農委會漁業特刊第五十三號、魚病研究專集(十六)
COA Fisheries Series No. 53, Reports on Fish Disease Research (XVI) 47-53, 1995

草蝦弧菌 *Vibrio damsela* 細胞外產物之被動免疫研究

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本研究利用 *Vibrio damsela* 進行其細胞外產物對草蝦致病性的探討。此具病原性菌株係於民國77年草蝦大量死亡時自病体中分離而得。將試驗菌株細胞外產物於紐西蘭白兔中獲取抗血清後，進行初步毒性中和及被動免疫試驗。試驗用草蝦重約10g，蓄養於打氣之海水水槽並餵以市售飼料。每個試驗組使用蝦數各爲10尾。

兔子抗 *V. damsela* 細胞外產物之抗血清與等容量之 *V. damsela* 細胞外產物（500μg protein/ml）或菌懸液（1×10^8 CFU/ml）混合後以背部肌肉注射方式打入0.1 ml於約10g重之草蝦各10尾，　各組採二重複試驗。結果試驗蝦皆活存，而同樣劑量但未混合抗血清之對照試驗組蝦皆於2日內死亡，顯示細胞外產物之毒性能被抗血清所中和，　且兔子之抗細胞外產物抗血清可保護蝦體免於 *V. damsela* 之致死性攻擊。

由以上結果得知，兔子抗 *V. damsela* 細胞外產物抗血清對該菌之細胞外產物及活菌皆各具毒性中和及被動免疫效果，本研究首次證實可於草蝦中以哺乳動物之抗血清進行毒性中和試驗及被動免疫試驗之研究，此結果可供蝦類致病機制及疾病防治之參考。

前　言

弧菌症爲海水養殖水產生物最嚴重的疾病之一（Austin and Austin, 1987; Lightner, 1988），有關草蝦弧菌症的相關研究在最近幾年來因國內及東南亞地區養殖蝦大量死亡而興起，已發表之文獻中多數集中於菌株的分離、症狀的描述（Anderson et al., 1988; Lightner, 1988; Lavilla-Pitogo et al., 1990; Liu, 1990; Song et al., 1990; Ruangpan and Kitao, 1991; Chen et al., 1992; Song and Lee, 1993; Song et al., 1993; Jiravanichpaisal and Miyazaki, 1994.）以及檢疫防治對策（Lightner, 1988; Song and Sung, 1990; Adams, 1991; Itami and Takahashi,　1991），但是本病症至目前爲止仍未能有效防治且不同種類弧菌症亦相繼出現。爲了草蝦養殖產業的繼續維持，　因此有必要在對相關弧菌本身致病性及可能毒力因子進行初步了解後（Lee and Chen, 1994），再針對弧菌細胞外產物進行其在草蝦中之免疫相關試驗。

Itami等人（1989），Itami and Takahashi（1991）及Song and Sung（1990）曾發表有關以弧菌菌體對斑節蝦或草蝦進行疫苗試驗之結果，但僅屬於實驗室階段成果，目前仍未實際應用於現場之防治且確切成效仍待評估。由於目前仍無法肯定蝦類具有記憶性之免疫反應，在養殖魚類之相關研究中已有以被動免疫試驗探討細菌之保護性抗原文獻（(Harrell et al., 1975; McCarthy et al., 1983; Olivier et al., 1985; Ellis et al., 1988; Marquis and Lallier, 1989，但養殖蝦類則仍未有此方面之研究，因此本研究進行毒性中和及被動免疫試驗。於紐西蘭白兔先獲取抗弧菌細胞外產物之抗血清，　再進行有關此抗血清在草蝦體內對致死性之弧菌細胞外產物是否具有毒性中和效果及對致死性之弧菌活菌攻擊是否具被動免疫保護效果，以供疾病防

治之參考。

材料與方法

菌株來源與保存

本研究使用菌株 *Vibrio damsela* (VD1) 由台大動物系陳秀男教授提供，係於民國77年國內草蝦發生大量死亡時分離自罹病草蝦肝胰腺（Chen et al., 1992; Lee and Chen, 1994）。菌株之保存方式爲先於tryptone soya agar (TSA, ＋2.5% NaCl, Oxoid）上純培養細菌24小時，溫度爲26℃，再以滅菌過之生理食鹽水（PBS, pH7.4）配成菌懸液，於加入10%滅菌過甘油後裝入滅菌過之1.5 ml eppendorf，於－70℃凍藏。

菌體與細胞外產物之製備

將細菌培養於TSA（＋2.5% NaCl），於26℃，24小時後，以PBS收集細菌配成菌懸液再經PBS澱洗三次後即爲菌體。取上述條件培養之細菌 2 swabs以 5 ml PBS配成菌懸液，再傾倒並均勻塗佈於覆蓋鋪有無菌cellophane之TSA（＋2.5% NaCl）上，於上述條件培養，再以PBS收取菌懸液，4 ℃下以3500 g 離心 1 小時後將上澄液以0.22 μ m過濾膜除菌（Lee and Ellis, 1990, 1991）。將ammonium sulfate緩慢加入此上澄過濾液中至70%飽和濃度，2 － 3 小時後，以25,000 g 離心30分鐘，取沉澱物溶於 5 ml去離子水，於 4 ℃以去離子水透析兩天。透析後之細胞外產物再以12,000 g 離心10分鐘，去除不溶物後分裝於eppendorf中貯存於－20℃備用。

蛋白質含量測定

採用Bradford method (1976) 測定細胞外產物之蛋白質含量。將樣品0.1 ml加入 5 ml之Bio-Rad Protein Assay Kit solution，於 595 nm波長測吸光值，以牛血清白蛋白（bovine serum albumin, BSA）爲標準蛋白質。

紐西蘭白兔抗細胞外產物抗血清之獲取

將 3 ml（1.78 mg protein）*V. damsela* 細胞外產物以 3 % formalin處理，於25℃放置48小時後，經以PBS過夜透析後再加入 3 ml完全佐劑（Freund's complete adjuvant; FCA）乳化後，經皮下注射入紐西蘭白兔。六週後再追加同樣劑量但FCA則改爲不完全佐劑（Freund's incomplete adjuvant; FIA），再經兩週後採取血清裝於1.5 ml eppendorf中，保存於－70℃備用（Lee and Ellis, 1990）。

草蝦毒性中和試驗與被動免疫

試驗用草蝦

體重約10 g ，爲自孵化後即蓄養於打氣海水水槽之健康蝦子，並餵以市售飼料。每試驗及控制組使用蝦數皆爲10隻。每一實驗組皆採取二重複進行。

細胞外產物毒性中和試驗

抗 *V. damsela* 細胞外產物抗血清與等容量之 *V. damsela* （500 μ g/ml）混合，各以背部肌肉注射方式打入蝦體（0.1 ml），觀察期間爲一週。

被動免疫

抗 *V. damsela* 細胞外產物抗血清與等容量之 *V. damsela* 菌懸液（1×10^8 CFU/ml）混合，各以背部肌肉注射方式打入蝦體（0.1 ml），觀察期間爲兩週。

結果與討論

兔子抗 *V. damsela* 細胞外產物之抗血清可中和各該菌細胞外產物之毒性（Table 1），本毒性中和試驗使用之細胞外產物劑量2.5μg/g prawn約爲其對草蝦最低致死劑量0.53μg/g prawn (Lee and Chen, 1994) 之5倍，此結果顯示此抗血清對草蝦無毒性且可藉由其中所含抗細胞外產物毒性因子之抗體保護蝦體免於死亡。此一實驗結果，係首次證實能夠以於哺乳動物獲取之抗血清進行蝦體之毒性中和試驗。

Table 1. Neutralization of ECP lethal toxicity for tiger prawn weighing about 10 g by incubation with rabbit antisera to the ECP of *Vibrio damsela* (VD1)

Treatment	ECP dose injected (μg protein/ g prawn)	Lethal test
PBS+ECP (1:1 v/v)	2.5	10/10
Rabbit normal serum+ECP (1:1 v/v)	2.5	10/10
Rabbit anti−ECP antiserum+ECP (1:1 v/v)	2.5	0/10

* The lethal tests were conducted in duplicate and observed for one week, dead prawns were all observed within the first 2 days.

由Table 2之結果得知，兔子抗 *V. damsela* 細胞外產物之抗血清可保護草蝦免於該菌之致死性攻擊，本攻擊試驗所使用之細菌劑量5×10^6 CFU/g prawn約爲其對草蝦最低致死劑量4.96×10^6 CFU/g prawn (Lee and Chen, 1994)，此結果顯示本細菌在草蝦所造成的死亡可能係完全以產生致死性之細胞外產物爲主。此實驗結果亦係首次證實能夠以於哺乳動物獲取之抗血清進行蝦體之被動免疫試驗。

毒性中和試驗及被動免疫試驗已久被用於哺乳動物疾病相關之研究，在養殖魚類中則較少且多集中於鮭鱒類（Harrell et al., 1975; McCarthy et al.,1983; Olivier et al., 1985; Marquis and Lallier, 1989），本研究首次以養殖草蝦爲對象進行研究且證實其可行性，在被動免疫實驗中爲減少因多次注射可能引起蝦體緊迫（stress），遂採取將兔子抗血清與菌體混合後注射入蝦體之方式進行，此爲與先注射入抗血清再以細菌攻擊方式略爲不同之處。

Table 2. Passive immunization for tiger prawn weighing about 10 g using rabbit antisera to the ECP of *V. damsela* (VD1)

Treatments	Bacteria challenged (CFU/ g prawn)	Lethal test
PBS+bacteria (1:1 v/v)	5×10^5	10/10
Rabbit normal serum +bacteria (1:1 v/v)	5×10^5	10/10
Rabbit anti−ECP antiserum+bacteria (1:1 v/v)	5×10^5	0/10

* The lethal tests were conducted in duplicate and observed for two weeks, dead prawns were all observed within the first 2 days.

Itami等人（1989）認爲以弧菌菌體製備疫苗在斑節蝦獲得實驗室階段之免疫效果，又 Itami and Takahashi (1991) 也認爲加入弧菌疫苗製備微膠粒飼料，對草蝦苗具免疫效果，但 Song and Sung (1990) 認爲以弧菌疫苗在草蝦僅能增進成長而已。由於目前仍無法證實蝦類能被疫苗誘導產生特異性免疫反應，且未有田間試驗效果之相關報告，因此此類疫苗之實際效果仍待評估。而依據本研究之初步結果似乎採用哺乳動物或脊椎動物（因具記憶性免疫反應）對相關病原弧菌之細胞外產物所產生的抗血清可提供防治效果，而如何廉價地獲取及混入飼料經口投與之效果等問題仍有待探討。

誌　謝

本研究承蒙行政院農業委員會資助研究經費（83-科技-2.16-漁-06(2)），特申謝忱；又於實驗期間，承蒙臺大動物系陳秀男教授研究室提供菌種及本系劉秉忠老師惠予協助，謹此致謝。

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Passive Immunization Study in Tiger Prawns (*Penaeus monodon*) Using Rabbit Antiserum to the Extracellular Products of *Vibrio damsela*

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This study investigates the pathogenicity of *Vibrio damsela* VD1 isolated from diseased tiger prawn (*Penaeus monodon*) in 1988 in Taiwan. Rabbit antiserum to the extracellular products (ECP) of VD1 strain were obtained and used for toxicity neutralization and passive immunization in tiger prawn. Ten prawns weighing about 10 g raised in air-lifted sea water were used in each treatment.

Rabbit antiserum to the ECP of *V. damsela* was mixed with an equal volume of ECP (500 μ g protein/ml) or bacterial suspension (1×10^8 CFU/ml) prior to the intramuscular (i.m.) injection of 0.1 ml of the mixture into the prawn. All the tested groups were conducted in duplicate. The prawns of control group (with same dosage of ECP or bacterial cells) were dead within 2 days while the prawns of experimental group were survived. These results indicate that the toxicity of ECP of *V. damsela* to prawn can be neutralized by rabbit antiserum to the ECP, and the lethal challenge of *V. damsela* to prawn can be prevented by rabbit antiserum to the ECP. In conclusion, toxicity neutralization of the ECP of *V. damsela* and passive immunization against bacterial challenge of *V. damsela* can be provided by rabbit antisera to the ECP of *V. damsela*. This is the first study using mammal antisera to investigate toxicity neutralization and passive immunization in prawns, the results could be useful for study concerning virulence mechanism and disease control in prawns.