

Full Length Research Paper

Phenotypic and genotypic characterization of isolates of *Dermatophilus congolensis* from cattle, sheep and goats in Jos, Nigeria

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Accepted 18 January, 2019

Dermatophilus congolensis is the causative agent of dermatophilosis an economically important disease of livestock, and also an agent of zoonotic importance. The disease has been reported worldwide, with a wide host range which includes domestic, wild and aquatic animals. This study was therefore undertaken to characterize isolates of the organism from cattle, sheep and goats in Nigeria. All the isolates, except two sheep isolates fermented glucose and sucrose. The whole cell protein profiles of the isolates were similar at about 62 and 20 KDa, but different at other levels. The polymerase chain reaction (PCR) of the partial 16SrRNA gene amplified all the isolates, but not other organisms included. The multiple sequence alignment of the PCR amplicons sequences showed an identity of between 98.5 and 100% across all the isolates. There was also a sequence similarity of between 99.2 and 99.76% between the isolates and the partial sequence of the type strain of *D. congolensis* DSM 44180T in the Genbank. Based on these techniques it may be concluded that all the isolates are the same with minor differences which were not enough to speciate them.

Key words: *Dermatophilus congolensis*, phenotypic, genotypic, sds-page, polymerase chain reaction, sequencing, multiple sequence analysis.

INTRODUCTION

Dermatophilus congolensis is the causative agent of dermatophilosis an economically important disease of livestock, and also an agent of zoonotic importance (Zaria, 1993; Burd et al., 2007). The disease has been reported worldwide, with a wide host range. Austwick (1958) and other researchers, concluded that only one species of the organism was responsible for the disease. This has however, not deterred scientists in their quest to find out if other species of this organism exist. (Masters et al. (1995) reported the identification of a new species isolated from chelonids in Australia.

In 1958 based on the work of (Austwick, 1958) and other researchers, it was decided that only one species of

the organism was responsible for the disease. This has however, not deterred scientists in their quest to find out if other species of this organism exist, (Masters et al., 1995) reported the identification of a new species isolated from chelonids in Australia. Similarly (Buenviaje et al. 2000), reported the isolation of a species from crocodiles and suggested that it should be considered as a new species.

The organism has been described as a Gram positive, pleomorphic, branching, filamentous, actinomycete with rows of coccoid cells transversing the entire length of the filament transversely and horizontally Chodnik (1956). Biochemically the organism isolated from different animal species has been found to have slight variations which are not consistent. Varying results have been reported by different researchers with maltose, sucrose, galactose and fructose and other reagents (Van Sacegham, 1934; Macadam and Haalstra, 1971; Gordon, 1976).

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Different researchers have tried to establish the protein profiles of isolates from different animal species with the aim of differentiating the isolates by the use of polyacrylamide gelelectrophoresis (PAGE) and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Gogolewski et al., 1992; Ellis et al., 1993; Masters et al., 1995; Kruger et al., 1998; Makinde and Gyles, 1999). The application of polymerase chain reaction in dermatophilosis research has been in detection of the organism (Buenviaje et al., 2000; Han et al., 2007). It has also been used to clone a serine protease gene, (Mine and Canegie, 1997). Larasa et al., (2002) reported the use of a simple Random polymorphic DNA genotyping method for field isolates of *Dermatophilus congolensis* and suggested that using this technique; they found genotypic variation between isolates, which correlated with host species. Larasa et al., (2002) also used other methods in their attempt to type isolates of *Dermatophilus congolensis* by evaluation of randomly amplified polymorphic DNA (RAPD) and Pulsed Field Gel Electrophoresis (PFGE) techniques for molecular typing of *Dermatophilus congolensis* and concluded that both methods were good for molecular typing. In this paper we report the use of traditional sugar characterization methods, sodium dodecyl sulphate polyacrylamide gel electrophoresis, PCR and DNA sequencing to characterize isolates of *D. congolensis* from cattle, sheep and goats in Jos, Nigeria.

MATERIALS AND METHODS

D. congolensis Isolates

Skin scabs from clinically infected Dermatophilosis animals (cattle, sheep and goats) in Jos metropolis mainly around the abattoir, were collected in clean bijou bottles, labelled and brought to the laboratory and used for the isolation of *D. congolensis*.

Cultural isolation

The organisms (*D. congolensis*), were isolated from the skin scabs collected as described by Haalstra (1965), with slight modification. Briefly the samples were pulverized and suspended in distilled water in Bijou bottles and incubated at 37°C for 45 min, under 10% CO₂. These were then brought out and a loop full from each suspension was then plated out on 10% blood agar containing 1000 iu /ml polymixin B. The plates were then incubated at 37°C for 48 to 72 h under 10% CO₂. Nine isolates, three each from cattle, sheep and goats were then inoculated into brain heart infusion broth in duplicates and kept for further studies.

Sugar fermentation

This was done as previously described (Cowan and Steel, 2004), in the following sugars Glucose, Fructose, Maltose, Galactose, Lactose, Sucrose, Xylose, Sorbitol, Mannitol and Dulcitol and incubated at 37°C and observed for five days for any activity by the organisms.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) preparation of whole cell proteins

Cultures of the isolates from the blood agar plates were harvested with a wire loop directly into eppendorf tubes and washed three times in phosphate buffered saline (PBS) pH 7.2 by centrifugation at 10,000 g for five minutes. The pelleted washed cells were suspended in sample treatment buffer (double working strength of sample buffer) containing 125 mM Tris- HCl, 4% SDS, 2% Mercaptoethanol, 20%W/V glycerol and then boiled for 10 min. The suspension was then centrifuged at 14,000 g for five min and the supernatant transferred to fresh Eppendorf tubes. Sample buffer (Laemmli, 1970) containing 2% SDS, 4% Mercaptoethanol, 10%W/V glycerol, 0.1% Bromophenol blue dissolved in 0.625 mM Tris HCl pH 6.8 was added to the supernatant and used as *D. congolensis* whole cell proteins.

SDS-PAGE gel electrophoresis

A discontinuous SDS- PAGE was performed with 4% stacking gel and a 12% separating gel. The various extracts were solubilized by boiling for 5 min at 100°C in Laemmli solution. The samples were loaded at 20 l (approximately 10 mg protein/ml as determined by Binchoninic protein assay) per lane and separated in 0.75 mm thick gel slabs in the mini protein 11Dual slab gel (Biorad laboratories Rockville NY). Electrophoresis was carried out at a constant voltage of 200 volts for 45 min until the tracking dye was approximately 1 cm from the bottom of the gel. Pre-stained molecular weight marker (Amresco Inc. Solon OH) containing Myosin 200 KDa, b-Galactosidase 120 KDa, Bovine serum 91 KDa, Glutamate 62 KDa, Ovalbumin 46 KDa, Carbonic anhydrase 38 KDa and Lysozyme 19 KDa) were included as reference proteins. Bands were visualized by fixing gels and staining for 1 h in a solution of 0.2% coomassie blue R. 250 (Biorad Laboratories) in 50% methanol and 10% acetic acid. Apparent molecular weights were determined by comparison with known molecular weight of the reference protein standards.

Polymerase chain reaction of isolates

DNA extraction

Genomic DNA was extracted using the high pure PCR template preparation Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions.

PCR amplification

Polymerase chain reaction was carried out according to the method of (Han et al., 2007), with modifications. The primers were designed from the 16SrRNA gene of an isolate of *Dermatophilus congolensis* in the GenBank (Han et al., 2007). The primers were 5'-ACATGCAAGTCGAACGATGA-3' and 5'-ACGCTCGCACCTACGTATT-3'. The amplification was targeted at a 500bp fragment of the 16SrRNA gene of the organism. The amplification was targeted at a 500bp fragment of the 16SrRNA gene of the organism. *D. congolensis* isolates isolated from cattle, sheep and goats above were used for the amplification. *Staphylococcus aureus*, *Escherichia coli* and *Salmonella spp* were included as non specific DNA templates. Ten microlitres of PCR products were electrophoresed in 1.5% agarose gel containing 10 l of 10 mg/ml Ethidium bromide at 80 volts for 45 min. One hundred base pair marker (Roche, Mannheim, Germany) was used as a molecular size marker. DNA amplifications were examined and photographed using Bio imaging system (Syngene VWR international Japan).

Table 1. Biochemical reactions of *D. congolensis* isolates from cattle, sheep and goats.

Sugars	Isolates								
	C	C2	C6	S1	S2	S3	G1	G2	G3
Glucose	+	+	+	-	+	-	+	+	+
Maltose	+	+	-	-	-	-	w	+	+
Sucrose	+	+	+	-	+	-	+	+	+
Lactose	-	+	-	-	-	-	-	+	-
Galactose	-	+	-	-	-	-	-	w	-
Fructose	+	w	-	-	-	-	w	+	-
Dulcitol	-	-	-	-	-	-	-	-	-
Mannitol	-	-	-	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-	-	-	-
Xylose	-	-	-	-	-	-	-	-	-

+ positive reaction,-No reaction, W weak reaction.

Sequence analysis

The PCR amplicons (products) were sequenced at inqaba biotechnical company South Africa. The 16S rRNA sequencing was performed using ABI Prism Big-Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA). The machine used was the 3130XL Genetic Analyser from ABI. The results obtained after the sequencing were edited and deposited in the Genbank and accession numbers assigned to them as follows: [FJ 708616 for isolate C, FJ 708617 for isolate C2, FJ 708618 for isolate C6, FJ 708619 for isolate S1, FJ 708620 for isolate S2, FJ 708621 for isolate S3, FJ 708622 for isolate G1, FJ 708623 for isolate G2 and FJ 708624 for isolate G3].

Bioinformatics analysis

The results of the sequences were edited and aligned using ClustalW a software for multiple sequence alignment from Georgetown university website [http://pir.georgetown.edu/]. The sequences from all the isolates that is cattle, sheep and goats were aligned. Sequences were aligned against each other to determine identity and divergence. The sequences of each isolate were then blasted (aligned), according to Altschul, (1990) against existing sequences in the GenBank anchored by National Center for Biotechnology Information (NCBI). The Tamura-Nei neighbour joining complete deletion method of the computer program, Molecular Engineering Genetic Analysis (MEGA) version 4.1 (Tamura et al., 2007) was used to obtain a phylogenetic tree. Bootstrap confidence values were obtained with 1,000 resamplings.

RESULTS

Biochemical reactions

The results of the biochemical reactions of the isolates of *D. congolensis* is presented in Table 1.

Protein profile analysis of SDS PAGE

Protein profiles of the *D. congolensis* isolates, including

the molecular weight standard containing reference proteins [Myosin 200 KDa, b-Galactosidase 120 KDa, Bovine serum 91 KDa, Glutamate 62 KDa, Ovalbumin 46 KDa, Carbonic anhydrase 38 KDa and Lysozyme 19 KDa] showed protein bands on the sodium dodecyl sulphate polyacrylamide electrophoresis gel that can be visualized. This is presented on Figure 1.

PCR amplification

The results of the amplification of the *D. congolensis* isolates from cattle, sheep and goats are presented in Figure 2. It shows the amplification of the partial 16S rRNA gene of a band at approximately 500 base pairs (bp).

Multiple sequence alignment

The result of the multiple sequence alignment is presented in Figure 3. It shows sequences of eight isolates with areas of similarities and differences. One out of the nine isolates is included due to the challenges of sequencing.

Phylogenetic analysis

Figure 4 shows a phylogenetic tree constructed with the isolates of the organism with bootstrap values.

DISCUSSION

In this study, a general limited sugar activity was observed for all the isolates under consideration, which was more pronounced with the sheep isolates, as shown

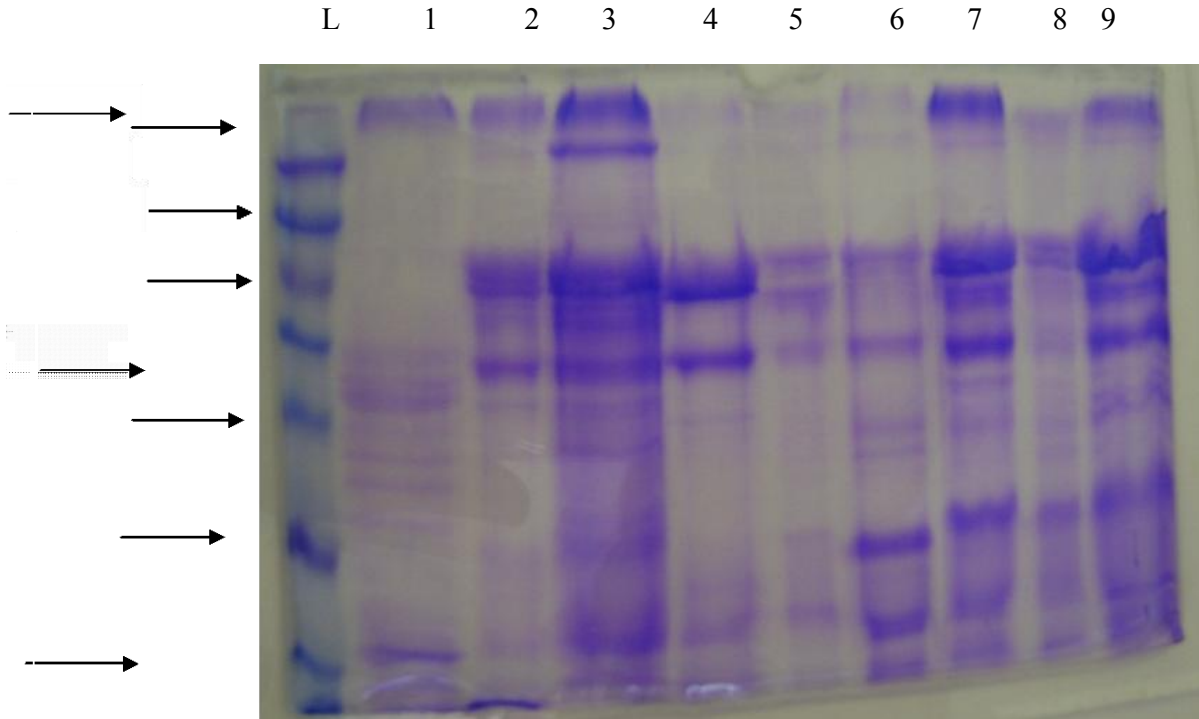


Figure 1. *Dermatophilus congolensis* isolates ran on a 12%gel. The isolates are labeled 1-9. The ladder is labeled L, Lane1 cattle isolate C, Lane 2 cattle isolate C2, Lane 3 cattle isolate C6, Lane 4 Sheep isolate S1, Lane 5 sheep isolate S2, Lane 6 Sheep isolate S3, 7 Lane Goat isolate G1, Lane 8 Goat isolate G2 and Lane 9 Goat isolate G3.

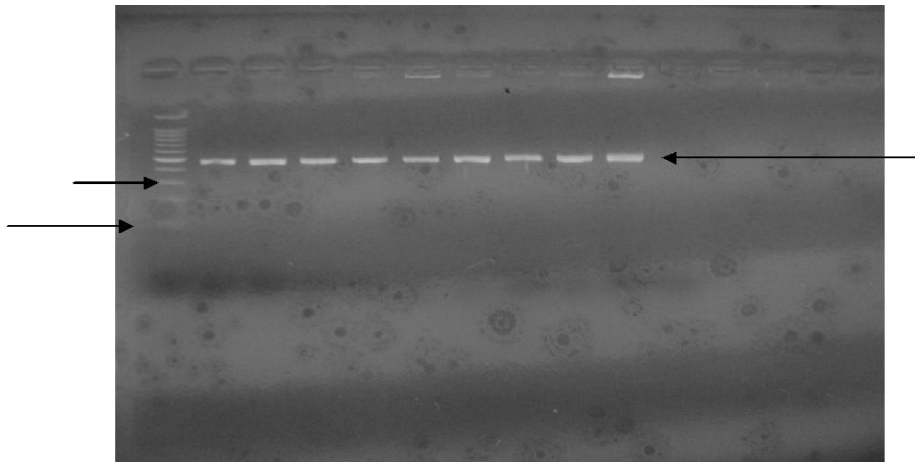


Figure 2. PCR amplification of a fragment of 16S rRNA gene from *D. congolensis* isolates. M 100 bp ladder, lanes 1 – 3 cattle isolates (C, C2 and C6). Lanes 4 – 6. Sheep isolates (S1, S2 and S3) and lane 7 - 9 Goat isolates (G1 G2 and G3), and lane 10 -12 negative controls (*Staphylococcus aureus*, is in lane 10, *E. coli* lane 11 and *Salmonella* spp lane 12. Legend for phylogenetic tree.

on Table 1. This observation is in agreement with Zaria, (1993). On the other hand, all the isolates were fermented by the following sugars; glucose, fructose, maltose, lactose, galactose and sucrose in a non defined pattern from isolates of a particular animal species or

isolates from different animal species with a particular sugar. At least one isolate from each animal species fermented glucose, except two sheep isolates. The fermentation of most of the isolates with glucose is in agreement with previous researchers (Gordon, 1964;

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C_  -ATGCAAGGCGAACGATGAAGCCCAGCTTGGTGGGTTAGTGGCGAACGGGTGAGTA 60 S1 -
ATGCAAGGCGAACGATGAAGCCCAGCTTGGTGGGTTAGTGGCGAACGGGTGAGTA 60 G2 -
ATGCAAGTTCGAACGATGAAGCCCAGCTTGGTGGGTTAGTGGCGAACGGGTGAGTA 60 G3
AATGCAAGGCGAACGATGAAGCCCAGCTTGGTGGGTTAGTGGCGAACGGGTGAGTA 60 C2 -
ATGCAAGGCGAACGATGAAGCCCAGCTTGGTGGGTTAGTGGCGAACGGGTGAGTA 60 G1 --
TGCAAGGCGAACGATGAAGCCCAGCTTGGTGGGTTAGTGGCGAACGGGTGAGTA 60 S2 --
TGCAAGTCGAACGATGAAGCCCAGCTTGGTGGGTTAGTGGCGAACGGGTGAGTA 60 S3 -----
AAGGCGAACGATGAAGCCCAGCTTGGTGGGTTAGTGGCGAACGGGTGAGTA 60

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C_  ACACGTGAGTAATCTGCCCTCACTTTGGGATAAGCCCCGGAACGGGGTCTAATACTGA 120 S1
ACACGTGAGTAATCTGCCCTCACTTTGGGATAAGCCCCGGAACGGGGTCTAATACTGA 120 G2
ACACGTGAGTAATCTGCCCTCACTTTGGGATAAGCCCCGGAACGGGGTCTAATACTGA 120 G3
ACACGTGAGTAATCTGCCCTCACTTTGGGATAAGCCCCGGAACGGGGTCTAATACTGA 120 C2
ACACGTGAGTAATCTACCCCTCACTTTGGGATAAGCCCCGGAACGGGGTCTAATACTGA 120 G1
ACACGTGAGTAATCTGCCCTCACTTTGGGATAAGCCCCGGAACGGGGTCTAATACTGA 120 S2
ACACGTGAGTAATCTGCCCTCACTTTGGGATAAGCCCCGGAACGGGGTCTAATACTGA 120 S3
ACACGTGAGTAATCTGCCCTCACTTTGGGATAAGCCCCGGAACGGGGTCTAATACTGA 120

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Figure 3. Multiple sequence alignment for all the 8 isolates (cattle, sheep and goats isolates). 8 areas of non identities for all the 8 isolates (np 1- 5, 9, 76 and 80).

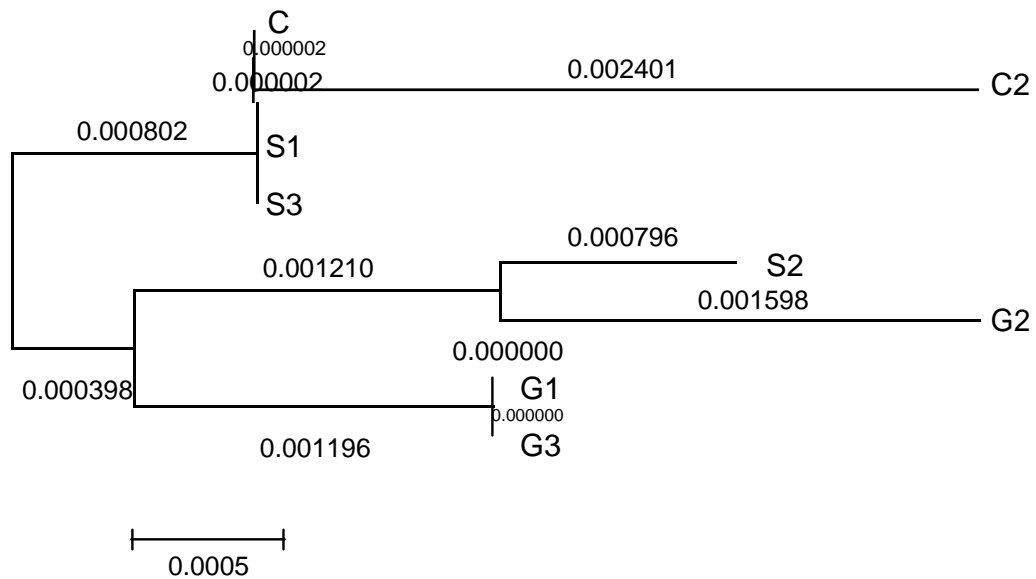


Figure 4. phylogenetic tree of the isolates of *Dermatophilus congolensis* from cattle sheep and goats; C and C2 cattle isolates, S1, S2 and S3 sheep isolates, G1, G2 and G3 goat isolates.

Macadam and Haalstra 1971; Adlan and Obeid 1977; Abu, 1978; Cottral, 1978; Ellis et al., 1993; Masters et al., 1995; Buenvieje 1997; Gordon, 1974; Samuel et al., 1998; kruger et al., 1998; Burd et al., 2007). The inability of the two sheep isolates to ferment glucose and other sugars could be due to their genetic make-up.

The reaction of the isolates to maltose from previous workers ranged from positive to variable. Samuel et al. (1998) recorded only positive reactions from camel isolates and not from cattle and sheep isolates, while we recorded positive reactions from cattle and goats isolates. Gordon (1964), Cottral (1977), Gordon, (1974) and kruger (1998) reported positive reactions with maltose, while Macadam and Haalstra (1971), Abu (1978), Ellis et al. (1993) and Masters et al. (1995) reported variable reactions with maltose.

The reaction of the isolates to sucrose followed the same pattern as in glucose. Except Samuel et al. (1998) who reported positive reaction from sucrose, other researchers either reported negative reactions (Gordon, 1964; Cottral, 1978; Ellis et al., 1993; Gordon, 1974; Masters et al., 1995; kruger et al., 1998; Burd et al., 2007) or variable results from sucrose (Macadam and Haalstra, 1971; Adlan and Obed, 1977; Abu, 1978).

Most of the literature consulted showed that all the researchers reported negative results with lactose (Macadam, 1964; Abu, 1978; Cottral, 1978; Ellis et al., 1993; Gordon, 1974; kruger et al., 1998; Buenvieje et al., 2000; Masters et al., 1995; Burd et al., 2007). This however, did not agree with my observation as one isolate from cattle and one from goat were positive. Macadam and Haalstra (1971) and Gordon, (1974), reported positive reactions with galactose, which agreed with our observations, and did not agree with kruger et al. (1998) who did not observe any reaction with galactose.

Though Zaria (1993) quoting others workers reported a prompt reaction with fructose after 48 h, it was however, not so in this case, as the fermentation of fructose was observed after 120 h. There was a consistency in the biochemical behaviour of all isolates of *D. congolensis* from cattle, sheep and goats with respect to four sugars (dulcitol, mannitol, sorbitol and xylose). They were all not fermented by any of the isolates. This is in agreement with previous research findings (Gordon, 1974; Ellis et al., 1993; Masters et al., 1995; Buenviaje et al., 1997), though Macadam and Haalstra (1971) reported a positive reaction to mannitol.

In summary, the sugar fermentations of all the isolates did not present a defined pattern from isolates of a particular animal species or isolates from different animal species to a particular sugar. This seems to agree with what has been observed that different researchers used different sugars for their characterization of *D. congolensis* isolates, with varying results as these results were not consistent even within different isolates of the same species as is observed in this study.

The study on protein profiles of the organism by

different researchers showed varying results that were not consistent with animal species and researchers. The same was observed in this study. Though bands were observed in all the isolates, however differences in the protein bands from one isolate to the other exists Figure 1. Two bands are common to all the isolates seen at about 62 and 20 KDa, indicating the relatedness between the isolates. Gogolewski et al. (1998) similarly observed two bands at about 30 and 76 KDa in ovine isolates of *D. congolensis*. Kruger et al. (1998) observed bands of between 30 and 97 KDa in horse isolates of *D. congolensis*. Makinde and Gyles (1999) have also reported the occurrence of protein bands between 16 and 62 KDa from cattle, sheep and horse isolates. Shaibu and Adetosoye (2008) observed bands between 21 and 72 KDa among cattle isolates of *D. congolensis*. There are other bands that are indicative of the relationship of the isolates; these are the band at about 120 KDa which occurred in 7/9 of the isolates. Others are the band at about 76 KDa which occurred in 5/9 of the isolates and the band at about 75 KDa which occurred in 6/9 of the isolates. These bands in the sheep isolates tend to agree with what Gogolewski et al. (1998) observed. The differences in the molecular weights of protein bands observed by different researchers is probably based on methods of measurements and calculations, as these are based on relative mobility of the proteins which are influence by many factors such, pH of water used, ionic properties of the water, source and age of reagents used. It is suggested that standard extracts of known proteins from characterized organisms be used as standard protein markers or positive controls.

The results obtained from the amplification of a 500bp segment of the 16S ribosomal RNA gene of *D. congolensis* from sheep isolates of *D. congolensis* in this study as seen on Figure 2, agrees with the work of Han et al. (2007), who used the same primers designed from the 16S ribosomal RNA of *D. congolensis* gene, to detect the organism from skin scabs of dermatophilosis infected sheep in china. The result obtained went further to show that the technique detected the organism in cattle and goats as well. This is an indication of the specificity of the primers, as other organisms that were not *D. congolensis* used as non specific DNA templates, were not amplified. This goes to confirm that PCR can be used as a diagnostic technique in the identification of *D. congolensis* isolates. This is also an indication that isolates of *D. congolensis* in various animal species derived may be closely related. PCR is a good diagnostic technique for *D. congolensis* isolates, as it has been able to detect *D. congolensis* from cattle, sheep and goats and discriminatory between *D. congolensis* isolates and other bacteria, tested in this study. The technique however, has been unable to discriminate between the different isolates of *D. congolensis* from cattle, sheep and goats. This is probably, because all the isolates amplified by the technique, are either the same species or this sequence

in all the isolates are conserved and therefore cannot be used to differentiate the isolates from the different animal species. It is suggested that other variable regions of the genome of *D. congolensis* be used to design primers for use in the possible differentiation of different species and RAPD as reported by Larasa et al. (2002).

From the results of the sequences and their alignments with each other, it was observed that the overall similarities between the sequences were over 98%. There were 8 areas of variations between the eight isolates from cattle, sheep and goats in the multiple sequence alignment done. These variations were noticed in nucleotide positions (np 1- 5, 9, 76 and 80). This is presented in Figure 4 and may be an indication of the degree of closeness of the isolates to each other. The observations of nucleotide variations within isolates that are closely related are not uncommon (Masters et al., 2003) as these variations could be due to mutations as a result of evolutionary changes, drug effects, and chemical or radiation effects. When the sequences of each isolate were blasted against partial sequences of the same 16S rRNA gene of *D. congolensis*, DSM 44180, (Stackebrandt and Schumann, 2000) Gene Bank accession no A1243918), revealed a 99% identity and higher.

The construction of a phylogenetic tree also indicated the close relationship between the isolates across species level. The isolates were clustered into two main groups as seen in Figure 4. Studies comparing isolates of *D. congolensis* at sequence level are rare. Buenviaje et al. (2000) compared sequences of *D. congolensis* and other animal species and got between 55 and 100% sequence similarities. In this case similarities between all the isolates across species were between 98.5 and 100%. This degree of closeness is an indication that all the isolates are the same and are probably strains. Though there are arguments for and against speciation Vs sequence variations in relation to one of the present molecular biology guidelines which suggest that 3% sequence variation of the 16S rRNA sequence is a threshold value to represent distinctly different bacterial species (Stackebrandt and Geobel, 1994; Clayton et al., 1995; Kolbert and Persing, 1999; Matsuda et al., 2006). However Fox et al. (1992) reported that lower variations may not mean identity.

A restriction enzyme map analysis of the sequences indicated that Hind1 and Ssp1 restriction enzymes, had sites that will give two bands which are almost at the same position in both restriction enzymes in the entire sequence lengths of all the isolates. This also shows the degree of closeness of the isolates.

Conclusions

It may be concluded that based on the techniques used in this study, all the isolates across the animal species are very closely related with only slight variations which were not enough to speciate them, but suggest the

existence of strains.

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