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Culture filtrate proteins of *Dermatophilus congolensis*

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Lors d'études antérieures sur les antigènes de *Dermatophilus congolensis*, très peu d'attention a été accordée aux hyphes et aux produits d'excrétion/sécrétion (PES) des bactéries en croissance active. Nous avons cultivé quatre isolats de *D. congolensis* dans un milieu liquide synthétique, sans sérum, à base de RPMI 1640. Les PES ont été préparés à partir du liquide de culture infectée, par diafiltration et ultrafiltration. Ces méthodes ont produit des quantités de PES suffisantes pour étudier les profils de polypeptides des quatre isolats par SDA-PAGE et Western immunoblotting. Les quatre isolats ont produit un grand nombre de polypeptides en culture, dont la plupart étaient produits par tous les quatre. Néanmoins, des polypeptides propres à chaque isolat étaient également produits. Des études par Western immunoblotting sur des sérums mélangés d'animaux du Ghana affectés de façon chronique, ont montré que certains polypeptides dans les PES d'un isolat du Ghana étaient antigéniques. Lorsque les mêmes sérums ont été testés sur les PES d'un isolat écossais, certains polypeptides du même poids moléculaire que ceux dans l'isolat ghanéen, ainsi que d'autres de poids moléculaires différents, ont été reconnus. Ceci indique que des isolats de *D. congolensis* de régions géographiques différentes produisent des PES avec des déterminants antigéniques communs.

Mots clés : *Dermatophilus congolensis* - Protéine - Technique de culture - Polypeptide.

INTRODUCTION

Dermatophilus congolensis is a Gram positive actinomycete bacterium which causes the skin disease dermatophilosis. It has a worldwide distribution, and a wide host range including a wide variety of animal species and man.

D. congolensis has a multiphasic-life cycle which was described by ROBERTS (8) and ABU-SAMRA (1). Infective zoospores or cocci give rise to branching hyphae which undergo longitudinal and transverse divisions to produce filaments containing cocci. The branching hyphae invade the living non-cornified layer of the epidermis, and may be regarded as the proliferative stage of the bacterium. The role and significance of hyphae and their excreted-secreted products (ESP) in the immunology and pathogenesis of this disease are yet to be fully determined. Apart from the investigation of exo-antigens

by KWAPINSKI (5), no detailed, systematic studies on hyphae and ESP of this organism have been carried out. However, several different approaches and media have been adopted in research on various *D. congolensis* antigens.

The main objective of this work was to study the protein composition of ESP produced by actively growing *D. congolensis* in a defined synthetic serum free medium by the use of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblotting. We have attempted to characterize and compare individual isolates from different geographical regions by examining their protein profiles. We have tested sera from chronically infected cattle for the presence of antibodies against ESP of two isolates to show that unique and cross-reactive antigens occur in these isolates.

MATERIALS AND METHODS

D. congolensis isolates

Four isolates of *D. congolensis* were used in this study (table I). They were recovered from naturally infected cattle and sheep. They had been passaged in blood agar and broth culture twice in our laboratory.

TABLE I Origin of *D. congolensis* isolates used in this study.

Isolate	Host	Origin
A5N	Sheep	Scotland, U.K.
Gh89	Cattle	Ghana, Africa
Zambia	Cattle	Zambia, Africa
Paynters, Antigua	Cattle	Antigua, West Indies

Growth conditions and preparation of ESP

Stabilates of the isolates were grown, without shaking, in 75 cm² non-inhibitory tissue culture flasks (Nunc) containing 15 ml of wholly synthetic serum free liquid culture medium [RPMI 1640, 0.05 % sodium pyruvate, 0.025 % sodium metabisulfate and 0.001 % ferrous sulfate] at

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37 °C in air containing 5-10 % CO₂ for 48 h. The ESP were collected from infected culture fluid of each *D. congolensis* isolate. Phenyl methyl sulphonyl fluoride (PMSF) and ethylene diamine tetra acetic acid (EDTA) were added at 1 mM and 5 mM final concentration, respectively. The culture fluid was centrifuged at 3,000 x g for 15 min at 4 °C to remove bacteria. The supernatants were filtered through a 0.45 µm Millipore low protein binding filter and then desalted and washed three times, by diafiltration, with 3 volumes of phosphate buffered saline (pH 7.4) containing PMSF and EDTA. Finally the supernatants were concentrated by ultrafiltration and stored at -20 °C until they were used. Millipore membranes with a 10,000 dalton cut-off were used for diafiltration and ultrafiltration.

Polyacrylamide Gel Electrophoresis (PAGE)

Separation of proteins in the ESP according to their molecular weight was achieved using 1.5 mm thick slab gels. A 4.5 % stacking gel, and a 7-20 % gradient separating gel were used. Samples were boiled for 5 min in sample buffer, containing 0.1 % dithiothreitol, 10 % SDS and a trace of bromophenol blue, and loaded into each gel track. Molecular weight markers (6-200 kDa) were run on the same gel. Electrophoresis was carried out at 100 volts overnight. Gels were fixed in ethanol and acetic acid. Several different ESP preparations of each isolate were prepared at different times and each preparation of ESP was run on at least 3 occasions.

Western immunoblotting

Proteins from SDS-PAGE gels of the A5N and Gh89 isolates were electrotransferred onto nitrocellulose membranes (0.45 µm pore size) using a Bio-Rad semidry transfer cell. The gels were soaked in transfer buffer for a few minutes beforehand. Electrophoresis was carried out at 200 mA for 90 min. After transfer the nitrocellulose membrane was blocked with 5 % dried milk powder in buffer overnight. The membrane was then divided as necessary, and probed overnight with gentle shaking at room temperature in a 1:50 dilution of pooled chronic sera from 9 dermatophilosis infected cattle from Ghana, West Africa. The membranes were washed 3 times for 15 min each with PBS-0.01 % Tween. The strips were screened for antibody binding by incubation with biotin conjugated goat anti-bovine IgG, the strips were then washed in PBS/Tween 3 times, 15 min/each. The blots were then incubated in streptavidin alkaline phosphatase in 4 % normal goat serum for 1 hour at 37 °C. Blots were washed again in PBS/Tween and incubated in the substrate bromo-chloro-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) to visualize the reaction.

RESULTS

The procedures for desalting, washing and concentrating the culture fluid and the use of protease inhibitors were necessary for detailed electrophoretic analysis of proteins in ESP. Preliminary results using untreated culture fluid were poor and desalting by dialysis followed by freeze-drying produced indistinct protein bands. The results shown here are all of samples that were treated by diafiltration and ultrafiltration. As shown in figures 1 and 2, it is evident that the isolates used in this study produced excreted-secreted products (ESP) during their growth in supplemented RPMI 1640. The ESP obtained by this method were satisfactory for the purposes of characterizing *D. congolensis* isolates by their SDS-PAGE polypeptide profiles and for testing their antigenicity by Western immunoblotting. There was a satisfactory reproducibility between different ESP preparations of each isolate. The different isolates produced a number of peptides of the same molecular weight and several that were unique to each isolate.

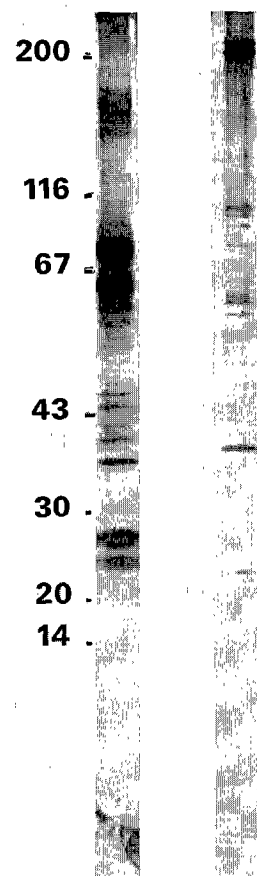


Figure 1 : The ESP of a Scottish isolate (lane 1) and a Ghanaian isolate (lane 2) separated by SDS-PAGE. The Gh89 ESP was diluted to reveal the bands above the 200 kDa marker. Molecular weight standards are shown on the left.

Figure 2 shows the polypeptide profiles of the ESP of the four *D. congolensis* isolates. The points of note are that bands shared by all four isolates were numerous between 15 and 70 kDa, for example the bands at 25, 29, 36, 44, 66 and 70 kDa.

The Ghanaian isolate, Gh89 in lane 2 of figure 1 had double bands with molecular weight of more than 200 kDa, and in lane 3 of figure 2 had bands at 94, 80 and 82 kDa bands that were not found in any of the other 3 isolates. The Scottish isolate, A5N in lane 1 of figure 1 and lane 2 of figure 2 had unique bands at 120, 84, 76, 70 and 58 kDa.

The Antiguan and Zambian isolates in lanes 4 and 5 of figure 2 respectively had very similar profiles as detailed by the silver staining ; the only unique bands were at 67 kDa for the Antiguan isolate and the 69 kDa in the Zambian isolate.

Figure 3 shows the Western immunoblotting results of ESP of Gh89 (lane 1) and A5N (lane 2) tested using pooled chronic cattle sera. The sera contained antibodies against a number of antigenic bands in the ESP of both of the isolates for example bands at 25, 27, 33 and 47 kDa were antigenic and are common to both isolates. The difference between them being that in the Gh89 ESP one

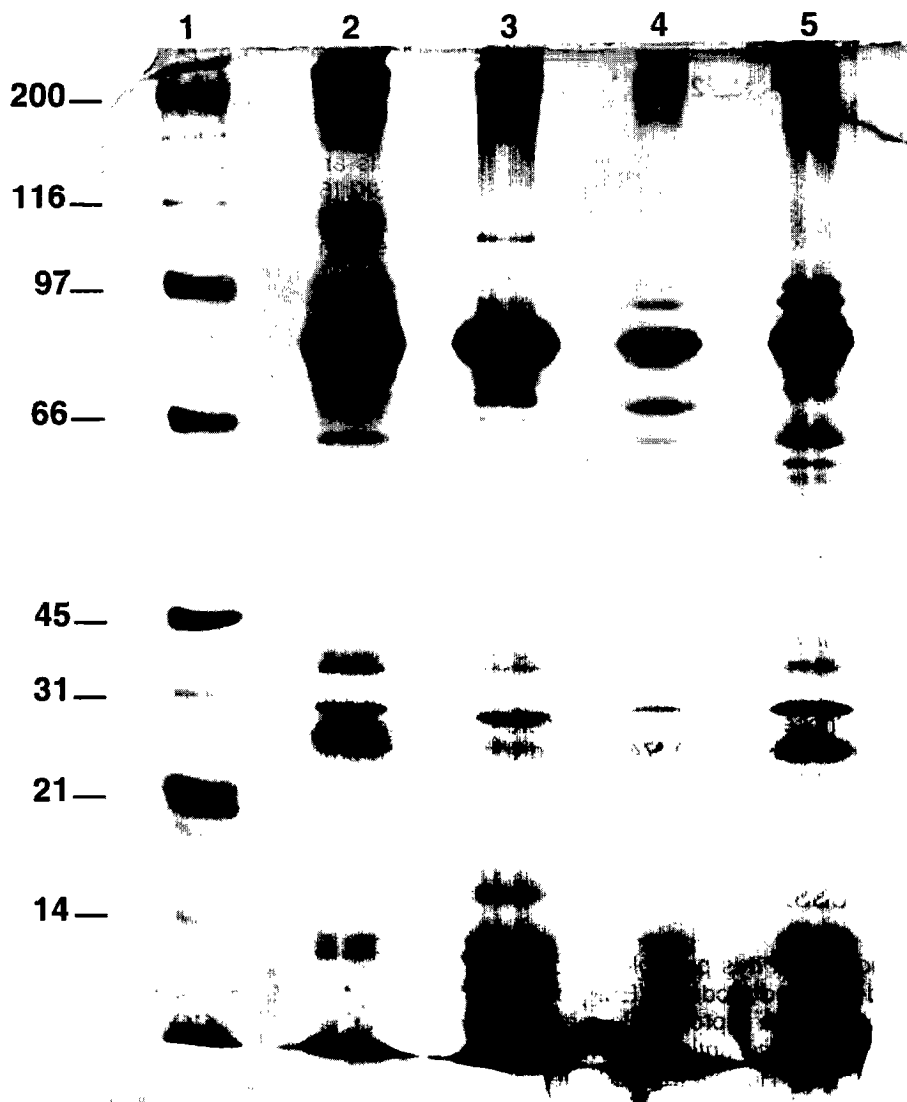


Figure 2 : The ESP of four isolates separated by SDS-PAGE. Lane 2, Scottish ; lane 3, Ghanaian ; lane 4, Antiguan ; lane 5, Zambian. Molecular weight standards are shown on the left.

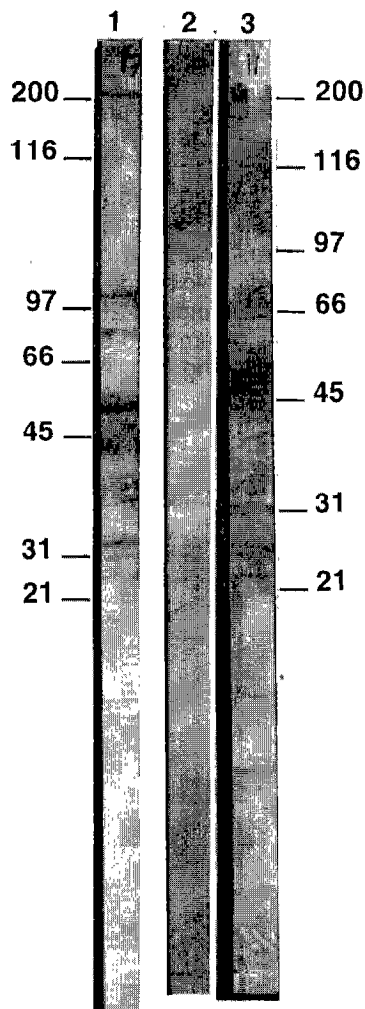


Figure 3 : Western immunoblot of ESP of Ghanaian (lane 1) and Scottish (lane 3) isolates both probed with pooled sera from chronically infected cattle from Ghana. The 2 lanes were taken from different blots, the molecular weight standards are shown to the left and right. Lane 2 is an example of a control using ESP from the Scottish isolate probed with commercial newborn calf serum.

band above 200 kDa, and those at 91 and 59 kDa were antigenic and in A5N ESP bands at 120, 74, 70 and a large band at 52 kDa were antigenic.

DISCUSSION

In this study we have shown that it is possible to collect and concentrate excreted-secreted products (ESP) of different *D. congolensis* isolates in a reproducible manner. Researchers working *D. congolensis* antigens have referred to their complexity (5, 7, 10). However there have not been any attempts to pursue their characterization and there has not been a common approach to antigen preparation. We have attempted to overcome some of these

problems by cultivating this organism under controlled conditions in a synthetic serum free liquid culture medium, the main component of which is standardized and commercially available. The medium may be suitable for a wide range of studies on the biology of *D. congolensis*.

Other researchers working on ESP of actinomycetes, such as FIFIS et al. (3), have used prolonged periods of cultivation in which some extracellular products may be degraded. We have overcome this problem by using a short period of culture, by the addition of protease inhibitors to all stages of protein preparation, minimising the time taken to concentrate the ESP and keeping the temperature of the solutions low. Diafiltration and ultrafiltration as a method of ESP preparation, is superior to dialysis and concentration by freeze drying.

All four isolates of *D. congolensis* studied produced a number of proteins in their ESP, the majority of which are common to all isolates. However, we have also observed differences in the polypeptide profiles of the isolates. Our results are therefore in agreement with those of KWA-PINSKI (5) and the recent studies of MAKINDE (6), HOW and LLOYD (4) and SUTHERLAND et al. (9) that there is more than one strain of this organism.

Our Western immunoblotting results illustrate that in sera against one of those isolates there were antibodies to common antigens of the same molecular weights in both isolates. In addition there were bands in both isolates that were unique, indicating that A5N has antigenic determinants in common with Gh89, but they occur on peptides of different molecular weights. It can be concluded that the ESP of isolates probably have both shared and common antigens. If the unique antigens are host protective then this could be one reason why attempts to produce an effective vaccine against dermatophilosis have not been successful.

Proteins secreted by many pathogens play an important role in virulence and tissue invasion as they can be toxins or enzymes. Antibody production is often directed against these proteins. Growing mycobacteria have been shown to release proteins into their surroundings and there is evidence that these antigens evoke protective T-cell responses (2). Therefore it is of considerable importance to determine the role and significance of hyphae and ESP in the immunology of *D. congolensis* infections.

CONCLUSION

Four isolates of *D. congolensis* from different geographical regions were grown in a serum free synthetic culture medium based on RPMI 1640.

Diafiltration and ultrafiltration were used to desalt, wash and concentrate culture fluid containing excreted-secreted products (ESP) of each isolate. Proteins in the ESP were separated by SDS-PAGE and tested for antigenicity by immunoblotting with pooled sera from chronically infected cattle.

The excreted-secreted products of the four isolates contained a large number of polypeptides. Many of these were produced by all four isolates. Other polypeptides appeared to be uniquely produced by only one isolate.

Pooled chronic sera contained antibodies against a number of ESP polypeptides from the isolate the animals were exposed to and from an isolate from a different geographical area. This indicates that ESP of different isolates have shared antigenic determinants.

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In previous studies on the antigens of *Dermatophilus congolensis* very little attention has been given to the hyphae and to excreted-secreted products (ESP) of actively growing bacteria. In this study we have grown four isolates of *D. congolensis* in a serum free synthetic liquid culture medium based on RPMI 1640. Diafiltration and ultrafiltration were used to prepare ESP from infected culture fluid. These methods produced sufficient quantities of ESP that the polypeptide profiles of the four isolates could be examined by SDS-PAGE and Western immunoblotting. The four isolates produced a large number of polypeptides in culture, most of which were produced by all four isolates. However each isolate produced polypeptides that were unique to it. Western immunoblotting studies using pooled sera from chronically affected animals from Ghana showed that a number of polypeptides in ESP of a Ghanaian isolate were antigenic. When the same sera was tested against ESP from a Scottish isolate a number of polypeptides of the same molecular weight as those in the Ghanaian isolate and some at different molecular weights were recognized. This indicates that isolates of *D. congolensis* from different geographical areas produce ESP with shared antigenic determinants.

Key words : *Dermatophilus congolensis* - Protein - Culture technique - Polypeptide.

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EL JACK (M.A.), AMBROSE (N.). Cultivos de filtrados proteínicos de *Dermatophilus congolensis*. *Revue Elev. Méd. vét. Pays trop.*, 1992, **46** (1-2) : 257-261

En estudios anteriores sobre los antígenos de *Dermatophilus congolensis*, se ha dado poca importancia a las hifas y a los productos de expresión y secreción (ESP) de las bacterias en crecimiento activo. En el presente trabajo, se cultivaron cuatro muestras de aislamientos de *D. congolensis* en medios de cultivo no séricos, líquidos y sintéticos, basados en RPMI 1640. Para preparar los ESP, a partir de cultivos líquidos, se utilizó tanto la diafiltración como la ultrafiltración. Estos métodos produjeron suficiente cantidad de ESP como para permitir el examen del perfil polipeptídico de los cuatro aislamientos, gracias a la inmunotinción de SDS-PAGE y al Western blotting. El cultivo de los cuatro aislamientos produjo una gran cantidad de polipéptidos, la mayoría de los cuales fue producida por cada una de las cuatro muestras. A pesar de esto, también se observó que cada muestra produjo algunos polipéptidos propios. La tinción mediante el Western blotting de un pool de sueros provenientes de animales ganenses portadores de una infección crónica, mostró que varios de los polipéptidos de los ESP de las muestras ganenses presentaban características antigénicas. Cuando estos mismos sueros fueron sometidos a un examen contra ESP provenientes de un aislamiento escocés, se identificaron varios polipéptidos, tanto de mismo peso molecular que aquellos del aislamiento ganense, como de peso molecular diferente. Esto indica que los aislamientos de *D. congolensis* provenientes de diferentes zonas geográficas producen ESP con determinantes antigénicos comunes.

Palabras claves : *Dermatophilus congolensis* - Proteína - Técnica de cultivo - Polipéptido.