山羊關節炎腦炎監測之研究(1)

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摘要

本試驗旨在進行山羊關節炎腦炎病毒（Caprine arthritis-encephalitis virus, CAEV）血清監控及建立巢式聚合酶連鎖反應（nested polymerase chain reaction, nested PCR）篩檢法，以提供控制本病方法之參考。第一階段以台東某羊場所飼養努比亞種羊 131 頭血樣進行檢測，使用酵素結合免疫吸附試驗（Enzyme linked immunosorbent assay，ELISA）分析血清樣本，調查結果顯示 CAE 陽性率在 0－1 歲、1－2 歲及 3 歲以上之不同年齡層，分別為 85.7%（72/84）、92.5%（25/27）及 100%（20/20），顯示羊隻年齡與 CAEV 陽性比率呈顯著相關（χ^2＝15.24, P＜0.0005）。 檢測 0－1 歲齡母羊及公羊，CAEV 其陽性率分別為 85.3%（35/41）及 86.0%（37/43），結果顯示在 0－1 歲年齡層之陽性率與性別無關。第二階段將 ELISA 檢測結果陰性血清反應羊隻共 14 頭，進一步採血萃取 DNA，利用 nested PCR 檢測定序結果陽性反應 2 頭，利用 nested PCR 的方式確實可增幅出 CAEV 之 gag 片段。

關鍵詞：山羊、山羊關節炎腦炎、酵素結合免疫吸附法、巢式聚合酶連鎖反應。

緒言

山羊關節炎腦炎（Caprine arthritis-encephalitis；CAE）為反轉錄病毒中之慢病毒（lentvirus），屬於 Retroviridae 之 Lentivirnae 亞科，是一種山羊慢性進行性疾病（Cork *et al*., 1974）。CAEV 主要標的細胞為單核細胞 (monocytes）和巨噬細胞（macrophages）（Narayan *et al*., 1983; Ravazzolo *et al*., 2001），此病會造成持續性感染狀態，感染初期並無大量病毒產生，病毒需經反轉錄過程才會形成嵌入型原病毒 DNA（integrated proviral DNA），而感染後誘發抗體產生時間因個體會有所差異，大部份感染羊隻都不會有臨床症狀，但會呈持續性感染狀態，以及長期傳播病毒帶原狀態（Rimstad *et al*., 1993; Hanson *et al*., 1996），而本病毒屬一種多症狀的疾病，除造成山羊之關節炎外，亦可造成間質性肺炎，非化膿性腦脊髓炎及乳房硬固等不同綜合病症（Adams *et al*., 1983; Nord and Adnoy, 1997）。目前已知 CAE 病毒傳染的方式，主要為垂直傳染，即 CAE 病毒藉由初乳或乳汁（Adams *et al*., 1983），次要的感染方式為水平感

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染，同欄舍羊隻間長期接觸而造成感染（Phelps and Smith, 1993; Rowe and East, 1997）。本疾病因感染後至症狀出現警覺不易，尚無藥物或疫苗可治療，而為養羊事業重大困擾疾病之一（Luengo *et al*., 2004; Peterhans *et al*., 2004; Reina *et al*., 2009）。目前檢測方式以血清診斷為主，如瓊脂凝膠免疫擴散法 (agal gel immunodiffusion, AGID)、ELISA 等，早期以 AGID 最常用，但敏感度較差（Herrmann *et al*., 2003; de Andres *et al*., 2005），近年市面上可購得 CAE ELISA 檢測套組已逐漸取代 AGID 法。經檢測呈抗體陽性之羊隻，大致可確定已受病毒感染。但檢測呈抗體陰性羊隻，尚不能認定其未受感染，原因是感染至抗體產生有所延遲，致有些仍無法測得抗體陽性反應，實際上該羊隻可能已受到感染（Adams *et al*., 1983; East *et al*., 1993; Rimstad *et al*., 1993）。本研究使用 CAE ELISA 檢測套組及 PCR 檢測技術，透過 CAE 病毒核苷酸引子序列定位，確認山羊是否帶有 CAE 病毒，以利逐年降低帶有 CAE 病毒不良羊隻，隔離飼養無 CAE 病毒羊隻，期望降低發生率及改善山羊產業經濟損失。

材料與方法

I. 血清樣品採集

自東部某一種羊場之外觀健康無病徵之努比亞種羊 125 頭，記錄動物年齡及性別。由頸靜脈採集血樣，俟其凝固後，經 1100 × g 離心 20 分鐘，分離血清，保存於−20℃ 備測。

II. 血清中 CAE 抗體評估:

抗體評估以市售 CAE ELISA 商業套組（CHEKIT CAEV/MVV Antibody Test Kit, IDEXX Laboratories, Inc. USA）依其操作方法進行檢測。其血清抗體之陽、陰性標準依下列公式計算後判定。

Value (%)＝OD sample – OD negative / OD positive – OD negative X 100%

Negative＜30%, Suspect≧30 to＜40%, Positive≧0 % 其中：OD sample 為樣品測出吸光值，OD negative 為陰性控制組測出吸光值，OD positive 為陽性控制組測出吸光值，Negative 為判定陰性，Suspect 為判定疑似，Positive 為判定陽性。

III. CAE 之 nested PCR 檢測：

(i) DNA 萃取

ELISA 檢測陰性羊隻進一步採取 3 – 5 mL 全血，利用 DNA 純化套（QIAamp DNA Mini kit” Qiagen, Courtaboeuf, France）依照其操作步驟進行 DNA 之萃取及純化，之後進行 nested PCR，檢測病毒核苷酸。

(ii) CAEV gags 序列引子設計：

CAEV proviral DNA 檢測參考 Fieni 等（2002）之方法，使用 nested PCR 方式確認 gag 區域之部份片段，引子序列由生工有限公司合成（MD Bio,Inc），第一組 PCR 使用 CAEV gags 序列引子 P1（5`-caagcagcaggagggagaagctg-3`）及 P2（5`-tcctacccccataatttgatccac-3`）預期產物 296bp，第二組 PCR 反應，使用引子序列為 P3 （5`-gttccagcaactgcaaacagtagcaatg-3`）及 P4（5`-acctttctgcttcttcatttaatttccc-3`）預期產物 184bp。

(iii) PCR 及電泳：

使用萃取 DNA 做為模板進行 nested PCR。PCR 反應液含有 2 μM DNA，10 μM 的 P1 與 P2 引子各 1 μL，Taq DNA 聚合酶（Roche, Germany）0.4 μL（5 U/ μL），10 × PCR buffer 5 μL，10mM dNTP 1 μL 及 2dH2O 40 μL，PCR 反應總體積約為 50 μL。PCR 條件為 94℃ 5min ; 94 ℃ 30 sec，55 ℃ 40 sec 與 72℃ 45sec，循環 30 次最後為 72℃ 5 min extension，完成第一組 PCR 可得一條 296 bp 的產物，取 2 μL 第一組 PCR 產物當模版，進行第二組 PCR 反應，使用引子 P3 及 P4 濃度為 10 μM 各 取 1 μL，PCR 條件為 94℃ 5min ; 94℃ 30 sec，52℃ 40 sec 與 72℃ 45sec，循環 30 次。電泳分析可得一條 184 bp 序列大小產物，取 7 μL 產物並加入 2 μ L 的 6 × Loading buffer，

置入 1% 瓊脂醣膠片內，於 0.5 × 之 Tris-acetate-EDTA（TAE）緩衝液中進行電泳，條件為 70 Volt 45 min，電泳後將膠片染色，並於紫外燈箱上觀察紀錄結果。

IV. DNA 序列分析與比對：

取 DNA 凝膠送至核苷酸定序服務公司定序確認，再進行序列測定與分析。完成 DNA 序列分析的資料利用 NCBI 進行 Basic Local Alignment Search Tool（BLAST）比對，以了解序列的正確性與比較網路資料庫中的相似序列。

V. 統計分析

相關數據利用 SAS 套裝軟體（Statistical Analysis System; SAS, 2005）進行統計分析，並以卡方檢定（Chi-square Test）比較因子間之相關性。

結果與討論

本研究第一階段使用 ELISA 方式，監測山羊 CAE 病毒狀況，血清學評估採 CAEV/MVV Antibody Test Kit（CHEKIT）商業套組，結果如表 1 所示；其中 0－1 歲齡羊隻 84 頭，CAEV 陽性反應為 85.7%（72/84），1－2 歲齡羊隻 27 頭，陽性反應為 92.5%（25/27），而 3 歲齡以上羊隻 20 頭，陽性反應為 100%（20/20），結果顯示羊隻年齡與陽性比例呈顯著相關（χ2=15.24, P＜0.0005）。就 0－1 歲齡羊隻，依性別分類檢測 CAEV 結果如表 2 所示，其中母羊檢測陽性反應為 85.3%（35/41），公羊檢測陽性反應為 86.0%（37/43），結果顯示羊隻之陽性率與性別無關。關於 CAE 診斷，因 CAEV 感染羊隻經長期飼養後才會陸續出現臨床症狀，而在感染初期並無可辨識之臨床及肉眼症狀，故世界動物衛生組織（World organization for animal health; OIE）建議採行血清學測試，例如 AGID、ELISA，而不是採行病毒分離或臨床症狀觀察（Knowles and Herrman, 2008）。目前市面上可購得 CAE ELISA 套組，其操作方便且可檢測大量血清樣本，在診斷應用上更廣泛、普遍。許多研究關於 CAEV 感染與品種、年齡和飼養方法之間的關聯性探討（East *et al*., 1987; Cutlip *et al*., 1992; Nord *et al*., 1998; Aslantas *et al*., 2005; Elfahal *et al*., 2010; Mohamed *et al*., 2012）

第二階段以 ELISA 酵素分析陰性血清反應羊隻為樣本，進一步採血萃取 DNA 檢測，透過 nested PCR 檢測 CAEV gag 基因，利用兩組專一性引子，去偵測 gag 基因片段，經完成第二組 PCR 反應產物，電泳分析可得一條 184 bp 產物，檢測 PCR 產物回收，進行定序分析與比對以作為確認（圖 1）。序列分析資料利用 National Center for Biotechnology Information (NCBI)進行 DNA Basic Local Alignment Search Tool（BLAST）比對（圖 2），結果擴增片段為 CAEV gag 基因片段，定序結果陽性反應 2 頭，使用 nested PCR 的方式確實可增幅出 CAEV 之 gag 片段，進行定序與序列分析證實。

ELISA 血清反應檢測結果呈陽性的羊隻，被認為是已受病毒之感染；但陰性結果尚不能判定未受感染，原因是受病毒感染羊隻，剛開始不會大量複製病毒，但隨著年齡增加其血清轉陽性比率會逐漸被表現（Rowe *et al*.,1991），故早期研究必須經一段時間再重複檢測以確認感染情形（Mackenzie *et al*., 1987; Rowe *et al*., 1992; Rimstad *et al*., 1993）。近年來國外之研究，診斷 CAE 常並用其他檢測技術，如分子生物技術 PCR 或 nested PCR（Brinkhof et al., 2010; Barquero et al., 2011; Eltahir *et al.,* 2006; Elfahal *et al*., 2010; Gil et al., 2006; Konishi *et al*., 2011），以增加其特異性（specificity）及敏感性（sensitivity）。應用 PCR 檢測 CAEV gag 核苷酸片段，不僅用於血樣、亦可用於組織培養（Clavijo and Thorsen, 1995; Daltabuit Test *et al*., 1999; Adebayo *et al*., 2008; Ali Al Ahmad *et al*., 2006, 2012），生殖系統樣品（子宮、卵巢或輸卵管）（Zanoni *et al*., 1991; Fieni *et al*., 2002, 2003; Ali Al Ahmad *et al*., 2008, 2012）、生殖細胞（Fieni *et al*., 2012）及乳汁樣品等檢測（Reddy *et al*., 1993; Rimstad *et al*., 1993; Zanoni *et al*., 1996; Leroux *et al*., 1997; Wagter *et al.,* 1998; Brinkhof *et al*., 2010），可作為 CAEV 感染時之不同來源之樣品檢測。

CAE 最早是 Cork *et al.*（1974）檢測美國華盛頓州乳羊場之仔羊所發現。Crawford *et al.*（1981）使用 AGID 共檢測 24 州之 1, 160 頭山羊，結果 81% 羊隻呈現血清抗體陽性反應，顯示感染程度嚴重。本省於民國 70 年代陸續自美國引進山羊，可能亦已大量引進本病，而至 1993 年養豬科學研究所以電子顯微鏡發現 CAE 病毒顆粒始證實本病之存在(Loung *et al.*,1993)。其後學者之研究，以台灣中南部及東部羊場進行血清檢測結果盛行率高達 75.9%; 顯示本病已廣泛感染於本省各地（Lee *et al.*, 1996）。2010 年中華民國養羊協會建請本場設定執行方案，建構 CAE free 場模式，配合種畜團隊研究計畫執行 CAE 監測，以解決山羊種畜亟需處理之問題。目前 CAEV 之血清學調查，顯示發生率澳洲為 82%（Grewal *et al.*, 1986）、美國 81%（Crawford *et al.*, 1981）、挪威 49.5 %（Nord *et al.*, 1998）、巴西 36.5% （Garcla *et al.*, 1992），有些國家並已開始對羊群進行「無山羊關節炎腦炎感染」之管控計畫。日本學者之研究，自 2002-2006 年進行清除 CAEV 計畫，施行管控三策略包括（1）仔羊出生立刻隔離（2）世代分開管理（3）周期性進行檢測，並去除陽性羊隻，結果使 2002 年之 CAEV 陽性率 60.8%，至 2006 年仔羊陽性反應者降至 5.36%（11/205），且於施行清除計畫後並顯著提升乳產量，由此證實管控三策略確可有效建立從感染的 CAEV 羊群成為無 CAEV 之羊群（Konishi *et al.*, 2011）。另外，也有國外研究要求羊群中所有的成年羊隻，每 6 個月接受檢驗，初乳之充分消毒與滅菌，然而血清學測試為陰性之仔羊當飼養至年齡大時血清學測試仍會轉陽性。 因此根除 CAEV 之傳染需要有效的隔離規畫，並配合持續的血清學評估（Leitner *et al.*, 2010）。由於本病在台灣血清陽性率極高，而目前並無具價值之治療策略可運用，唯一可行之控制措施，可採用本試驗所建立血清 ELISA 及 nested PCR 方式篩選監控羊群，並隔離或淘汰血清呈陽性反應的羊隻，而新生仔羊與應母畜隔離，並餵予經 56℃、1 小時加熱處理的初乳，逐步建立陰性羊群，以降低陽性率，減少經濟上的損失。

表 1. 不同年齡山羊之關節炎腦炎發生頻率

Table 1. Frequency of caprine arthritis encephalitis in different age

Age	Number of goats		
	Examined	Positive (%)	Negative (%)
0-1 years	84	72(85.7)	12(14.3)
1-2 years	27	25(92.5)	2(7.4)
Over3 years	20	20(100)	0(0)

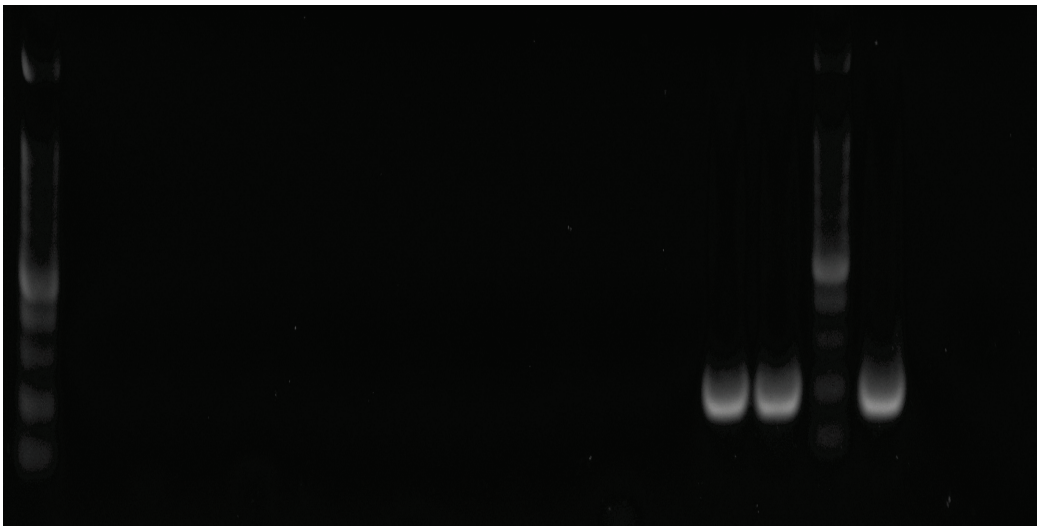
Chi-Square was significant difference between age. ($P < 0.0005$).

表 2. 不同性別山羊之關節炎腦炎發生頻率

Table 2. Frequency of caprine arthritis encephalitis in different sex

Sex	Number of goats		
	Examined	Positive (%)	Negative (%)
0-1 ♀	41	35(85.3)	6(14.6)
0-1 ♂	43	37(86.0)	6(14.0)

Chi-Square was not significant difference between sex. ($P > 0.05$).



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

圖 1. 以 PCR 反應檢測山羊 CAEV gag 基因片段表現。 Lane 1 與 16 為 100bp ladder marker; Lane2 至 Lane15 為檢測樣品; Lane17 為陽性對照（184bp）、Lane18C 為陰性對照、 Lane19 空白對照。

Fig. 1. PCR analysis of CAEV gag gene in goats. Lane 1 and 16: 100bp ladder marker, lane 2-15: sample, Lane17: positive controsl(184bp), Lane18: negative control, Lane19: H_2O blank

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Query  5     GCATGGCCTCGTGTCTGAGGA-TTTGAA-GGCAGCTGGCATATTATGCCACTACCTGGAC  62
             ||||||||||||||||||||| |||||| ||||| |||||||||||| ||||||||||
Sbjct  1027  GCATGGCCTCGTGTCTGAGGACTTTGAAAGGCAGTTGGCATATTATGCTACTACCTGGAC  1086

Query  63    AAGTAAAGATATACTAGAAGTATTGGCCATGATGCCTGGGAATAGAGCTCAGAAGGAGCT  122
             ||||||||| ||||||||||||||||||||||||||| |||||||||| |||||| |
Sbjct  1087  AAGTAAAGACATACTAGAAGTATTGGCCATGATGCCTGGAAATAGAGCTCAAAAGGAGTT  1146

Query  123   AATTCAAGGGAAATTAAATGCACAAGCAGAA  153
             |||||||||||||||||| | ||||||||
Sbjct  1147  AATTCAAGGGAAATTAAATGAAGAAGCAGAA  1177
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圖 2. 山羊 CAEV 基因的序列比對

Fig. 2. Alignment and comparison of goat CAEV DNA sequences

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Studies on Caprine arthritis-encephalitis monitoring(1)

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Abstract

The objectives of this experiment are to perform CAE virus (CAEV) monitoring in serum and to use a screening method named “nested polymerase chain reaction” (nested PCR) as a reference for the control of CAE. In the first stage, blood samples of 131 Nubian goats from a Taitung goat farm were used for test. The method of Enzyme Linked Immunosorbent Assay (ELISA) was used to analyze the serum samples. In the results, The frequency of positive results in 0-1 yrs, 1-2 yrs, and above 3 yrs were 85.7% (72/84), 92.5% (25/27), 100% (20/20),respectively. The results indicates a significant positive correlation ($\chi 2=15.24$, $P < 0.0005$) between different ranges of age in goat. In the 0-1 yrs., the frequency of positive results in does and buck were 85.3% (35/41) and 86.6% (37/43), respectively. There were no correlation between dos and buck of goat in the CAE positive results ($P>0.05$). In the second, the DNAs were extracted from the blood of the 14 ELISA-negative goats and used nested PCR for viral detection. There were showed 2 positive results, and then nested PCR results were confirmed by sequence analysis.

Key words: Goat, Caprine arthritis-encephalitis, Enzyme linked immunosorbent assay, Nested PCR.

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