The Sudan J. Vet. Res. (2006): 21:
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Molecular Characterization of Some Sudanese isolates of *Mycoplasma*

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ملخص البحث

أمكن بوضوح روية حزم البروتين لبعض مستعمرات العترات GL 19, GF 19 بعد إستخلاصها وفصلها بتقنية الرحلان الكهربائى لهلام الإكريلأمايد المتعدد (SDS- PAGE) و صبغها بالكوماسي الأزرق. أما إختبار تحليل اللطخة الغربية (Western-blot analysis) فقد تم إجرائه في تجربتين:-

التجربة الأولي كانت بمقارنة البروتين الموجود على الغشاء السايتوبلازمي لعترات مجموعة المفطورة المستفطرة(*M. mycoides* cluster) وتمت بهذه الاختبارات معرفة وتفريق المفطورة التيسية تحت نوع إلتهاب رئة الماعز (*M.capricolum* subsp. *Capripneumoniae*) عن بقية عترات مجموعة المفطورة المستفطرة وذلك لوجود حزمة بروتين بحجم جزيئي بين 7.6 و 18 كيلو دالتون ولا توجد هذه الحزمه في بقية عترات افراد تلك المجموعة علماً بأن هذه النتيجة لم يسبق تسجيليها من قبل. لذا نوصي باستخدام اختبار اللطخة الغربية في التعرف على هوية المفطورة التيسية تحت نوع إلتهاب رئة الماعز لتشخيص الإلتهاب الرئوي البلوري الساري في الماعز. فمستعمرات العتره GL 19 الثلاث لهذه المفطورة والمستخدمة في هذا الاختبار أعطت نتائج متطابقة حيث تحللت كل مستعمرة إلى حوالي 35-50 حزم من البروتين يقع معظمها بين الأوزان الجزئية 42.2 و 132 كيلو دالتون. أما بقية عترات أنواع المجموعة فقد أعطت أربع حزم واضحة من البروتين تركزت بين الآوزان الجزئية 71 و 91 كيلو دالتون.

أما التجربة الثانية فقد أجريت لمقارنة البروتين المتواجد على الغشاء السلايتوبلازمي للمستعمرات الكبيرة والصغيرة للعترتين GL 19 و GF 19 وكان الفرق كمياً حيث أعطت المستعمرات الكبيرة مقداراً أكبر من البروتين يقع معظمه بين الأوزان الجزئية 35.1 و 132 كيلو دالتون بينما أعطت المستعمرات الصغيرة مقداراً أقل تركز بين الأوزان الجزئية 42.2 و 91 كيلو دالتون.

استخدمت تقنية التقسيم المرحلى بواسطة Triton x-114 كخطوة اولي لمعرفة لمكونات السطحية للعترة GL 19 للمفطورة التيسية تحت نوع التهاب رئة الماعز والتى اكدت الصفة المميزة للمستعمرة الصغيرة لهذه العترة والتى وجدت بواسطة اختبار تحليل اللطخه الغربيه لكل بروتين الخلية. اظهرت كل البروتنيات التي كثفت فى الطبقة غير المحبة للماء تفاعلاً مناعياً في اختبار تحليل اللطخة الغربية وهى تقريباً نفس البروتينات المولدة للمناعة (ومسئولة عن عملية الالتصاق) للمستعمرة الصغيرة التي تمت دراساتها. وهذه الحقيقة قد تؤكد الفرضية القائلة ان المستعمرات الصغيرة هى الممرضة.

Summary

Protein bands separated from some colonies of strains GL19 and GF19 were easily visualised on SDS-PAGE, when stained with Coomassie-blue Stain. Western-blot analysis of soluble proteins was done in two experiments:

In the first experiment, the analysis was carried out on members of the *Mycoplasma mycoides* cluster. A distinct protein band was found between molecular weights 7.6 and 18 KDa, in the three colonies of GL 19 and it was completely absent in all other members of the *M. mycoides* cluster. Therefore, Western-blot analysis is strongly recommended as an identifying test for *M. capricolum* subsp. *capripneumoniae* as well as a differential diagnostic test for CCPP. Moreover, the protein bands of three GL 19 colonies showed 35-50 identical distinct protein bands, the majority of them were between molecular weights 45.2 and 132 KDa, while only 2-4 distinct bands were produced by the other members of the *M. mycoides* cluster and they were approximately at molecular weights 71-91 KDa. The second experiment compared some of the large and small colonies of strains GL 19 and GF 19. A degree of difference between the two types of colonies was established, confined mainly to quantitative variation of protein bands. The large colonies from both strains provided more deviating patterns and a protein bands of molecular weights of 35.1 to 132 KDa while most of the protein bands from the small colonies were between 45.2 and 91 KDa.

TritonX-114 phase partitioning was employed as first step towards identifying hydrophobic surface constituents of *M. capricolum* subsp. *capripneumoniae* strain GL19 which confirmed the characteristic strain features of the small colonies found with whole cell protein in Western-blot analysis. Virtually all of the proteins enriched in the hydrophobic phase exhibited immune reactivity in Western-blot analysis, were actually the same immunogenic proteins (responsible for adherence) of the small colonies of the candidate strain, a fact that might confirm the assumption that the small colonies are the pathogenic types.

Introduction

Mycoplasmas are considered to be host specific. Goats harbour a number of species (DaMassa *et al*., 1992), the most important of them is *Mycoplasma capricolum* subsp. *Capripneumoniae*, the causal agent of contagious caprine pleuropneumonia (CCPP) and belongs to the "*Mycoplasma mycoides* cluster". The following six mycoplasmas designated as species or subspecies are included into the cluster: *M. mycoides* subsp. *mycoides* biotype sc, *M. mycoides* subsp. *mycoides* biotype lc, *M. mycoides* subsp. *capri* (MC), *M. capricolum* subsp. *capricolum* (CC), *M. capricolum* subsp. *capripneumoniae* (MCCP)

and *M. sp.* group 7 of Leach (B7). These are significant animal pathogens (Erno, 1987), closely related, serologically cross-reactive and have similar biochemical features (Cottew *et al.*, 1987; Erno 1987).

It has become apparent that many mycoplasma species possess membrane surface protein antigens that vary in their expression (phase-variation), structural features and size-variation (Behrens *et al.*, 1994; Rosengarten *et al.*, 1994; Wise *et al.*, 1993; Yogev *et al.*, 1994). These are the tools of pathogenesis of all *mycoplasma* infections. However, little is known about the mechanism involved in the pathogenesis of *M. capricolum* subsp. *capripneumoniae*. Therefore, further investigations directed towards identifying and characterising this agent's major antigen, are urgently needed highly to provide a basis for future work on its pathogencity factors and molecular mechanisms. Furthermore, this could help setting up diagnostic criteria that distinguish between strains of high and low virulence.

This study was undertaken to examine the membrane protein antigens of *M. capricolum* subsp. c*apripneumoniae*, to transfer (blotting) proteins on nitro-cellulose membrane, and to identify the hydrophobic proteins of the lipid bilayer by antibodies (Western blot-analysis) and to immunoblotting .

Materials and Methods

SDS-PAGE five ml cultures of morphological different colonies of GL 19 and GF19 strains representing *M. capricolum* subsp. *capripneumonae* were used in this experiment. Each culture was centrifuged, washed and mixed with the loading buffer (1:1v/v). The mixture was heated at 95°C for 5 min.

The sample and standard protein weight marker were loaded into 9% . Electrophoresis was done at 150 volt or 30mA for mini gel or 25mA for big gel. The gel was stained with coomassie blue for 30 min.

Western blot analysis:

Cultures from different clones of strains GL19 and GF19 and from each of the other 5 members of the "*M. mycoides* cluster", were included in the test.

Two experiments were run, to compare the members of the *M. mycoides* cluster with GL19 and GF19. The proteins from each member of the *M. mycoides* cluster and clonies of strain GL 19 representing *M. capricolum* subsp. *capripneumoniae*, were prepared

and separated on SDS-PAGE (Experiment 1). Proteins from morphologically different colonies of strains GL19 and GF19 were also separated on SDS-PAGE (9%). The separated protein bands were transferred to nitrocellulose membrane (82 mm diameter, 0.45 µm pore size). The blot was run for a minimum 1 hr with 100 V. Rabbit hyperimmune serum (primary antibody 1:1000) was used against *M. capricolum* subsp. c*apripneumoniae* and anti-goat secondary antibody conjugate (1:2000). ABTS (2.2azin) substrate was used.

Triton X-114 phase fractionation:

Broth cultures form two different colony sizes of strain GL19 were used. One to two mg of mycoplasma proteins were obtained by centrifugation of one ml culture (10^7-10^9 CFU/ml) of each colony size. The pellets were washed 3 times with PBS/ PMSF solution [PBS containing 0.5mm protease inhibitor (Phenyl methyl sulfonyl fluoride, PMSF].

The pellet from each colony size was suspended into 1000 µl cold 1% triton X-114, incubated for 2-4 hrs at 4°C followed by centrifugation. Triton insoluble pellet was transferred to a new tube, then incubated at 37°C for 5 min, followed by centrifugation. The type aqueous phase (A9-phase) was transferred to a new tube, while the triton X-phase (TX-phase) was left.

Some practical factors must be taken into consideration. Having a sufficient detergent concentration to solublise reasonable quantities of mycoplasmal components, the following were done 1) Establishing a volume ratio of the partitioned phases that allows efficient re-extraction (washing) of TX phase. 2) Maintenance of a constant volume of TX phase during sequential washing steps. 3) the concentration of the TX phase after partitioning should be approximately 10-11% w/v (Bordier, 1981).

Purification of the TX-Phase:

900 µl ice cold PBS/ PMSF was added to the TX-Phase, vortexed and incubated on ice for 5min; then incubated at 37°C for 5 minutes and centrifuged at RT/ 8000g for 5 min. The supernant was discarded. This step was repeated 4 times; followed by addition of 900 µl ice cold PBS/PMSF, vortexed and incubated on ice for 5 minutes and centrifuged at 4°C / 12000 g for 5 minutes. Triton-insoluble pellet for suspended in a new tube, incubated at 37°C for 5 minutes, and then centrifuged at RT/8000 g/5 min. The supernatant was then discarded.

Methanol Precipitation of the TX-Phase:

900 µl cold methanol (-20°C) was added to the TX-Phase, incubated overnight at -80°C, centrifuged at 4°C/12000 g/10 min and the methanol was then discarded and the pellet suspended in loading buffer and used for loading SDS-PAGE gel.

Purification of the AQ-Phase: 100µl ice cold 10% TX-114 was added to the 900 µl AQ-Phase, vortexed, incubated on ice/5 min, incubated at 37°C for 5 min and centrifuged at RT/8000 g for 5 minutes. The upper AQ-Phase was transferred into a new tube. All above steps were repeated 4 times. four parts AQ-Phase + one part 5x loading buffer was used for loading SDS-PAGE gel.

Results

SDS-PAGE:

Protein bands separated from some morphologically different clones of the strains GL19 and GF19 were easily visualised on SDS-PAGE gel, when stained by Coomassie-blue (Fig. 1).

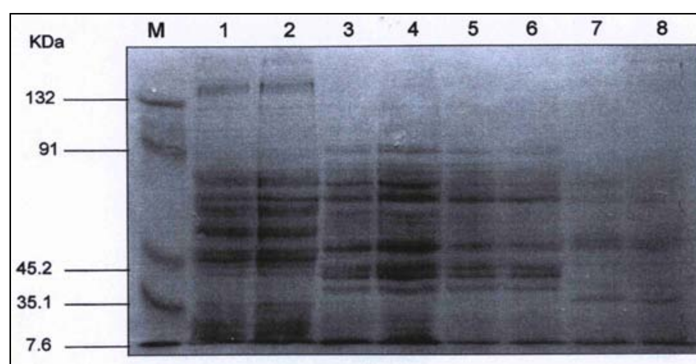

Fig. 1: Protein profiles of Mycoplasma strains GL19 & GF19 Stained with Coomassie Brilliant Blue. Lane M= Molecular marker; lanes 1and 2

large colonies of GL 19; lanes 3, 5 and 6 small colonies of GF 19; lane 4 a large colony of GF 19 and lanes 7 and 8 small colonies of strain GL 19.

Western-blot analysis:

Using the Mycoplasmas of "*M. mycoides* cluster", the protein bands of three clones from strain GL19, produced 35-50 distinct bands. Major part of them lay between molecular weights 35.1 and 132 KDa. Distinct protein band KDa was seen between molecular weight 7.6 and 18 which was completely absent in all other members of the "*M. mycoides* cluster**"**. Two to four distinct bands were produced by the other members of the "*M. mycoides* cluster" lied approximately at molecular weights 71 and 91 KDa. (Fig.2). Morphologically, the different colonies from strains GL19 and GF19, showed interesting results; large colony from the candidate strains revealed identical pattern of protein bands obtained from 3 clones of GL19, used with the other members of the "*M. mycoides* cluster" as seen in Fig. (3), while small colony (SC) from both candidates produced intensive bands with a molecular weight 45.2-91 KDa (Fig. 2; 3).

Triton X-114 phase partitioning:

The hydrophobic membrane protein fractions of strain GL19 as prepared by TX-114 phase partitioning, and analysed by SDS-PAGE (TX-Phase), confirmed the characteristic feature of the small colonies strain whole cell protein in Western-blot analysis.(with molecular weights of 35.1-91 KDa), while protein with the hydrophilic characteristics (AQ-Phase), were of molecular weights (35.1-132 KDa.) similar to those of LC from the two candidate strains seen in Western-blot analysis (Fig. 4).

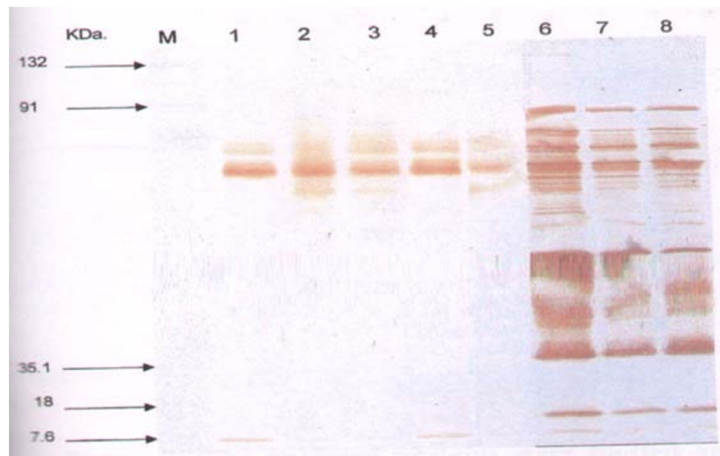

Fig. 2: Western-blot analysis of M. mycoides cluster antigen stained with hyperi-mmune serum of GL 19 strain. Lane M molecular marker; lane 1 *M. mycoides* subs. *mycoides* (SC); lane 2 *M. mycoides* subs. *mycoides* (LC); lane 3 1 *M. mycoides* subs. *mycoides capri* (MC); lane 4 *M. mycoides* subs. *capricolum* (CC); lane 5 Mycoplasma group 7 of each (B7) and lanes 6, 7 and 8 colonies from GL 19 strain.

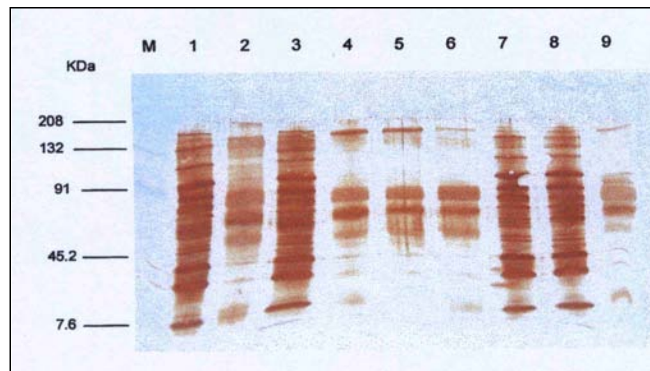

Fig. 3: Western-blot analysis comparing morphologically different colonies from strains GL19 and GF19. Lane M= protein Marker; Lane 1,3,7 & 8= GL19 (large colonies); Lane 2,4,5,6 & 9: colonies from GF19 (small colonies). The analysed proteins were blotted on nitro-cellulose membrane and visulaized by staining with the hyperimmune serum raised against strain GL19 (MCCP)

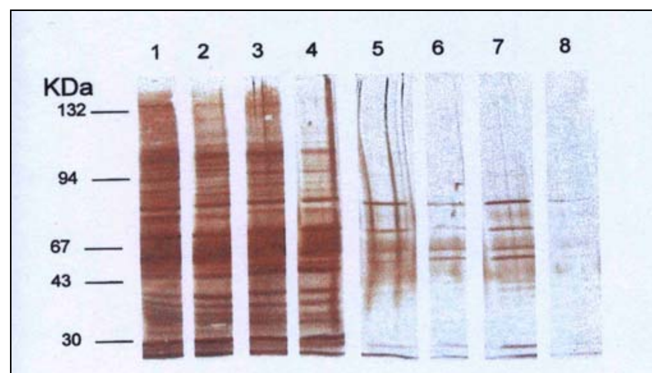

Fig. 4: Western blot. analysis of hydrophobic and hydrophilic proteins of GL19 strain fractionated by Triton X 114. Lanes: 1,2,3,4 =AQ-Phase of the 4 colonies; Lanes= 5,6,7,8 = TX-Phase of the same 4 colonies, probed with the hyperimmune serum raised against strain GL19 (*MCCP*).

Discussion

The proteins of the six members of the "*M. mycoides* cluster", analysed by the SDS-PAGE, and immunoblotted on nitro-cellulose paper, were compared after staining with hyperimmune serum raised against strain GL19. As shown from the results, 20-35 immunogenic protein bands were obtained from MCCP strain GL19, with the homologous rabbit hyperimmune serum. This result is in agreement with those of Rodwell (1982) and Hans *et al*. (1984). This hyperimmune serum lane 3 and 4 reacted strongly with proteins of the other members of "*M. mycoides* cluster". In addition, these results coincide with the findings of Olsson *et al*. (1990) and Kanyi Kibe *et al*., (1985), who found that four major protein antigens were shared between MCCP, SC, LC, MC and bovine group 7.

The reaction of this hyperimmune serum with strain GL19 (MCCP), revealed a distinct protein band present between molecular weight 7.6 and 18 KDa, which was completely absent in all other members of the "*M. mycoides* cluster". Such a finding was not recorded before and can be used for differential diagnosis of MCCP and hence the diagnosis of contagious caprine pleuropneumonia (CCPP). Moreover, the hyperimmune serum used revealed a protein band between molecular weights 18 and 35.1 KDa, from the analysis of the reference strain of *Mycoplasma* bovine group 7, which was not detected in the homologous strain. This may indicate heterogeneity among the field GL19, a phenomenon which was previously found within strains of MCCP and *M. capricolum* ,CC (Costas *et a*l., 1987).

Despite the close serological relationship between *M. mycoides* subsp. *mycoides* LC and *M. mycoides* subsp. *mycoides* SC (Rodwell, 1982), immuno-blotting has indicated that, LC is more related to *M. mycoides* subsp. *capri* (MC) than to SC. This finding was also reported by Rodwell (1982) where he used (1-D) SDS-PAGE and Olsson *et al.* (1990) with (2-D) SDS-PAGE in combination with immunoblotting. Nevertheless, LC and SC could not be clearly distinguished as two separate groups in this study; such result is in agreement with the findings of Leach *et al.* (1989) and Costas *et al.* (1987). From our immunoblotting, it was difficult to find a difference between the pattern of *M. capricolum* (CC) and SC. This finding was observed by Olsson *et al.* (1990) and confirms the relationship between CC and SC reported by Rodwell (1982).

The analysis with the SDS–PAGE has indicated that inter-species heterogeneity within the strains of MCC, investigated in this study, is mainly confined to quantitative variations of certain protein bands. Variations of electrophoretic protein patterns within a given species are known for many prokaryotes and have also been reported within "*M. mycoides* cluster" (Costas *et al.*, 1987), M. *bovis* (Sachse *et al.*, 1992) and *M. gallisepticum* (Khan *et al.*, 1990). Costas *et a*l. (1987) have decreased the some mentioned heterogeneity in protein patterns of MCCP and CC, when they used a computerised numerical analysis of SDS-PAGE for several strains of "*M. mycoides* cluster".
For an assessment of the scope of interspecies antigen variability, we compared whole-cell protein SDS-PAGE and the immunobloted patterns for the GL19 and GF19 strains. The main conclusion is that antigenic variation within these strains is mostly confined to quantitative differences in the concentration of certain antigen clusters, which varied between individual clones. It can be assumed that this phenomenon is part of a versatile and complex mechanism of surface antigen variation of mycoplasmas (Rosengarten and Wise, 1991). TritonX-114 phase partitioning of *M. capricolum* subsp. *capripneumoniae* was employed as first step towards identifying hydrophobic surface constituents, and not to compare clones of the two strains, thus we used clones of the strain GL19 only. Comparison suggests that virtually all of the proteins enriched in the hydrophobic phase exhibited immuno- reactivity in Western-blot assays, were actually the same immunogenic proteins (responsible for adherence) of the small colonies of the two candidate strains. This finding, infact,

confirms the assumption that the small colonies are the pathogenic types. On the other hand, the information provided by a protein pattern is quite extensive with regard to all major antigens, thus making whole-cell protein analysis a suitable tool for research in the field of epizootiology, pathogenesis and immunology.

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