Journal of Animal and Veterinary Advances 11 (20): 3823-3826, 2012
ISSN: 1680-5593

Rapid Detection of *Edwardsiella ictaluri* from Channel Catfish Tissue and Water Samples by PCR Amplification

Jesus Genaro Sanchez-Martinez, Roberto Perez-Castaneda, Ma. De la Luz Vazquez Sauceda, Jaime Rabago Castro and Gabriel Aguirre-Guzman
Facultad de Medicina Veterinaria y Zootecnia, Universidad Autonoma De Tamaulipas, Carretera Victoria-Mante Km. 5, Cd. Victoria, 87000 Tam, Mexico

Abstract: Polymerase Chain Reaction (PCR) primers were designed to detect *Edwardsiella ictaluri* in tissue and water samples. Three pairs of primers were designed and tested against the *E. ictaluri* type strain (ATCC No. 33202). The primers for pEI1 and EIl6s were very sensitive for the detection of *E. ictaluri* in water and tissue samples of fish experimentally infected with different bacterial dilutions. The PCR conditions described were optimized to obtain results in 5 h. This PCR has the potential of detecting low levels of *E. ictaluri* in tissue and water samples allowing for the timely detection of enteric septcaemia of catfish in farms.

Key words: PCR, detection, *E. ictaluri*, catfish, Ell6s

INTRODUCTION

Channel catfish, *Ictalurus punctatus* (Rafinesque) culture is a growing industry in Mexico. Enteric Septicaemia of Catfish (ESC), a bacterial disease that affects Channel catfish (Thune, 1993) has become the most devastating disease affecting catfish culture and is possibly the most common disease for this species. ESC was first described by Hawke (1979) and it virtually only affects channel catfish (Plumb and Sanchez, 1983) although, it has been reported in other catfish species, albeit with differences in the susceptibility to the disease (Wolters and Johnson, 1994). This disease is responsible for millions of dollars in losses and treatment costs (Klesius and Sealy, 1995) in the United States with an incidence in small, medium and large farm operations. It is safe to presume that the incidence of this disease in Mexico may be similar. The etiological agent of ESC is the bacterium *Edwardsiella ictaluri* which has different strains (Mitchell and Goodwin, 1999). Techniques available for the identification of this species depend on its biochemical characterization after culture which is time consuming. Because ESC is an acute disease and a timely detection is necessary for its effective control, the object of this study was to develop a PCR Method for the rapid detection of *E. ictaluri* in water and catfish tissue.

MATERIALS AND METHODS

This study used the *Edwardsiella ictaluri* type strain (ATCC NO. 33202), originally isolated by Hawke (1979). Three methods of DNA extraction of total bacterial DNA were tested: Proteinase K extraction (Docker *et al*., 1997); DNA extraction with a commercial kit (High Pure PCR Template Preparation kit [Roche]) following the manufacturer instructions and extraction by boiling using a modification of a protocol proposed by Mohran *et al*. (1998). A colony of *E. ictaluri* was grown in brain heart infusion broth, the cells were harvested by centrifugation at 10,000 rpm for 10 min at 5°C and then washed twice in sterile PBS (Phosphate Buffered Saline). The bacterial cells were finally resuspended in 1000 μL PBS. Aliquots of 100 μL were used immediately for DNA extraction by any of the three methods, followed by PCR amplification. The aliquots not immediately used were stored at -20°C until used.

Three pairs of primers complementary to sequences in *E. ictaluri* DNA were designed using the program Primer 3 from DNA sequences submitted to GeneBank, specifically, the complete plasmid sequence of *E. ictaluri* plasmid pEI2 (GeneBank AF244084), the complete sequence of *E. ictaluri* l6S ribosomal *RNA* gene, (GeneBank AF3l0622) and *E. ictaluri* plasmid pEIl, complete plasmid sequence (GeneBank AF244083). The primers tested were designated as pEI2f and pEI2r, pEI1f and pEI1fr, EI16sf and EI16sr. The sensitivity of the assay and the capability of DNA extraction from bacteria present in the water were assessed by seeding non-sterile fresh water with *E. ictaluri.* An overnight culture of *E. ictaluri* was harvested by centrifugation and washed twice in sterile PBS. The pellet was resuspended in 1000 μL of PBS. Serial dilutions of the bacterial cells were made with

Corresponding Author: J.G. Sanchez-Martinez, Facultad de Medicina Veterinaria y Zootecnia, Universidad Autonoma de Tamaulipas, Carretera Victoria-Mante Km. 5, Cd. Victoria, 87000 Tam, Mexico

non-sterile water (1:10, 1:100, 1:1000, 1:10,000, 1:100,000, 1:1,000,000, 1:10,000,000 and 1:100,000,000). About 100 μL sterile PBS were added to 900 μL water and served as a negative control.

Naive channel catfish tissue was seeded with *E. ictaluri* cells to test for the detection of the bacteria in tissue. Catfish brain and kidney were chosen as target tissues and seeded using a modification of the protocol described by Wiklund. The excised organs were macerated and mixed with sterile PBS in a 50% (w/v) suspension; 50 μL aliquots of each organ suspension were inoculated with an equal volume of the serial dilutions of the bacterial cell suspension. Washed bacteria, non-seeded organs and sterile water were used as positive and negative controls for the PCR.

PCR amplifications were prepared using a commercial kit, Taq&Go (Qbiogen) following the manufacturer's instructions in a 50 μL reaction mixture with 50 pmol of each primer (the sequence and positions of primers are shown in Table 1) and 5 μL of extracted DNA. The PCR reactions were performed in a Thermo Hybaid PCR Sprint Thermocycler (Thermo Hybaid, Ashford, Middlesex, UK).

Table 1: Sequences and positions of primers for the amplification of *E. ictaluri*

Primer (bp)	Position	Sequence (5'-3')	Product length
pEI2f	98-117	TGT GCT CAC CGA AAC TTG AG	408
pEI2r	505-486	CAC TCT CCC CAG ACC AAC AT	
pEI1f	2403-2422	AAA GGC AGA CAA AAG GCA GA	432
pEI1r	2834-2815	TCT GCT CCA GTC GAT GTG AC	
EI16sf	222-241	CGG ACG GGT GAG TAA TGT CT	405
EI16sr	626-607	TTA GCC GGT GCT TCT TCT GT	

Table 2: Detection of *E. ictaluri* DNA in experimentally infected water with different dilutions of the bacteria using 3 different pairs of primers

Bacterial dilution	pEI1	pEI2	EI16s
1:1	x	x	x
1:10	x	x	x
1:100	x		x
1:1,000	x		x
1:10,000	x		x
1:100,000	x		x
1:1,000,000	x		x
1:10,000,000	x		x
1:100,000,000	x		x

Table 3: Detection of *E. ictaluri* DNA in experimentally infected tissue with different dilutions of the bacteria using 3 different pairs of primers

	Brain			Kidney		
Bacterial dilution	pEI1	pEI2	EI16s	pEI1	pEI2	EI16s
1:1	x	x	x	x		x
1:10	x	x	x	x		x
1:100	x	x	x	x		x
1:1,000	x	x	x	x		x
1:10,000	x	x	x	x		x
1:100,000	x		x	x		x
1:1,000,000	x		x			

A touchdown PCR was used for all 3 pairs of primers. Initial denaturation was 5 min at 95°C, followed by 30 amplification cycles of 94°C denaturation for 30 sec; 62°C (-0.5°C for each successive cycle) annealing for 30 sec and 72°C extension for 1 min, followed by a final extension step of 10 min at 72°C. The amplified PCR products were electophoresed in 1% agarose gels and visualized by UV translumination after ethidium bromide staining. Sensitivity assays were performed in water (Table 2) and seeded the naive tissue (Table 3).

RESULTS

Total bacterial DNA yield from the three extraction methods tested was suitable for PCR. All primers tested generated amplicons of the expected size from DNA obtained from *E. ictaluri* (Fig. 1). When tested against other fish bacteria no DNA was amplified and is thus considered specific. The thermal profile used in which a touchdown PCR uses an initial alignment temperature higher than that of the melting Temperature (Tm) of the primers (but decreases with each successive cycle) is useful to avoid non-specific amplification of DNA (Sambrook and Russell, 2001). DNA extraction by the boiling method was the fastest for PCR from water samples seeded with the bacteria. For tissue DNA, both proteinase K and the commercial kit yielded DNA suitable for PCR. A modification of the proteinase K Method that boiled the tissue samples for 10 min (in lieu of an 18 h incubation) prior to phenol chloroform extraction and precipitation, yielded DNA suitable for PCR and was as

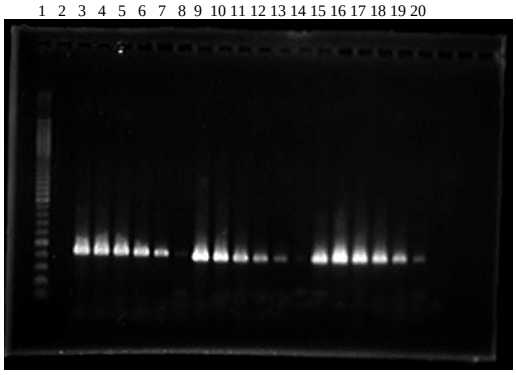

Fig. 1: About 1% agarose gel electrophoresis showing the amplification pattern of the ADN extracted from pure cultures of *Edwardsiella ictaluri* at different dilutions, Lane 1: Molecular weight marker (100 pb); Lane 2: empty; lane 3-8, *E. ictaluri* DNA amplified with the primer pair pEI1f::pEI1r; Lanes 9-14: *E. ictaluri* DNA amplified with the primer pair pEI2f::pEI2r; Lanes 15-20: *E. ictaluri* DNA amplified with the primer pair EI16sf::EI16sr

fast as the comercial extraction kit. The primers for pEI1 and EI16s detected the presence of *E. ictaluri* in all the bacterial dilutions in water however, the primer for pEI2 had a very low detection threshold (Table 2). *Edwardsiella ictaluri* was rapidly detected in water by PCR using the pEI1 and EI16s primers even at the highest dilutions.

DISCUSSION

The detection of *E. ictaluri* in water by PCR could be a useful alternative after further studies for monitoring culture tank water, especially if samples can be taken from relatively large (100 mL) water volumes, to concentrate the bacteria present before extraction by boiling. This is important, given that isolation of *E. ictaluri* from water samples using agar plates is not easy, since this bacterium is very fastidious and may be overgrown by other bacteria present in the water. In seeded tissue samples, differences in the sensitivity of detection were observed, depending on the pair of primers and the organ used (Table 3). The results obtained by seeding of brain and kidney tissues with different dilutions of *E. ictaluri* indicate that the primers for pEI1 and EI16s are the most sensitive and were able to detect the presence of the bacterium DNA at a dilution of 1:1,000,000. This sensitivity is similar to that reported by other researchers with other primer pairs (Bilodeau *et al*., 2003). However, the primers for pEI2 had a lower detection threshold in brain and did not detect the DNA in kidney. The primer pair designed against plasmid pEI1 was very sensitive, agreeing with the suggestion that the *E. ictaluri* plasmids may be useful for the identification of the bacteria in infected channel catfish (Lobb and Rhoades, 1987), especially since they are thought to be specific enough to be useful for the identification of the bacterium (Speyerer and Boyle, 1987) and appear to be conserved among the different strains of the bacterium (Lobb *et al*., 1993). Plasmid sequences have been successfully used for the design of primers for the identification of other fish bacteria and their diagnosis (Aoki *et al*., 1996). Of the pairs of primers designed, pEI2 was the least effective, since it had a low threshold of detection of *E. Ictaluri* DNA in water and brain and could not detect the DNA in kidney.

The PCR conditions described in the present study have been optimized so that by using the boiling method for water samples and the commercial kit for catfish tissue, results can be obtained in 5 h after simple submission versus 3 or 4 days when using traditional methods which is comparable to the time frame obtained by other researchers (Bader *et al*., 2003) with other species of bacteria. This is important, since traditional methods of detection which use culture and biochemical identification besides being lengthy, many times are not able to discriminate among different isolates of the same species (Bader *et al*., 1998) while PCR can discriminate among serologically homogeneous strains (Lobb *et al*., 1993). The method described in this study allows the identification of the etiological agent of ESC before the first signs of disease are evident which makes it a viable option for the monitoring of tanks or cages. Furthermore, the levels of detection of *E. Ictaluri* by PCR are comparable to those reported by Bilodeau *et al*. (2003) when compared to traditional diagnostic techniques which use bacterial culture but are obtained in a fraction of the time. The primers developed in this study have been tested against two different natural outbreaks of ESC in cultured channel catfish resulting in the rapid detection of *E. ictaluri* which was later isolated from the different cases and typified by biochemical conventional methods. Catfish juveniles are often transported between different culture points and test procedures with high sensitivity are needed to prevent the dissemination or introduction of infected fish.

CONCLUSION

The PCR-based test for the detection of *E. ictaluri* described here has suitable sensitivity and specificity for discriminating between infected and uninfected catfish during culture.

ACKNOWLEDGEMENT

This study was possible thanks to the Consejo Nacional de Ciencia y Tecnologia (CONACyT), grant No. CONACyT-2002-C01-39555. Researchers thank FOMIX-Cotacyt Tamaulipas for the publication costs of this study.

REFERENCES

Aoki, T., D. Ikeda, T. Katagari and I. Hirono, 1996. Rapid detection of the fish pathogenic bacterium *Pasturella piscida* by polymerase chain reaction targeting nucleotide sequences of the species specific plasmid bZP1. Fish Pathol., 32: 143-151.

Bader, J.A., C.A. Shoemaker and P.H. Klesius, 2003. Rapid detection of columnaris disease in channel catfish (*Ictalurus punctatus*) with a new species specific 16S rRNA gene based PCR primer for *Flavobacterium columnare*. J. Microcbiol. Met., 52: 209-220.

Bader, J.A., C.A. Shoemaker, P.H. Klesius, M.A. Connolly and J.M. Barbaree, 1998. Genomic subtyping of *Edwardsiella ictaluri* isolated from diseased channel catfish by arbitrarily primed polymerase chain reaction. J. Aquat. Anim. Health, 10: 22-27.

Bilodeau, A.L., G.C. Waldbieser, J.S. Terhune, D.J. Wise and W.R. Wolters, 2003. A real-time polymerase chain reaction assay of the bacterium *Edwardsiella ictaluri* in channel catfish. J. Aquat. Anim. Health, 15: 80-86.

Docker, M.F., R.H. Devlin, J. Richard, J. Khattra and M.L. Kent, 1997. Sensitive and specific polymerase chain reaction assay for detection of *Loma salmonae* (Microsporea). Dis. Aquat. Organ., 29: 41-48.

Hawke, J.P., 1979. A bacterium associated with disease of pond cultured channel chatfish. J. Fish. Res. Board Can., 36: 1508-1512.

Klesius, P.H. and W.M. Sealy, 1995. Characteristics of serum antibody in enteric septicemia of catfish. J. Aquat. Anim. Health, 7: 205-210.

Lobb, C.J. and M. Rhoades, 1987. Rapid plasmid analysis for identification of Edwardsiella ictaluri from infected channel catfish (*Ictalurus punctatus*). Applied Environ. Microbiol., 53: 1267-1272.

Lobb, C.J., S.H. Ghaffari, J.R. Hayman and D.T. Thompson, 1993. Plasmid and serological differences between *Edwardsiella ictaluri* strains. Applied Environ. Microbiol., 59: 2830-2836.

Mitchell, A.J. and A.E. Goodwin, 1999. Evidence that Enteric Septicemia of Catfish (ESC) was present in Arkansas by the late 1960s: New insights into the epidemiology of ESC. J. Aquat. Anim. Health, 11: 175-178.

Mohran, Z.S., R.R. Arthur, B.A. Oyofo, L.F. Peruski, M.O. Wasfy, T.F. Ismail and J.R. Murphy, 1998. Differentiation of *Campylobacter* isolates on the basis of sensitivity to boiling in water as measured by PCR detectable DNA. Applied Environ. Microbiol., 64: 363-365.

Plumb, J.A. and D.J. Sanchez, 1983. Susceptibility of five species of fish to *Edwardsiella ictaluri*. J. Fish Dis., 6: 261-266.

Sambrook, J. and D.W. Russell, 2001. Chapter 8: *In vitro* Amplificaction of DNA by the Polymerase Chain Reaction. In: Molecular Cloning: A Laboratory Manual, Sambrook, J. and D.W. Russell (Eds.)., 3rd Edn., Vol. 2, Cold Spring Harbor Laboratory Press, New York, pp: 8.18-8.113.

Speyerer, P.D. and J.A. Boyle, 1987. The plasmid profile of *Edwardsiella ictaluri*. J. Fish Dis., 10: 461-469.

Thune, R.L., 1993. Bacterial Diseases of Catfishes. In: Fish Medicine, Stoskopf, M.K. (Ed.)., WB Saunders Co., Philadelphia, pp: 511-520.

Wolters, W.R. and M.R. Johnson, 1994. Enteric septicemia resistance in blue catfish and three channel catfish strains. J. Aquat. Anim. Health, 6: 329-334.