

以乳膠微粒凝聚法快速鑑定弧菌

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摘 要

目前已知的弧菌(vibrios)有30種(species)以上,其中9種可能引起人類疾病或食品中毒事件之發生。在製備腸炎弧菌(*Vibrio parahaemolyticus*)莢膜(capsule)抗體時,以13株混合並經加熱殺死之腸炎弧菌菌體(即莢膜抗原)免疫兔子得到之抗血清,發現這些抗體與大部分測試之弧菌屬細菌(*Vibrio* spp.)均有交叉反應。抗血清經過 *Aeromonas media* CCRC 12211 吸附(absorption)後,被覆(coating)於乳膠微粒上,當試劑與弧菌混合時,即產生凝聚反應,反應結果在1至2分鐘內可由肉眼判讀。此乳膠微粒凝聚法對弧菌(32種123株)大部分(99.2%)皆呈現正反應,對其他細菌(48種69株)則均為負反應。此種乳膠微粒凝聚法具有很高的敏感度(sensitivity)及專一性(specificity),可做為弧菌快速鑑定之用。

前 言

弧菌(vibrios)屬於革蘭氏陰性、不產孢,具有鞭毛及運動性之細菌,目前已知有30種(species)以上,其中9種(包括 *V. cholerae*、*V. mimicus*、*V. vulnificus*、*V. parahaemolyticus* 等)是人類病原菌⁽¹⁸⁾。弧菌存在於近海沿岸地區及鹽份適中之陸地水域,並且常附著於水生動物上(如魚類、蚶、蝦等)藉著食物鏈之方式傳染給人類,造成人類之疾病。因此,無論在臨床或食品衛生安全上,弧菌都是重要的微生物⁽¹⁴⁾。

目前已有一些方法,可以針對個別的弧菌或毒素進行快速檢測。如檢測 *V. cholerae* O1型脂多醣體(lipopolysaccharide)的酵素免疫分析法(enzyme immunoassay, EIA)^(2,7,17)、偵測 *V. cholerae* 毒素之乳膠微粒凝聚法(latex agglutination test)^(3,17)及檢測 *V. cholerae* O1血清型之螢光免疫分析法(fluorescent immunoassay)^(5,19); 檢測腸炎弧菌(*V. parahaemolyticus*)菌體之乳膠微粒凝聚法⁽⁶⁾、Kanagawa現象之EIA法⁽⁸⁾和DNA探針(probe)法^(1,12,16)及檢測 *V. vulnificus*的DNA探針法⁽¹⁵⁾等。但是,愈來愈多的報告顯示有更多的弧菌具有致病性。因此,若能發展出一種可以快速鑑定弧菌屬細菌的方

法,可以在進行致病性弧菌鑑定前先行篩選。本文即在報告此過程與結果。

材料與方法

一、實驗菌株

本研究使用之123株(計32種)弧菌及69株(計48種)其他細菌(表一)均來自食品工業發展研究所(Food Industry Research and Development Institute)之菌種保存及研究中心(Culture Collection and Research Center, CCRC)。弧菌依其嗜鹽性之不同分別在 trypticase soy agar (TSA)、TSA-1% NaCl或TSA-3% NaCl於37°C培養18小時;其他細菌則以TSA培養18小時,以便進行乳膠微粒凝聚反應。

二、莢膜抗原之製備

根據Twedt等人⁽⁹⁾之定義,以簡單加熱法殺死的菌體稱為莢膜抗原(capsule antigen)。莢膜抗原的製備乃將13株腸炎弧菌[CCRC 10806 (K1, Kanagawa-positive)、12863 (K1, Kanagawa-positive)、12864 (K26, Kanagawa-positive)、12865 (K38, Kanagawa-positive)、12867 (K17, Kanagawa-

Table 1. Agglutination test of different bacteria with latex particles coated with anti-*Vibrio parahaemolyticus* capsule antibodies.

Bacteria	Number of isolates tested		
	Total	Positive	Negative
<i>Vibrio spp.</i>	60	59	1 ^a
<i>Aeromonas spp.</i>	5	5	0
<i>Escherichia coli</i>	1	0	1
<i>Staphylococcus aureus</i>	1	0	1

^a The strain causing false-negative reaction was *Vibrio parahaemolyticus* CCRC 12970.

positive)、12868 (K18, Kanagawa-positive)、12869 (K19, Kanagawa-negative)、12870 (K24, Kanagawa-negative)、12871 (K19, Kanagawa-negative)、12872 (K28, Kanagawa-negative)、12873 (K30, Kanagawa-negative)、12874(K32, Kanagawa-negative)和12877 (K8, Kanagawa-positive)]分別培養在TSA-3% NaCl斜面(slant),再以2 ml之3%無菌食鹽水將菌體懸浮,並將濃度調至620 nm之吸光值為0.1。分別取13株腸炎弧菌之菌體懸浮液1 ml混合,於60°C加熱10分鐘殺死細菌,這些殺死之菌體即為莢膜抗原,用來免疫兔子。

三、抗血清之製備

將製備好之13株混合腸炎弧菌莢膜抗原與等體積之不完全佐劑(Freund's incomplete adjuvant)乳化後,以皮下多點注射方式免疫兔子。於第4、7、11及15週進行追加注射(每一次免疫之抗原量為4.0 ml),並於第三次追加注射後開始採集血液。血液置於37°C 1小時及4°C過夜後,以6,000×g離心15分鐘去除血餅。血清於56°C水浴加熱30分鐘以破壞補體(complement)。

四、免疫球蛋白G (IgG)的純化

於4°C添加固體硫酸銨(ammonium sulfate)至血清中使達50%飽和度,以8,000×g離心15分鐘。沉澱部分以少量0.07M磷酸緩衝液(pH 6.3)溶解,並以相同的緩衝液進行透析(dialysis)。經過透析後,通入一事先經0.07M磷酸緩衝液(pH 6.3)平衡之DEAE-Sephacel陽離子交換管柱(2.6×26 cm)中,並以相同的緩衝液進行流洗(流速為36.5 ml/h)。收集第一個吸收峰(不與DEAE-Sephacel管柱

結合的分子),經Speed Vac (Savant, Farmingdale, NY, USA)真空乾燥後貯存於-20°C。

五、乳膠微粒(latex)之被覆(coating)處理

取直徑1.09μm的乳膠微粒(Japan Synthetic Rubber, Tokyo, Japan; Cat. no. L2101CY)0.25 ml,以磷酸生理緩衝液(phosphate-buffered saline, PBS, pH 7.2)清洗二次後,將乳膠微粒懸浮於5 ml的PBS中,並加入等體積之IgG溶液(濃度約為70μg/ml),於室溫下旋轉(end-over-end)反應3小時,再以PBS及乳膠稀釋液(PBS中含0.5%牛血清白蛋白及0.05%之sodium azide)分別清洗一次。最後,將乳膠微粒懸浮在5 ml的稀釋液中,貯存於4°C備用(此為測試組試劑)。對照組(control)試劑乃以未經免疫兔子之IgG進行乳膠微粒之被覆處理,其他步驟和測試組相同。

六、乳膠微粒凝聚反應(latex agglutination test)

以簽字筆在載玻片上畫出二個直徑約1.5公分的圓圈,並用接種環(loop)將培養在斜面培養基的細菌刮下,輕輕沾在畫好的圓圈內。取20 μl的對照組及測試組的乳膠微粒試劑,分別加入左右二個圓圈內,並以接種環將菌體塗開,使菌體充分懸浮在試劑中。來回搖晃玻片,使乳膠微粒和菌體充分混合,觀察在1分鐘內有否凝聚發生。在1分鐘之內發生凝聚反應者為正反應,否則為負反應。

七、莢膜抗體之吸附(absorption)

為了消除交叉反應(cross-reaction),取 *Aeromonas media* CCRC 12211冷凍乾燥菌體(約9×10⁸菌體),以PBS洗二次後,加入10 ml 1%的甲醛(formaldehyde),於室溫下反應30分鐘將菌體固定(fixation)。經6,000×g離心15分鐘後,菌體以PBS洗一次,並加入2 ml之抗體(濃度約0.2-0.4 mg/ml),於4°C反應過夜。以6,000×g離心15分鐘去除菌體後,取上清液通入Protein A-Sepharose (Pharmacia, Uppasala, Sweden)親和性管柱(0.5×8.5 cm),並以PBS將未結合之雜質洗出(流速為10.6 ml/h)。當280 nm吸光值趨近於0時,以0.1 M Glycine-HCl緩衝液(pH 2.5)將結合於管柱之IgG洗出(試管中預置1 ml 1 M Tris-HCl, pH 8.5,以中和洗液),再以PBS透析,乾燥後貯存於-20°C備用。

八、敏感度(sensitivity),專一性(specificity),偽陽性比例(false-positive rate)及偽陰性比例(false-negative rate)之定義

Table 2. Agglutination test of different bacteria with latex particles coated with anti-*Vibrio parahaemolyticus* capsule antibodies absorbed with *Aeromonas media* CCRC 12211.

Bacteria	No. of isolates tested	No. of positive	No. of negative
<i>Vibrio albensis</i>	1	1	0
<i>V. alginolyticus</i>	2	2	0
<i>V. algosus</i>	2	2	0
<i>V. anguillarum</i>	2	2	0
<i>V. campbellii</i> ^a	1	1	0
<i>V. carchariae</i>	1	1	0
<i>V. cholerae</i>	6	6	0
<i>V. costicola</i> ^a	3	3	0
<i>V. damsela</i> ^a	1	1	0
<i>V. diazotrophicus</i> ^a	2	2	0
<i>V. fischeri</i>	3	3	0
<i>V. fluvialis</i>	3	3	0
<i>V. harveyi</i> ^a	2	2	0
<i>V. liquefaciens</i>	1	1	0
<i>V. logei</i>	1	1	0
<i>V. marinagilis</i>	1	1	0
<i>V. marinofulvus</i>	1	1	0
<i>V. marinovulgaris</i>	1	1	0
<i>V. natriegens</i> ^a	2	2	0
<i>V. nereis</i> ^a	1	1	0
<i>V. nigrapulchritudo</i> ^a	1	1	0
<i>V. ordalii</i> ^a	2	2	0
<i>V. orientalis</i>	1	1	0
<i>V. parahaemolyticus</i> ^a	70	69	1
<i>V. pelagius</i> ^a	1	1	0
<i>V. percolans</i> ^a	1	1	0
<i>V. ponticus</i>	1	1	0
<i>V. proteolyticus</i>	1	1	0
<i>V. salmonicida</i> ^a	1	1	0
<i>V. splendidus</i>	2	2	0

(to be continued)

敏感度是指乳膠微粒凝聚反應結果呈現正反應之弧菌菌株數除以所有供檢測之弧菌菌株數。專一性是指乳膠微粒凝聚反應結果呈現負反應之非弧菌屬細菌(non-vibrios)菌株數除以所有檢測之非弧菌屬細菌菌株數⁽¹³⁾。偽陽性比例(false-positive rate)是指乳膠微粒凝聚反應結果呈現正反應之非弧菌屬細菌菌株數除以所有正反應之細菌菌株數(包括弧菌及非弧菌屬細菌)。偽陰性比例

(false-negative rate)則是乳膠微粒凝聚反應結果呈現負反應之弧菌屬細菌菌株數除以所有負反應之細菌菌株數(包括弧菌及非弧菌屬細菌)。

結果與討論

以13株混合腸炎弧菌莢膜抗原免疫兔子,所得

Table 2. Agglutination test of different bacteria with latex particles coated with anti-*Vibrio parahaemolyticus* capsule antibodies absorbed with *Aeromonas media* CCRC 12211.

Bacteria	No. of isolates tested	No. of positive	No. of negative
<i>V. tubiashii</i>	3	3	0
<i>V. vulnificus</i> ^a	2	2	0
<i>Alcaligenes</i> spp.	2	0	2
<i>Aeromonas</i> spp.	16	0	16
<i>Bacillus</i> spp.	3	0	3
<i>Enterobacter</i> spp.	4	0	4
<i>Escherichia</i> spp.	3	0	3
<i>Erwinia carotovora</i>	1	0	1
<i>Flavobacter</i> spp.	2	0	2
<i>Micrococcus</i> spp.	2	0	2
<i>Photobacterium</i> spp.	3	0	3
<i>Plesiomonas shigelloides</i>	2	0	2
<i>Proteus</i> spp.	6	0	6
<i>Pseudomonas</i> spp.	4	0	4
<i>Salmonella</i> spp.	4	0	4
<i>Shigella</i> spp.	6	0	6
<i>Staphylococcus</i> spp.	4	0	4
<i>Streptococcus thermophilus</i>	2	0	2
<i>Xanthomonas maltophilia</i>	1	0	1
<i>Yersinia</i> spp.	4	0	4
Total strains of			
<i>Vibrio</i> spp.	123	122	1
<i>Aeromonas</i> spp.	16	0	16
Other bacteria ^b	53	0	53

^aSpecies already tested in Table 1.^bBacteria other than *Vibrio* and *Aeromonas*.

之抗血清以DEAE-Sephacel陽離子交換管柱純化後,被覆於乳膠微粒上,並進行凝聚反應試驗。結果顯示(表一),60株(計14種)弧菌中只有1株(*Vibrio parahaemolyticus* CCRC 12970)呈現負反應,其餘均為正反應,顯示弧菌屬細菌之莢膜抗原具有相當程度之類似性。另外,根據Kabir^(10,11)的研究報告指出,革蘭氏陰性菌之外膜(outer membrane)雖有一些屬於種的特異性抗原(species-specific antigens),但也有許多是同屬(genus)細菌所共有的抗原,且帶有極強的抗原性,所以製備之莢膜抗體與測試之弧菌有良好的凝聚反應。Twedt等人⁽⁹⁾亦有類似的報告。由表一可以看出,5株*Aeromonas*屬細菌均有交叉反應發生,根據Bergey's Manual⁽⁴⁾的

記載,腸炎弧菌與*Aeromonas*屬細菌在血緣上關係密切,在DNA相似性(DNA relatedness)及生理生化特性上有許多類似之處。所以,被覆腸炎弧菌莢膜抗體之乳膠微粒與*Aeromonas*屬細菌產生凝聚反應,並非偶然。

為了去除*Aeromonas*細菌引起的交叉反應,將腸炎弧菌莢膜抗體以*Aeromonas media* CCRC 12211進行吸附。吸附後之抗體再以Protein A-Sepharose親和性管柱純化IgG,經被覆於乳膠微粒後再對不同菌株進行測試,結果如表二所示。測試123株(計32種)弧菌中依然只有*Vibrio parahaemolyticus* CCRC 12970呈現負反應,其餘122株弧菌皆為正反應,而69株(48種)其他非弧菌屬細菌(包

括16株*Aeromonas*屬細菌)則均無反應,顯示*Aeromonas*屬細菌之交叉反應已被去除。也就是說,腸炎弧菌莢膜抗體以*Aeromonas media* CCRC 12211進行吸附,再被覆於乳膠微粒後,與弧菌屬反應的敏感度為99.2%(122/123),專一性則接近100%(69/69),而偽陰性及偽陽性比例分別為1.4%(1/70)及0%(0/122)。

綜合上述,以*Aeromonas media* CCRC 12211進行吸附之腸炎弧菌莢膜抗體被覆於乳膠微粒後,可以與大部分測試之弧菌產生凝聚反應,且反應的專一性很高。顯示,以此法發展出來之乳膠微粒試劑可以作為弧菌快速檢測之用。

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A Latex Agglutination Test for the Rapid Identification of Vibrios

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ABSTRACT

A latex agglutination test for the rapid identification of vibrios was developed. Anti-capsule antisera were raised in rabbits by immunizing the animals with mixed capsule antigens of 13 strains of *Vibrio parahaemolyticus*. Latex particles coated with the anti-capsule antibodies were used for the agglutination test of vibrios and other bacteria. Cross-reactions with some species of *Aeromonas* spp. were found, and could be eliminated by absorption of anti-capsu-

le antibodies with *Aeromonas media* CCRC 12211. Latex coated with the absorbed and affinity-purified antibodies could agglutinate 99.2% strains of vibrios (32 species, 123 isolates), and no cross-reactions were found with other bacteria (48 species, 69 isolates). Due to the high sensitivity and specificity, the latex agglutination test could be used for rapid identification of vibrios.

Key Words : *Vibrio parahaemolyticus*, Latex agglutination test, Rapid identification, Vibrios.