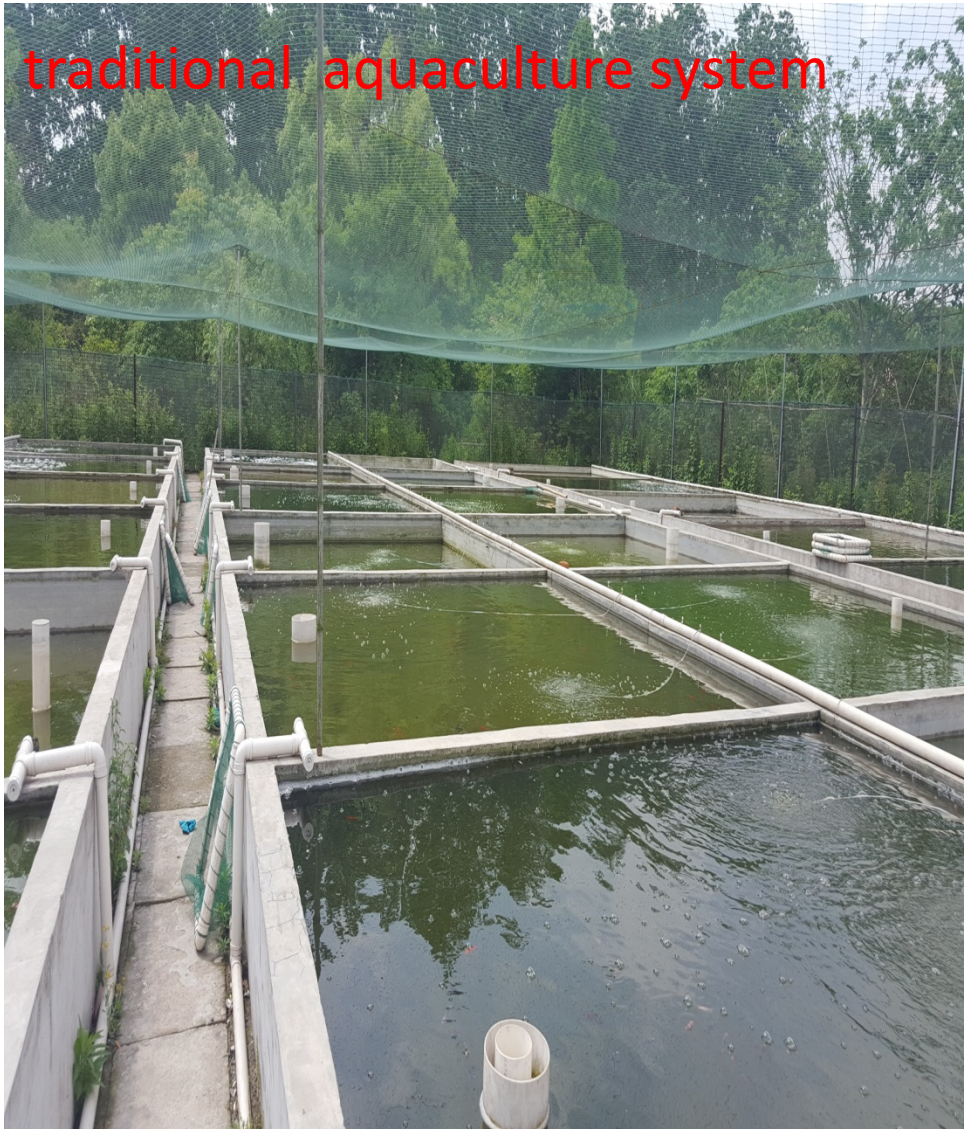
Fig7. PCR results for CrCB suspension infected by BKS of DGF from 4# tank

Example farm for prevention trial of CyHV-2

- Suzhou AquaBridge Aquarium Technology Co., Ltd is a registered company by AQSIQ for export of ornamental fish mainly goldfish.
- The company is under the control of local CIQ. The target surveillance and pre-export test is done by this laboratory.
- The company has developed a new system and good operation procedure for prevention of CyHV-2 and other diseases.

example farm for prevention trial of CyHV-2





example farm for prevention trial of CyHV-2

- Filter tank and aquaculture tank are integrated
- Water through 3 layers of filter is pumped into the aquaculture tank
- Green *Wolffia arrhiza serves as* plant feed source and to reduce organic ingredients(faeces, feed residue et al)
- **one species one pool from the fry stage**
- avoidance of introduction of diseases by self-breeding
- strict control policies and sound hygiene practices
- Isolated quarantine area for export of ornamental fish

Aquaculture tank