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# PCR for Direct Detection of *Edwardsiella tarda* from Infected Fish and Environmental Water by Application of the Hemolysin Gene

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**Jau-Der Chen and Shau-Yan Lai (1998)** PCR for direct detection of *Edwardsiella tarda* from infected fish and environmental water by application of the hemolysin gene. *Zoological Studies* **37**(3): 169-176. A DNA fragment associated with hemolysin production in the *Edwardsiella tarda* strain ET16 was labeled with non-radioactive DIG and used to probe chromosomal DNA from 40 *E. tarda* strains. The resulting hybridization patterns were classified into 2 groups, matching the group of strains which secreted or didn't secrete hemolysin, except for anomalous patterns produced by strain ET83 and reference strain ATCC 15947. Those strains yielding a 5.3-kb hybridization band, which corresponds to the original *Hin*dIII cloned fragment from the chromosome of ET16, were assigned to group I, while the presence of other hybridized *Hin*dIII bands from strains producing endo-hemolysin were characteristic of group II. When 2 oligomers selected from the beginning region of ORF II and the end region of ORF III were used as primers for PCR assay, a 1109-bp PCR product was generated by all 40 examined *E. tarda* strains. Thus, the hemolysin gene sequence spanning the region of ORF II and ORF III was concluded to be conservative. Live bacterial cells from the visceral organs and blood of infected fish, as well as bacteria in environmental water were detected by a direct PCR assay which yielded the 1109-bp fragment.

**Key words:** Edwardsiellosis, PCR diagnosis.

*Edwardsiella tarda* is a common pathogen which has been isolated from farmed fish such as eel, *Anguilla japonica* (Chen and Kou 1987, Chen et al. 1996) in Taiwan. The isolation of this microorganism from other infected fish species, for example, tilapia (Kubota et al. 1981), flounder (Nakatsugawa 1983), sea bream (Baxa et al. 1985), and striped bass (Herman and Bullock 1986) has also been reported. In addition, this pathogen can be isolated from mammals such as cetaceans (Buck et al. 1991), seals (Regalla 1982), and human beings (Vandepitte et al. 1983), and even from environmental sources such as human feces (Janda et al. 1991a,b, Janda and Abott 1993).

In the past, infection by this pathogen has led to epidemic outbreaks which have had a disastrous effect on aquaculture (Chen and Kou 1987). Outbreaks follow a general pattern in which at first only a few fish were infected by this bacteria, and in these the symptoms of edwardsiellosis appeared (Miyazaki and Egusa 1976a,b, Liu and Chien 1986). These fish are able to survive for a few days, but if antibiotics (Takemaru and Kusuda 1988, Nakano et al. 1989) are not applied promptly, fish being reared in the surrounding areas also become infected and the edwardsiellosis becomes epidemic (Chen and Kou 1987). Since early detection is vital for controlling this disease, methods for rapidly and accurately diagnosing edwardsiellosis would therefore be very useful.

The symptoms of edwardsiellosis include hemolysis and necrosis of the liver and kidney, and in most cases, these symptoms are very clear. However, when *E. tarda* is the causative agent, it is difficult to reach a diagnosis of edwardsiellosis based on observations of hemolysis, and this is especially true during the initial stage or in the case of a mild infection. In such a situation, the fluorescent antibody technique, first reported a de-

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cade ago, can rapidly diagnose edwardsiellosis (Kawahara and Kusuda 1987). However, 49 O-antigens, 37 H-antigens, and more than 148 H-O mixed antigens of this species have been reported (Edwards and Ewing 1972). This undermines the reliability of this method, since the accuracy of diagnosis depends on the serotypes of the examined bacteria.

Several virulent factors have been studied and documented by the use of different strains isolated from various infected organisms and environmental sources (Watson and White 1979, Ullah and Arai 1983a,b, Kusuda and Kitadai 1993, Suprapto et al. 1995 1996). One of these, hemolysin, was identified as an endotoxin because it is not secreted outside of the cell (Watson and White 1979, Ullah and Arai 1983a,b, Janda et al. 1991a,b, Janda and Abbott 1993). However, unlike any of the above reported *E. tarda* strains, *E. tarda* strain ET16 is able to export hemolysin across the cell wall. The hemolysin from this strain, and the cloning, characterization, and sequencing of its gene determinant have already been investigated (Chen et al. 1995, Chen and Huang 1996, Chen et al. 1996).

In the present study, we compare a collection of 40 *E. tarda* strains, most of which were isolated from infected fish from fish farms in Taiwan. In particular, we investigated: the ability of these 40 strains to transport/secrete hemolysin across the cell wall; the ability of a DIG-labeled probe derived from the hemolysin gene determinant of strain ET16 to hybridize with chromosomes from the other strains; and a PCR assay designed to produce a 1109-bp PCR product from the hemolysin gene in the region of ORFs II and III.

## MATERIALS AND METHODS

### Bacteria and culture media

*Edwardsiella tarda* strains (Table 1) were collected and provided by Dr. Chung (Department of Zoology, National Taiwan University). Typical *E. tarda* characteristics have been reported elsewhere (Kuo 1977). The 40 strains were grown on the surface of Rimler-Shotts selected medium (Shotts and Rimler 1973). Small green colonies with black centers were picked up and re-grown to ensure purity before being used in this study. Cells were grown at 37 °C to a density of (4 to 6) $\times 10^8$ cfu/ml in TSB broth (1.7% tryptone, 0.3% bactosoytone, 0.5% NaCl, 0.25% $K_2HPO_4$, 0.25% dextrose; Difco, Detroit, MI., USA) before use.

### Hemolytic activity assay

Hemolytic activity was determined by the appearance of a lytic zone on the surface of a TSB blood agar plate (Chen and Huang 1996). The hemolytic activity of the extracellular products (ECP) was measured using methods described elsewhere (Buckley and Howard 1988, Chen et al. 1996).

### Hybridization of DIG-labeled ET16 hemolysin gene with *E. tarda* DNA

Bacterial chromosomal DNA was isolated using the method described by Marmur (1961). Triton-X-100 (0.1%) was used as the detergent to lyse the cell walls of *E. tarda*. The digested genomic DNA was separated by agarose gel electrophoresis and transferred onto positively charged nylon membranes (Boehringer, Mannheim, Germany) using the method of Southern (1975). The hemolysin gene encoded on the *Hin*dIII fragment (5.3 kb) of the plasmid pETH3 (Chen et al. 1996) was recovered from the agarose gel after electrophoresis. It was labeled with the non-radioactive reagent, digoxigenin-11-2'-deoxy-uridine-5'-triphosphate (DIG; Boehringer, Mannheim, Germany) and used directly as a DNA probe. For each hybridization experiment, 50 ng of DIG-labeled DNA probe was used for blotting in accordance with the protocol provided by the manufacturer.

### Direct PCR assay of live *E. tarda* cells

*E. tarda* cells were picked up by use of a sterile loop ($6 \times 10^2$ to $2 \times 10^3$ cfu/ml) and directly inoculated into a 100-μl PCR reaction mixture which contained 100 pmol of each primer (forward primer: 5'-CCTTATAAATTACTCGCT, from 744 to 761 bp of ORF II; reverse primer: 5'-TTTGTGGAGTAACAGTTT, from 1850 to 1833 bp of ORF III [Chen et al. 1996]), 400 μM dNTP (Bio-Lab, Beverly, MA, USA), and buffer (10 mM Tris-HCl pH 8.8; 1.5 mM $MgCl_2$; 50 mM KCl; 0.1% triton-X-100) in an Eppendorff tube. PCR was started with an initial denaturation step of 5 min at 94 °C. Cells were lysed in the presence of Triton-X-100, and chromosomal DNA released into the PCR reaction mixture was used as the DNA template. Samples were then kept at room temperature for 5 min after which 1 unit of Vent DNA polymerase (Bio-Lab, Beverly, MA, USA), PrimeZyme (Tampa, FL, USA), or DynaZyme (Finnzymes OY, Finland) was added. PCR was performed for 35

**Table 1.** Strains of *Edwardsiella tarda* used in this study

| Strain | Hemolytic activity | | Source |
|---|---|---|---|
| | blood agar | ECP | |
| Group I | | | |
| ET2 | + | + | eel (*Anguilla japonica*); 781019-1K[a] |
| ET6 | + | + | eel (*Anguilla japonica*); 791209-10L |
| ET13 | + | + | eel (*Anguilla japonica*); 800123-6K |
| ET15 | + | + | eel (*Anguilla japonica*); 800123-4L |
| ET16 | + | + | eel (*Anguilla japonica*); 800123-5L |
| ET18 | + | + | eel (*Anguilla japonica*); 800131-2L |
| ET21 | + | + | eel (*Anguilla japonica*); 800423-3K1 |
| ET22 | + | + | eel (*Anguilla japonica*); 800423-1L |
| ET23 | + | + | eel (*Anguilla japonica*); 800423-2L |
| ET33 | + | + | tilapia (*Oreochromis* spp.); 801128-6K |
| ET89 | + | + | fish; AC466 |
| Group II | | | |
| ET1 | + | – | eel (*Anguilla japonica*); 760217-1G |
| ET3 | + | – | eel (*Anguilla japonica*); 781209-2L |
| ET4 | + | – | eel (*Anguilla japonica*); 791209-3L |
| ET14 | + | – | eel (*Anguilla japonica*); 800123-2L |
| ET20 | + | – | eel (*Anguilla japonica*); 800325-6L |
| ET24 | + | – | eel (*Anguilla japonica*); 801010-1L |
| ET26 | + | – | rainbow trout (*Salmo gairdneri*); 801029-2G |
| ET28 | + | – | rainbow trout (*Salmo gairdneri*); 801029-1S |
| ET30 | + | – | eel (*Anguilla japonica*); 801113-2K1 |
| ET51 | + | – | eel (*Anguilla japonica*); 810424-1K |
| ET52 | + | – | eel (*Anguilla japonica*); 810424-4K |
| ET53 | + | – | eel (*Anguilla japonica*); 810515-2K |
| ET54 | + | – | eel (*Anguilla japonica*); 810614-10 |
| ET60 | + | – | eel (*Anguilla japonica*); 810622-13 |
| ET61 | + | – | eel (*Anguilla japonica*); 810622-14 |
| ET62 | + | – | eel (*Anguilla japonica*); 810623-1 |
| ET63 | + | – | eel (*Anguilla japonica*); 810623-2 |
| ET64 | + | – | eel (*Anguilla japonica*); 811002-1K |
| ET68 | + | – | eel (*Anguilla japonica*); 811211-A |
| ET73 | + | – | eel (*Anguilla japonica*); 820318-8Ka |
| ET74 | + | – | eel (*Anguilla japonica*); 820318-1L |
| ET81 | + | – | eel (*Anguilla japonica*); 820429-1I |
| ET82 | + | – | eel (*Anguilla japonica*); 820429-1L |
| ET83 | + | – | eel (*Anguilla japonica*); 820501-4Lc |
| ET88 | + | – | eel (*Anguilla japonica*); AC457 |
| ET91 | + | – | eel (*Anguilla japonica*); K310 |
| ET92 | + | – | eel (*Anguilla japonica*); AK334 |
| ET93 | + | – | eel (*Anguilla japonica*); AT169-3B |
| ATCC 15947 | + | – | human feces, Kentucky, USA |
| | | | Culture Collection and Research Center, Taiwan, ROC; CCRC 10670 |

[a] The number after each species name was given by Dr. Chung (Dept. of Zoology, National Taiwan University) when these strains were isolated.

thermocycles with each cycle consisting of denaturing at 94 °C for 1 min, annealing at a range of 50 to 55 °C for 1 min, and extending at 72 °C for 1 min. Subsequently, 1/5 of the PCR products were analyzed by horizontal gel electrophoresis (1.0% agarose in 0.5X TBE) followed by ethidium bromide staining and $UV_{312}$ illumination.

### PCR detection of the hemolysin gene fragment in visceral organs of fish challenged by strain ET16

Healthy adult (600-700 g body weight) pond-reared tilapia, *Oreochromis niloticus*, were used in the challenge test. One milliliter of growing *E. tarda* cells at a density of $4 \times 10^8$ cfu/ml was injected directly into a blood vessel from the lateral line in the caudal region of the tilapia. Each fish was then placed in a 50-l tank containing growing ET16 cells ($1 \times 10^4$ cfu/ml), and after 30 min; the tilapia was dissected for PCR analysis. Blood extracted from the fish was diluted and plated on the surface of blood agar in order to count the number of cells that had been invaded by the bacterium. The liver, kidney, and intestine were incised with a sterile razor. A sterile loop was touched to these incisions and immediately used to inoculate a PCR reaction mixture in an Eppendorff tube. PCR analysis was then carried out as described above.

### Direct PCR detection from environmental water

In order to simulate conditions of an eel-culture pond (Chen and Kou 1987), growing cells of strain ET16 were poured into a 50-l tank to a final concentration density of $1 \times 10^4$ cfu/ml. Tilapia challenged by injection of ET16 bacteria as described above were placed in such a tank. A 1-ml sample of water was filtered through a sterile 0.2-μm filter membrane (Nalgene, Rochester, NY, USA). A small piece (0.2 $cm^2$) of this membrane was cut from the center and immersed in the PCR reaction mixture in an Eppendorff tube. The PCR assay was carried out as described above.

## RESULTS

### Hemolytic activities and relevant features of the examined *E. tarda* strains

All examined strains demonstrated hemolytic activity as determined by the formation of a distinct lytic zone on the surface of blood agar (Table 1). To establish whether or not hemolysin could cross the cell envelope, the hemolytic activities of ECP from these strains were also determined. As shown in Table 1, there were 11 examined *E. tarda* strains able to export hemolysin across the cell wall (group I). The other 29 strains did not secrete hemolysin into their ECP (group II).

### Hybridization patterns and polymorphism

The capability of exporting hemolysin across the cell wall might result from a difference[s] or mutation[s] on the hemolysin gene. When DNA of the ET16 strain was used as the standard for hybridization, 1 *Hin*dIII band (5.3 kb) should be observed. As expected, all 11 strains (including ET16) whose ECP showed hemolytic activity displayed 1 *Hin*dIII band (5.3 kb) (see Fig. 1). In addition, the identical hybridization patterns of the blotted *Eco*RI, *Cla*I, and *Xba*I bands were observed in these examined 11 strains. They were defined as group I.

Those strains producing hemolysin without the capability of its secretion into their ECP were categorized as group II. Differently patterned and/or absent hybridization bands would be expected to reflect different (i.e., secreting vs. non-secreting) hemolytic characteristics. Surprisingly, however, 26 strains assigned to group II, except strains ET24, ET83, and ATCC 15947, were found to show not only the exact same 5 *Hin*dIII blotted bands but also precisely the same patterns for blotted *Eco*RI, *Cla*I, and *Xba*I bands as well (see Fig. 1 for the characteristic pattern). The hybridization pattern of the non-secreting strain ET24 was the same as that of the secreting strain ET16. Reference strain ATCC 15947 showed 1 blotted *Hin*dIII band (5.3 kb) which is the same size as that of strain ET16. However, the blotted *Eco*RI, *Cla*I, and *Xba*I bands of reference strain ATCC 15947 had different hybridization patterns. In spite of the DNA being incompletely digested or unable to be separated by gel electrophoresis due to large restricted fragments, patterns of both ATCC 15947 and ET83 which differ from the characteristic patterns of group I and group II are also shown in Fig. 1.

### Identical PCR products

The 34-kDa hemolysin encoded by ORF III has been confirmed by recovery of hemolytic activity in situ after SDS-PAGE (Chen et al. 1996). Thus, the flank region of ORF III in all 40 examined strains might include the homologous sequence as

implied by the fact that all 40 examined strains could be hybridized by a DIG-labeled *Hin*dIII fragment (5.3 kb) from pETH3. When the 2 oligomers described in Materials and Methods were used as primers for PCR analysis, all tested strains generated the same-sized 1109-bp PCR fragments (see Fig. 2). This result strongly suggests that the hemolysin gene DNA between the beginning region of ORF II and the end region of ORF III is present in all 40 tested strains.

### PCR diagnosis of challenged infected fish and environmental water

Fish were dissected 30 min after being injected with live *E. tarda* strain ET16, and the number of bacteria in the blood was estimated as $4 \times 10^6$ cfu/ml. As expected, the 1109-bp PCR product was detected in samples taken from the liver, kidney, intestine, and blood of the tested fish as well as from environmental water (Fig. 3). No PCR product of this size was found when a DNA template from either healthy fish or normal culture water was used. This result suggests that live bacteria residing in the visceral organs of infected fish or environmental water can be easily detected by using the oligomers of the hemolysin gene as primers for PCR assay.

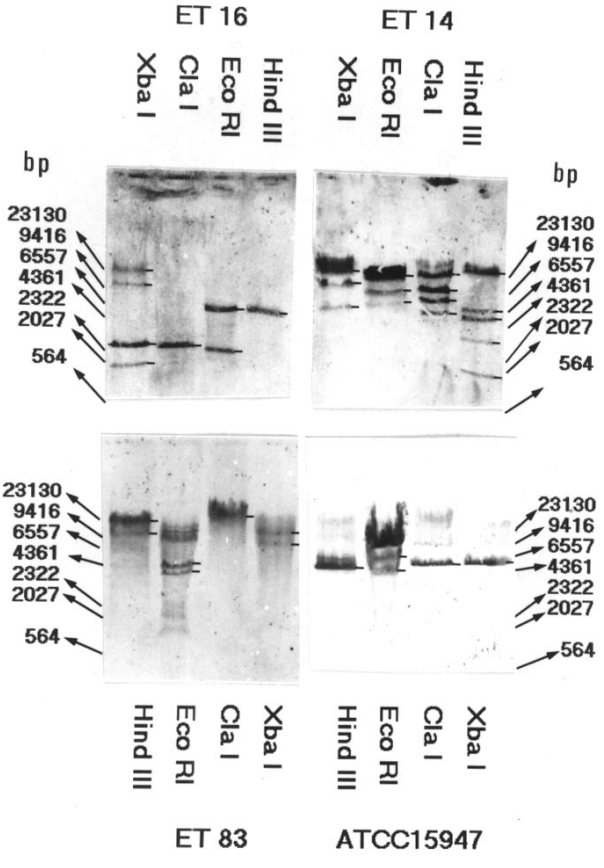


**Fig. 1.** Hybridization patterns of *Edwardsiella tarda* hemolysin gene. Chromosomal DNA extracted from 40 *E. tarda* strains was digested with *Hin*dIII, *Eco*RI, *Xba*I, or *Cla*I endonuclease, and the DNA digests were separated on 0.7% agarose gel. When electrophoresis was completed, they were transferred onto a positively charged nylon membrane and hybridized with a DIG-labeled ET16 hemolysin probe. One typical hybridization pattern from each group is shown: ET16 and ET14 are representative of groups I and II (see Table 1), respectively. The hybridization patterns of reference strain ATCC 15947 and ET83 are also presented. The clear hybridized bands including inseparable large fragments are marked by horizontal bars. Molecular size is indicated by base pairs of λ/*Hin*dIII.

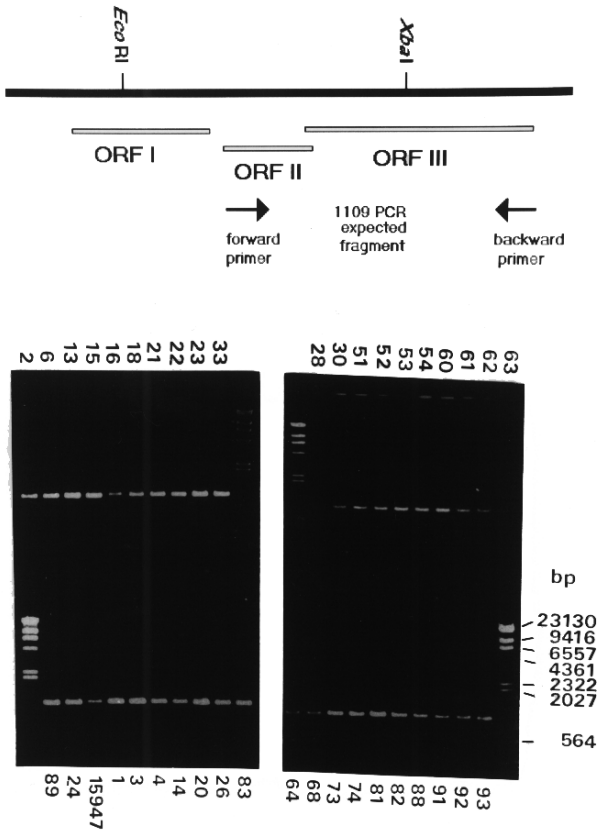


**Fig. 2.** PCR products amplified by using the conserved oligomers of the hemolysin gene as primers. Upper panel: DNA fragment containing the β-hemolysin gene and 2 restriction sites are shown as a solid line which is not drawn to scale. Three open bars below the solid line represent 3 ORFs. ORF III encodes a functional β-hemolysin gene. Forward and backward primers are shown by arrows. Lower panel: PCR products from the 40 examined strains were analyzed by 1% agarose gel electrophoresis. The expected 1109-bp fragment was found in every strain. Numbers represent ET strain numbers; marker columns use DNA of λ/*Hin*dIII as the size standard.

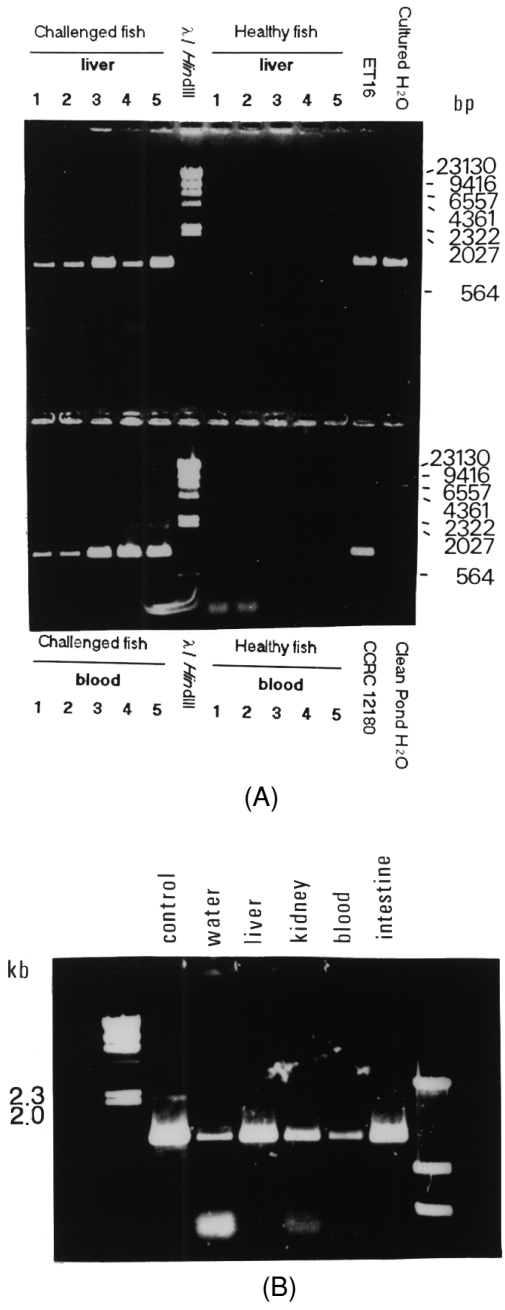


**Fig. 3**. Direct PCR diagnosis of live bacteria from challenged fish and environmental water. Samples for PCR analysis from environmental water and visceral organs of tested fish are described in Materials and Methods. (A) PCR samples from liver and blood of 5 tested fish and control fish were separated by 1% agarose gel electrophoresis. Live ET16 cells were used as a DNA template for a positive control to express the expected 1109-bp PCR fragment. The DNA from *Bacillus cereus* (CCRC 12810) was also used as a DNA template for PCR analysis. (B) One fish challenged by ET16 was dissected for sampling and analysis by PCR. The plasmid, pETH3 (Chen et al. 1996), was used as a DNA template for a positive control to express the expected 1109-bp PCR fragment. Standard sizes of DNA [pUCYP/*Rsa*I (37), and λ/*Hin*dIII] are indicated by numbers in kilo base pairs on both the right and left sides of the figure.

## DISCUSSION

Based on its ability to cross the cell envelope, hemolysin generated by *E. tarda* may be classified as either an endotoxin or exotoxin depending on which *E. tarda* strain is used as a source (see Table 1). This difference between strains is also reflected by the different hybridization patterns that result from probing by the DIG-labeled ET16 hemolysin gene (see Fig. 1), so that the examined *E. tarda* strains can be categorized into 2 groups. The strains in group I (which includes strain ET16) are able to secrete hemolysin across the cell wall. Strains that have this ability have so far only been isolated from infected fish from fish farms in Taiwan. The characteristics of the strains of group II, on the other hand, are similar to those reported for most other *E. tarda* strains by other laboratories (Watson and White 1979, Ullah and Arai 1983a,b, Janda et al. 1991a,b, Janda and Abott 1993, Kusuda and Kitadai 1993, Suprapto et al. 1995, 1996). This seems to indicate that members of this group are more common and widespread. Strain ET24 is anomalous in that it shows characteristics of group II while displaying the hybridization pattern of group I. A likely explanation of this discrepancy is the existence of other mutated region[s] of the chromosomal DNA, which affect the cell's ability to secrete hemolysin but which lie outside the reading frames (ORFs II and III) which are the focus of the present PCR assay. The hemolysin gene of reference strain ATCC 15947 which exhibited the same blotted *Hin*dIII band but different blotted *Eco*RI, *Xba*I, and *Cla*I bands from that of strain ET16 is a typical example of polymorphism. Only 1 strain (ET83) displays a different pattern from that of groups I and II, despite that its DNA is probably unable to be separated by gel electrophoresis due to large restricted fragments or is indigestible by restriction endonuclease.

Chromosomal DNA from all examined strains including ATCC 15947 was hybridized by the DIG-labeled ET16 hemolysin gene. This obviously argues that a homologous sequence of the *E. tarda* hemolysin gene must have been present in all examined strains. The different characteristic hybridization patterns of the 2 groups (see Fig. 1) might reflect differences at certain points in the gene structure that express hemolysin and/or its flank region. The fact that the same-sized 1109-bp PCR product was generated by all examined strains despite their different hybridization patterns provides strong evidence in support of this hypothesis. If there is a mutation within the hemolysin

gene determinant or in the control region flanking the hemolysin gene, this could plausibly account for the differences in hemolysin-secreting ability between the 2 groups. A comparative analysis of the hemolysin gene sequence from these strains might ultimately suggest a mechanism for these differences. Our future research will investigate these questions.

The use of fluorescent antibodies to diagnose edwardsiellosis induced by *E. tarda* becomes problematic in the presence of several serotypes. The homology of the hemolysin gene and the same-sized 1109-bp PCR product found in all examined strains suggests an alternative diagnostic approach. Direct PCR detection of the hemolysin gene of live bacteria is potentially simpler, more accurate, and rapid. Since direct probing for the presence of live *E. tarda* in infected fish and environmental water by PCR should be very reliable, it should be helpful in anticipating and preventing epidemics which otherwise occur so frequently on fish farms.

A comparison of sequence alignments showed the hemolysin gene of *E. tarda* ET16 to be most similar to the hblA gene of *Bacillus cereus* (EMBL/GenBank Data Library under accession number L20441; reference 10) with which it has 96% homology. *B. cereus* is usually isolated from contaminated foods and dairy products (Turnbull et al. 1979, Turnbull 1981). In the present study, we investigated the hblA gene of *B. cereus* (CCRC 12810) which could also be detected by this PCR assay (see Fig. 3A). Since, in any case, given the combination of symptoms of edwardsiellosis and the fact that *B. cereus* has never been isolated from infected fish, there would most likely be little danger of a false positive diagnosis if the methods proposed here were to be applied in aquaculture. We nonetheless cautiously predict that the generated 1109-bp PCR product described above will very likely be expressed by *E. tarda* at initial stages of diagnosis. Naturally, identification of *E. tarda* by other methods is still necessary.

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# 利用聚合酶連鎖反應法直接檢測愛德華氏菌溶血基因應用於愛德華氏菌感染病魚及養殖池水之迅速診斷

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愛德華氏菌的溶血基因核苷酸序列，依其上轉譯之遺傳密碼，可解讀出三個開放讀碼區，其中第三開放讀碼區已經由 SDS-PAGE 証實帶有 34 kDa 的溶血基因。當以帶有該菌的溶血基因 *Hin*dIII 片段用作探針，與台灣近十年來由養殖池感染病魚所採集到的 39 株愛德華氏病原菌作核苷酸雜交時，不論菌株是否釋出溶血外毒素至菌體外，或產生溶血內毒素保存於菌體內，皆可被愛德華氏菌溶血基因探針雜交檢測到。又以第二開放讀碼區的 5' 端及第三開放讀碼區的 3' 端，設計一對引子，以愛德華氏菌體直接加熱釋出核苷酸作模板，利用聚合酶連鎖反應法，可很敏銳的於電泳分離過的洋菜膠上觀察到形成預測的 1109 bp PCR 片段。因此更進一步嘗試以聚合酶連鎖反應法快速診斷愛德華氏病原菌存在於感染病魚體否時，於模擬愛德華氏病原菌注射感染吳郭魚試驗中，很快速的利用此法可檢測到愛德華氏菌進入感染魚的肝腎腸及血液中，顯示利用溶血基因普遍存在於本土分離愛德華氏菌之特性，以本實驗設計的引子用於快速診斷愛德華氏菌感染魚確屬快速可行的良好方法之一。

**關鍵詞：**愛德華氏菌症，聚合酶連鎖反應法直接檢測。

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