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With 2 figures in the text

Application of PCR and RFLP for Detection and Differentiation of two Sudanese *Mycoplasma* Isolates

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ملخص البحث

تم تعريف العزلتين GF19 , GL19 كعتـــرتين تحـــت النـــوع (subspecies) المفطـــورة *Mycoplamsa Capricolum* subsp. *Caprineumoniae* بإستخدام التفاعل المتسلسل للبوليمريز في الجين 16S-rRNA وإجراء إختبار القطع الخمائرى لخميرة محددة هي Pst 1 .نتج عن ذلك ثلاثة قطـــع مـــن الحامض النووى- احجامها 548 و420 و 128 زوج قاعدى من كل مستعمرة من المستعمرات الـــتي أختـــبرت للعزلتين المذكورتين أعلاه . بينما كان ناتج بقية أعضاء حزمة المفطورة المتفطـــرة (*M. mycoides* cluster) قطعتين فقط وبالحجمين 420و 128 زوج قاعدى.

أظهرت هذه الدراسة ان تسخين خلايا هذه المفطورة عند درجة حرارة 95 °م لمدة عشرة دقـــائق بدلاً عن إستخدام دارئ تحلل (lysis buffer) لإستخلاص الحامض النووى قد زاد من حساسية التفاعل المتسلسل للبوليمريز لتمايزهذا النوع من المفطورات.

Summary

Isolates GL19 and GF19 were genetically identified by the PCR of the 16 S rRNA and RFLP as *M. capricolum* subsp. *Capripneumoniae*.

Upon using the two primers CAP1 and CAP2, 548bp amplification product was obtained from all colonies used of the above two isolates. Following the Restriction Fragments Length Polymorphism (RFLP) of the Polymerase Chain Reaction (PCR) product from the two strains GL 19 and GF 19, three bands were obtained; one band represented intact PCR products of 548-bp from rrnB operon and the two other bands represented the two fragments of 420 - bp and 128 –bp from rrnA operon. Only two bands of 420bp and 128bp were obtained from the members, other than *M. capricolum* subsp. *Caprineumoniae*, of the "*M. mycoides* cluster".

A considerable improvement in the sensitivity of the PCR was obtained by replacing the step of using the lysis buffer with cooking of the culture at 95°C for 10 minutes.

Introduction

Mycoplasma biotype F38 (MacOwan and Minette, 1976) was later on classified as a subspecies of *M. capricolum* (Leach *et al.*, 1993). It is the etiological agent of contagious caprine pleuropneumonia (CCPP). More than 30 countries have declared that they have CCPP, but the organism has been isolated from goats only in 11 countries (Thiaucourt and Bölske, 1996).M. capricolum subsp. capripneumoniae MCCP,

belongs to the so called "Mycoplasma mycoides cluster" which includes M. mycoides subsp. mycoides biotype SC, M. mycoides subsp. mycoides biotype LC, M. mycoides subsp. Capri, M. capricolum subsp. Capricolum and Mycoplasma sp. group 7. All are closely related significant animal pathogens, show serological cross-reaction and have similar biochemical features (Cottew et al., 1987; Erno 1987). It is difficult to identify MCCP, which is the most fastidious member of the M. mycoides cluster, by conventional methods. A diagnostic method for CCPP based on PCR of the 16S rRNA genes product from MCCP and restriction enzyme analysis; a restriction fragment length polymorphism (RFLP) of the PCR product has been developed (Bascunana et al., 1994; Johansson et al., 1998).

Materials and Methods

The two *Mycoplasma* strains GF19 and GL19 used in this study were isolated from a case of CCPP in the Sudan. They were previously identified as F38-like species (Abbas, 1986)

DNA extraction, was carried out as described by Bernhard *et al.* (1997), with some modification, replacing the use of lysis buffer by the cooking of the cultures at 95°C for 10 min. (Abbas, 2002) The amplification was performed using the primers of Johansson *et al.* (1998) and for RFLP the PCR products were analysed directly without purification of DNA:

Results

548-bp amplification products had been observed in all clones of the two strains GL19 and GF19 (Fig. 1). After RFLP of the PCR products from MCCP, three bands were obtained, one band represented intact PCR product of 548-bp from the rrnB operon and the other two bands represented the two fragments of 420-bp and 128-bp from rRNA operon. Two bands of 420-bp and 128-bp were obtained from all other members of the *M. mycoides* cluster (Fig. 2).

Discussion

One could argue that identification of an important strain on the basis of a polymorphism is not very reliable and that other strains of MCCP may not have this polymorphism. However, ribosomal RNA genes are comparatively stable (Harvey and May, 1993) and MCCP appears to be a rather homogeneous subspecies (Christiansen and Erno, 1990; Kokotovic *et. al.*, 2000).

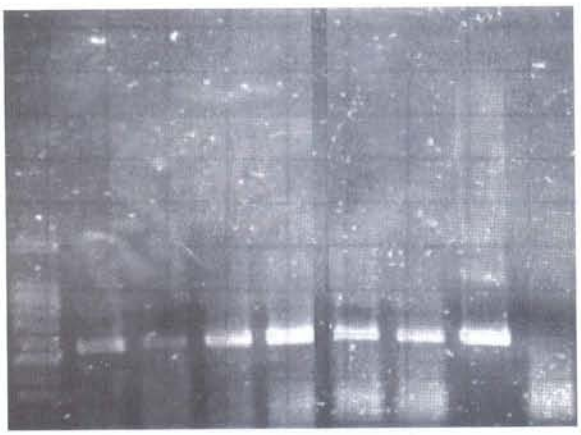

Fig. 1: *M. mycoides* cluster specific PCR system. Electrophoresis in 1.5% agarose gel of the PCR products using primers CAP 1- CAP 2 (Johansson *et al.*, 1998). Extracted DNAs, from strains GL 19; GF 19 (3 clones out of each), were the tested template and the extracted DNA of the Type strain of *M.capricolum* subsp. *capricolum* (Calf-kidney) was used as a positive control template. Lane M: 100-bp marker; lanes 1, 2 and 3 are clones from GL 19, LANE 4, 5 and 6; Clones from GF 19, lane 7; positive control *M.capricolum* subsp. *capricolum.* Lane N; Negative control.

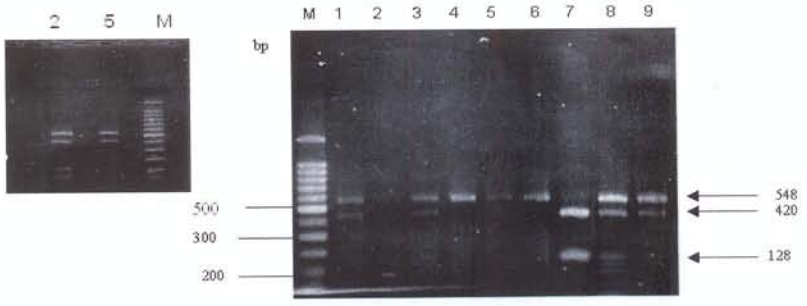

Fig. 2: Restriction enzyme analysis with Pst 1 of the *M. mycoides* cluster PCR products from strains GL19; GF 19 (clones from each) and *M. capricolu*m subsp. *capricolu*m (Calf) as a control. Electrophoresis in 1.5% agarose gel. The REA of the PCR products of both strains GL 19 and GF 19 (all clones), revealed three bands, (one band represents intact PCR product of 548-bp from rrnB operon and the other two bands represent the two fragments of 420-bp and 128-bp from rrnB operon), which is characteristic for *M.capricolum* subsp. *capripneumoniae*, while only two bands of 420-bp and 128-bp are obtained from *M.capricolum* subsp. *capricolum* which is a character for all other members of the *M. mycoides* cluster.

REA was repeated for clones 2 and 5 as shown above.

The results of this study showed by the combination of PCR and RFLP methods are in agreement with previous observations of others (Christiansen and Erno, 1990; Thiaucourt *et al.*, 1992; Bonnet *et al.*, 1993; Bascunana *et al.*, 1994; Bölske *et al.*, 1996). In this study, lyophilised pure cultures of GL19 and GF19 strains were used but it is very important to take into consideration the existence of some factors that influence PCR sensitivity, either positively or negatively. These factors may not only include the number of the examined organisms whether numerous or few in the sample, the amount of extracted DNA, or the condition of PCR, but also the effect of other bacterial flora, pH, storage duration and temperature of these samples.

A considerable improvement in the sensitivity of PCR over that of Bernhard *et al.* (1997) was obtained, when replacing the step of use of lysis buffer with cooking of cultures at 95°C for 10 min.; the former method may not allow release of considerable amount of DNA, so it decreases the sensitivity and reproducibility of PCR results. Moreover, the mentioned extraction methods have the additional drawback of being laborious and often involve the use of noxious chemicals

In future investigations, the influence of these factors should be highlighted. However, the comprehensive use of the PCR could open up a new possibility for the control of the diseases caused by mycoplasmas.

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