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PRELIMINARY STUDIES ON THE ANTIGEN CHARACTERIZATION OF BOVINE *BABESIA* SPECIES

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ABSTRACT

Studies on the antigen characterization of the various *Babesia* species were carried out using SDS-PAGE gel electrophoresis and Western blotting. The SDS-PAGE gel electrophoresis revealed several antigens of *Babesia* species separated according to their molecular weights. Notably, 3 plumpy bands were observed on all the *Babesia* species at molecular weights of 18-22, 50-55 and 60-64 KDa. Some common bands were observed between the *Babesia* species and the clean red blood cells. After immunoblotting the separated antigens with the positive bovine serum of *B. bigemina* (African), immunoreactive bands at certain molecular weights were illustrated: *B. bigemina* (Mexican) at 50 and 55 KDa, *B. bigemina* (African stabilate) at 45, 50 and 55 KDa, *B. bigemina* (African culture form) at 45 and 65 KDa and *B. bovis* at 20.1 KDa. The identification of the immunoreactive antigens provide information for which the production of monoclonal antibodies could be targetted. This could also be useful for the immunodiagnosis of the various isolates or strains of the different *Babesia* species

KEY WORDS: Antigen Characterization, Bovine *Babesia* species, Immunodiagnosis

Introduction

In Africa, *Babesia bovis* and *B. bigemina* are the two species of *Babesia* reported in cattle which are transmitted by *Boophilus microplus* and *Boophilus decoloratus* (Ristic, 1988). The distribution of *Babesia* is totally dependent on the respective distribution of these *Boophilus* species. These two *Babesia* species found in cattle cause bovine babesiosis.

Bovine babesiosis is manifested by fever, anaemia, occasional haemoglobinuria and the appearance of infecting protozoa in the erythrocytes of cattle. Babesiosis persists as one of the most important diseases of cattle in all tropical and semi tropical regions of the world where most developing countries are situated. Majority of the 1.2 billion cattle in the world are potentially exposed to one or more *Babesia* species (McCosker, 1981). Tick borne diseases, particularly babesiosis , constitute major constraints in the expected performance of these animals, thus limiting availability of animal protein for human consumption and potential supply of products for international markets. Babesiosis has caused serious loss of hides, meat and milk production in cattle (Beltran, 1975).

In many parts of Africa including Nigeria, diseases of cattle other than babesiosis which had serious impact on development of livestock include rinderpest, trypanosomiasis and contagious bovine pleuropneumonia. As these diseases are gradually coming under effective control, tick-borne diseases of cattle including babesiosis become more important. Babesiosis has not been given adequate attention in Nigeria in spite of the high relative abundance of ticks infecting this group of livestock.

Recent studies on antigen characterization of various *Babesia* species include those of Ali *et al.* (1993), Duzgun *et al.* (1991) and Figueroa *et al.*(1992) and

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Palmer *et al.*(1991). Proteins of *Babesia equi* piroplasm isolated from infected horse erythrocytes were characterized using the two dimensional Sodium Dodecyl Sulphate (SDS) polyacrylamide gel electrophoresis (Ali *et. al.*, 1993)

Because bovine babesiosis tend to develop resistance to drugs (Kuttler,1981, Fulton & Yorke,1941), it is necessary to carry out a preliminary antigen characterization so as to provide information on the molecular weight of antigen binding sites to antibodies. These immunoreactive antigens could be inoculated into experimental animals for the production of monoclonal antibodies. Antigen characterization is also a useful tool in the immunodiagnosis of the various isolates and strains of *Babesia* species.

MATERIALS AND METHODS

Preparation of 10% SDS-polyacrylamide gel

This was prepared based on the formula given in the supply kits by Life Technologies (GibcoBRL). To 10.3ml of 40% (w/v) Acrylamide : Bisacrylamide (37.5 : 1), 10ml of Resolving Gel buffer concentrate, 19.3ml of Distilled water and 400μl (0.4ml) of 10% Ammonium persulfate were added. After pouring the resolving gel, it was allowed to set for one hour before the stacking gel was poured.

The stacking gel was prepared by adding 1ml 40% Acrylamide : Bisacrylamide, 9ml of stacking gel buffer and 50μl of Ammonium Persulfate. After pouring, the comb was immediately inserted to provide lanes for the flow of the antigens.

Preparation of antigens (Winger *et al.*, 1987)

Eggs and larvae derived from infected and uninfected engorged *Boophilus decoloratus* were loaded on the various lanes of the SDS-PAGE gel. They were earlier prepared by crushing 3mg of each sample in phosphate buffered saline (PBS). Equal volume of each sample in PBS and reducing buffer were boiled in a waterbath for 5 minutes to break the polypeptide linkage. The samples were then centrifuged for two minutes and the supernatant were loaded on the various lanes in the SDS-PAGE gel apparatus. This was run using the SDS-PAGE tank buffer for ionic exchange with a power packed equipment (Shandon, Southern, Vokam SAE 2761) at 60mA for 3 to 4 hours.

The kinetes were derived by draining the haemolymph of the engorged female infected ticks (at 3+ degree of infectivity) within 10 days of their dropping from the calf. Antigens of *Babesia* species were merozoite fractions isolated from the red blood cells after *in vitro* cultivation at high parasitaemia using methods described by Winger *et al.* 1987. The various species of *Babesia* cultured included *B. bigemina* (African), *B. bovis* (Kwanyange), *B. bigemina* (Mexican), *B. bigemina* (African stabilate) and *B. divergens*

The clean red blood cells derived from calf C59 obtained from the Central Veterinary Farm, Weybridge, Surrey was further prepared for the 10% SDS-PAGE gel electrophoresis using methods described by Winger *et al.* 1987. This calf was isolated and maintained in this farm to ensure that it was devoid of any infection throughout the duration of this study. Blood from this calf provided clean red blood cells for loading on the SDS-PAGE gel.

Immunoblotting technique.

The antigens derived from the kinetes and the species of *Babesia* were loaded accordingly on the gel and transferred to a single nitrocellulose membrane by Western Blotting. The nitrocellulose membrane was reacted with positive antibodies of *B. bigemina* (African) positive bovine immune serum. The membrane was incubated in filtered 5% (w/v) non fat dried milk in Tris Buffered Saline (TBS) overnight. Johnson *et al.*(1984) recommended this as the best and least expensive blocking solution. This blocked the non specific binding sites thus preventing the binding of irrelevant proteins. On the next day, the nitrocellulose membranes were rinsed in TBS 3 times at 10 minutes interval in a shaker. The primary

antibodies containing the positive bovine serum of *B. bigemina* was reacted with the nitrocellulose membrane at 1 : 200 dilution in TBS. They were left on a Shaker at room temperature for one hour. It was then rinsed in TTBS (TBS with Tween) three times at 10 minutes interval in a Shaker.

The membrane was then incubated with secondary immunological reagent. Since primary antibodies incubated earlier was of bovine origin, the nitrocellulose membrane was incubated with Anti-Bovine IgG (Alkaline Phosphatase Conjugate) at 1 in 1000 dilution in TTBS. The incubation lasted for one hour at room temperature with gentle agitation on a shaker. The membranes were rinsed again three times in TTBS at 10 minutes interval in a shaker. The fourth wash was done with 0.15M Tris-Cl (pH 9.5) for 10 minutes. The nitrocellulose membrane was stained using the alkaline phosphatase colour reaction. Photographs were taken to provide a permanent record of the experiments.

RESULTS

Silver staining of SDS-PAGE protein profile of the *Babesia* species, the clean RBC, the kinetes and the infected and uninfected tick materials indicated the presence of antigens (protein bands) separated through the disruption of their polypeptide linkage by their relative molecular weights. Lanes 5-10 had over 18 protein bands each. In lane 3, 10 plumpy bands were noticed. Three plumpy bands were noticed in all the *Babesia* species at molecular weights of 18-22, 50-55, 60-64KDa. At least 5 common bands were observed between the *Babesia* species and the clean RBC (plate 1).

After immunoblotting the separated proteins with the positive bovine serum of *B. bigemina* (African), some immunoreactive bands were apparent. In lane 6, there was immunoprecipitation of the antigens and antibodies of *B. bigemina* (African stabilate form) at molecular weights of 45, 50 and 55KDa while in lane 7, the *B. bigemina* (African Culture form) was immunoprecipitated at molecular weights of 45 and 65KDa. In lane 8, *B. bigemina*

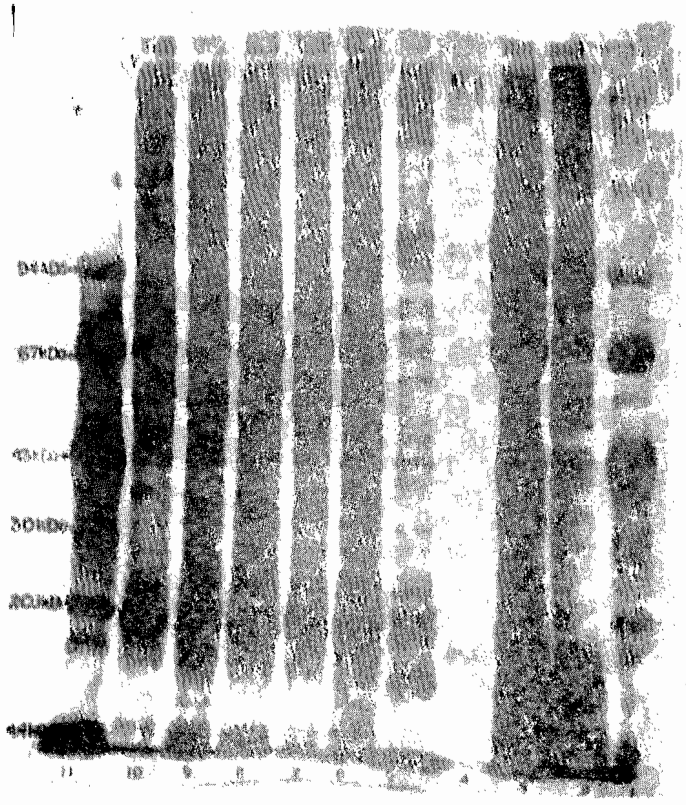

PLATE 1 : SDS-PAGE Protein Profiles of the various species of *Babesia*, the clean RBC, the kinetes and the infected and uninfected tick materials with silver staining. **Lanes 1 and 11** = Molecular weight markers, **Lane 2** = Uninfected tick materials, **Lane 3** = Infected tick materials, **Lane 4** = Kinetes, **Lane 5** = Clean Red Blood Cells, **Lane 6** = *B. bigemina* African stabilate, **Lane 7** = *B. bigemina* African culture forms, **Lane 8** = *B. bigemina* Mexican, **Lane 9** = *B. bovis* (Kwanyange), **Lane 10** = *B. divergens*

(Mexican) also had immunoreactive bands at molecular weights of 50 and 55KDa while *B. bovis* (Kwanyange) in lane 9 showed an immunoreactive band at 20.1 KDa.

A common band was found at the range between 17.5-19.5 KDa for all the *Babesia* species from lanes 6-10. Moreover, another common band was observed on all the *Babesia* species between 60-64 KDa. There was no immunoreactive band on *B. divergens*, kinetes and the clean RBC (plate 2).

DISCUSSION

The presence of the common protein bands on the SDS-PAGE gel on both the red blood cells and the *Babesia* species in the silver stained gel indicated that there was some level of contamination of the

merozoites with the red blood cells during their isolation process. Shimizu *et. al.*(1992) carried out SDS-PAGE analysis and found some little contamination of the merozoite fraction of *Babesia ovata* with its sheep erythrocyte component. After immunoblotting, the red blood cells did not show any band indicating that there was no reaction with the positive *B. bigemina* (African) serum. This showed that the red blood cells loaded on the SDS-PAGE gel were devoid of any contamination with pathogens especially *Babesia* species. This was used as a form of negative control since all the *Babesia* species were isolated from RBC.

B. bigemina (Mexican) had immunoreactive bands at molecular weights of 50 and 55 KDa, while *B. bigemina* (African Stabilate) had immunoreactive bands at molecular weights of 45, 50 and 55 KDa. This agrees with some of the bands of Duzgun *et al.*(1991) who carried out monoclonal antibodies binding to the surface of live Mexico isolate *B. bigemina* merozoite and found four parasite-encoded surface antigens at 36, 45, 55 and 58 KDa. The presence of the various immunoreactive bands on the various isolates of *Babesia* could be used as an index for the immunodiagnosis of these isolates. This is because different *Babesia* isolates possess specific parasite epitopes which primary antibodies can recognize.

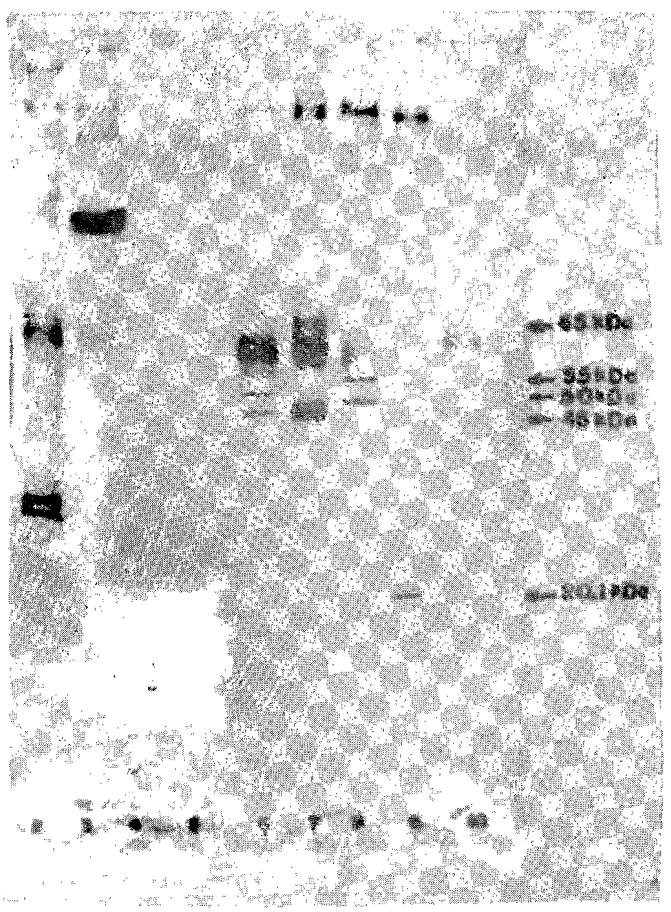

PLATE 2 : Western Blotting of the SDS-PAGE protein profiles of the various species of *Babesia*, the clean RBC, the kinetes and the infected and uninfected ticks. Lanes 2-10 were immunoblotted with the positive bovine serum of *B. bigemina* (African). **Lane 2** - Uninfected tick materials, **Lane 3** - Infected tick materials, **Lane 4** - kinetes, **Lane 5** - RBC, **Lane 6** - *B. bigemina* African stabilates, **Lane 7** - *B. bigemina* African culture forms, **Lane 8** - *B. bigemina* Mexican, **Lane 9** - *B. bovis* Kwanyange, **Lane 10** - *B. divergens*

After immunoblotting, a comparison of the banding pattern of *B. bigemina* stabilate and culture forms of the African isolates indicated that the culture forms lost some bands at the region of 50KDa. The stabilate forms of the parasites were obtained directly from the calves while the culture forms were grown in the laboratory for a longer period of time. It was either that the parasite no longer needed the protein or that the configuration of the protein changed during its maintenance in culture, such that the serum no longer recognised the protein (antigens) during the immunoprecipitation (Posnett and Metaferia, 1995, Personal communication). In the case of the stabilate forms of *B. bigemina* (African), the protein still maintained its integrity and did not alter its configuration since it came directly from the calf. The loss of the 50KDa band by *B. bigemina* culture forms seems consistent with changes in antigenic profiles resulting in some loss of epitopes during artificial media passages. Virulence properties of parental strain are often lost during this process.

There was no cross reaction of the positive *B. bigemina* (African) serum with the kinete stage of the parasite. This was probably because not enough amount of kinetes were loaded on the gel, due to the fact that it is extremely difficult to drain kinetes from tick haemolymph. The common protein bands at the regions of 60-64 KDa in the SDS-PAGE analysis were not parasite

specific. In other immunoblots involving the positive bovine serum of *B. bigemina* (African isolate), Posnett and Metaferia (1995), found the same bands at 60 - 64 KDa and concluded that they were not of parasite origin. These proteins were thus derived from the positive immune serum of the calf.

Future work by researchers would be aimed at extracting the antigens at their various molecular weights and injecting into laboratory animals to produce monoclonal antibodies. This would be very useful in the production of vaccines against bovine babesiosis that is becoming widespread and the pathogen resistant to chemotherapeutic agents such as tryphan blue, Imidocarb, Quinoline and Acridine derivatives.

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