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Application of New Sero- Diagnostic Techniques and Molecular Assays to Characterize Recent Mycoplasma Isolates from Ruminants in Nigeria

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A B S T R A C T

This study investigated the presence of contagious bovine pleuropneumonia (CBPP) and contagious caprine pneumonia (CCPP) in ruminants to confirm and identify the mycoplasma species present. A comprehensive approach was employed, involving serological and molecular assays. A total of 342 cattle and 319 goats were tested, with 42 cattle and 23 goats showing positive serum samples, along with six stored cultures. These samples were analyzed using various serological assays, including IDEXX CBPP and CCPP cELISAs, Immunoblot, CapriLAT, BoviLAT, and the Complement Fixation Test (CFT). Molecular techniques such as PCR-DGGE, species-specific PCRs, and real-time PCR kits were also utilized. The results indicated serological evidence of CCPP and CBPP infections, with some samples positive for *M. agalactiae* and *M. ovipneumoniae*. Due to culture contamination, the real-time PCR results were often borderline. However, the study suggests mixed infections in goats and highlights the importance of storing purified colony isolates and expanding sampling regions to identify diagnostic biomarkers and potential vaccine traits for mycoplasmas in Nigerian isolates.

Introduction

The application of new sero-diagnostic techniques and molecular assays for characterizing recent Mycoplasma isolates from ruminants in Nigeria is an evolving field that integrates advanced methodologies to enhance the accuracy and efficiency of diagnosis. Mycoplasmas are significant pathogens in livestock, particularly ruminants, leading to various diseases that can affect productivity and animal welfare. Recent studies have highlighted the importance of molecular techniques, particularly Polymerase Chain Reaction (PCR), in the rapid and specific identification of Mycoplasma species, which is crucial given the limitations of traditional culture methods that are often time-consuming and less sensitive [1,2].

Molecular diagnostics, such as PCR, have been validated as effective tools for detecting Mycoplasma species in various animal populations. For instance, Amores [2] demonstrated that PCR is a reliable method for detecting *Mycoplasmaagalactiae* and *Mycoplasmamycoides* subsp. capri in goats, showing a clear advantage over culture methods [2]. Similarly, the developed multiplex RT-qPCR assay that allows for the simultaneous detection of multiple pathogens, including *Mycoplasma capricolum*, thereby streamlining the diagnostic process [3].

These advancements are particularly relevant in Nigeria, where the prevalence of Mycoplasma infections in ruminants necessitates efficient diagnostic strategies to manage and control outbreaks. In addition to PCR, serological methods are also gaining traction in the diagnosis of Mycoplasma infections. The use of enzyme-linked immunosorbent assays (ELISA) has been explored for detecting antibodies against Mycoplasma species, providing an alternative approach to confirm exposure and infection status in ruminants. However, specific references supporting the use of ELISA for Mycoplasma detection in ruminants were not found in the provided references, indicating a need for further investigation in this area.

Furthermore, the characterization of Mycoplasma isolates through advanced techniques such as Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) has shown promise in differentiating between species and subspecies [4,5]. This method not only enhances the speed of identification but also reduces the reliance on traditional culture methods, which can be labor-intensive and may not always yield viable isolates. The application of MALDI-TOF MS in veterinary diagnostics has been validated as a superior tool for identifying Mycoplasma species isolated from animals, including ruminants [5].

The pathogenicity of *Mycoplasma bovis*, a critical pathogen in cattle, has also been studied in Nigeria, where recent isolates have been shown to cause mastitis and other diseases [6,7]. Understanding the virulence factors and antimicrobial susceptibility profiles of these isolates is essential for developing effective treatment protocols. Research indicates that Mycoplasma species exhibit varying degrees of resistance to antibiotics, necessitating ongoing surveillance and susceptibility testing to guide therapeutic decisions [8,9].

In conclusion, the application of new sero-diagnostic techniques and molecular assays is pivotal in characterizing recent Mycoplasma isolates from ruminants in Nigeria. The integration of PCR, serological methods, and advanced identification techniques like MALDI-TOF MS not only enhances diagnostic accuracy but also contributes to better management of Mycoplasma-related diseases in livestock. Continued research and development in this area are essential to address the challenges posed by Mycoplasma infections in ruminant populations.

Methods

The methodology employed for the serological and molecular characterization of Mycoplasma isolates involved a comprehensive and systematic approach, integrating various diagnostic techniques to ensure accurate identification and analysis. To begin with, serological techniques were utilized to detect the presence of specific Mycoplasma species. The CCPP (Caprine Contagious Pleuropneumonia) and CBPP (Cattle Bovine Pleuropneumonia) were assessed using IDEXX competition ELISA kits at the Animal and Plant Health Agency (APHA). Following the manufacturer's guidelines, results were interpreted based on a signal-to-positive percentage (SPI%) threshold of less than 55%, indicating a positive result. Additionally, the WOAH-approved Complement Fixation Test (CFT) was conducted at APHA, adhering to WOAH guidelines. The CFT antigen was prepared in-house from strains F38 (*M. capricolum* subsp. *capripneumoniae*) and B103 (*M. mycoides* subsp. *mycoides*). These strains were washed, inactivated, and resuspended in a complement fixation diluent, with results validated against positive and negative control sera. Further serological testing included the CapriLAT and boviLAT latex agglutination tests, which were

performed according to the manufacturers' instructions. A subset of serum samples was also subjected to the WOAH-approved immunoblotting test and an in-house ELISA specifically designed for M. ovipneumoniae. In parallel with serological testing, culture techniques were employed to isolate and identify Mycoplasma species.

Upon receipt of samples derived from cattle and goats suspected of harboring *M. mycoides* subsp. *mycoides* (Mmm) or *M. capricolum* subsp. *capripneumoniae* (Mccp), the cultures were visually inspected. Approximately 0.3-0.5 ml of each sample was transferred to Eaton's broth for DNA preparation, while the remaining culture was diluted and filtered to minimize contamination. The cultures were then incubated at 37°C (±2°C) in a microaerobic environment enriched with 5% CO2. For PCR analysis, 0.5 ml of direct cultures and 1 ml of enriched cultures were centrifuged, resuspended in ATL buffer with proteinase K, and subjected to a heat lysis step. DNA was subsequently extracted using the Maxwell 16 system with a Blood kit. To analyze the extracted DNA, PCR-DGGE (Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis) was performed.

This involved using standard and endemic/exotic ruminant mycoplasma species DNA control markers. Specific PCR assays were conducted according to established protocols, and selected goat samples were tested with a commercial VetMAX™ Mycoplasma agalactiae and M. mycoides cluster kit. Any PCR-DGGE products that required further identification were sent for sequencing. The analysis of bovine samples revealed that out of 42 samples tested for CBPP using the IDEXX ELISA, 18 were positive. The BoviLAT test confirmed 16 positive samples, while the immunoblotting test identified 6 positives among 23 selected samples. Notably, four samples tested positive across all three serological tests, with additional samples pending further testing to clarify their status.

In terms of culture results, none of the 32 cultured samples showed signs of mycoplasma-like growth on Eaton's broth or agar. Visual inspection indicated unexpected brownish coloration in many cultures, suggesting potential contamination. Finally, the PCR-DGGE and specific PCR results indicated that five samples displayed bands similar to *M. bovis* and *M. canis* controls; however, all were negative in specific PCR tests for these species. Contaminants were identified in several samples, leading to the conclusion that no Mycoplasma species could be confirmed in any of the tested samples.

Results

Out of the 23 samples tested for CCPP (*M. capricolum* subsp. *capripneumoniae*) antibodies using the IDEXX ELISA, only two samples, C513 and C523, returned positive results. Notably, these two samples were part of a group of four that also tested positive for CCPP via the immunoblotting test (IBT), which included C510, C513, C521, and C523. Interestingly, both C513 and C523, which were positive in both the ELISA and IBT tests, yielded negative results in the CapriLAT test. This discrepancy may suggest a decline in IgM response or indicate that these samples were from animals with non-recent exposure to the pathogen. Sample C513, however, also tested positive for *M. capricolum* using the complement fixation test (CFT). The other two IBT-positive samples, C510 and C521, were found to be positive for CCPP in the CapriLAT test, suggesting that these animals might be in the early stages of infection. The remaining 19 samples tested negative in the CapriLAT assay.

Additionally, seven samples (C51, C52, C53, C59, C512, C513, and C518) were evaluated using the APHA in-house ELISA for M. ovipneumoniae, and all were found to be positive. Sample C513 exhibited reactivity across multiple serological tests (*M. capricolum*, *M. ovipneumoniae*, and *M. conjunctivae*). However, this could potentially be attributed to cross-reactivity among these serological tests, particularly between the CFT and the M. ovipneumoniae ELISA, which utilize crude antigens and may lack specificity for closely related organisms within the M. mycoides cluster.

None of the 16 samples cultured exhibited any signs of mycoplasma-like growth on both liquid and solid Eaton media. Also, among the 15 samples tested using Mycoides Cluster endpoint PCR, none returned positive results. Although some bands were observed in the DGGE-PCR analysis, none corresponded to *M. mycoides* subsp. *mycoides*, *M. capricolum*, *M. capripneumoniae*, *M. agalactiae*, or *M. bovis*. Specific endpoint PCR assays targeting these pathogens also did not yield any positive bands. In a comparative validation exercise using the VetMax M. agalactiae/Mycoplasma mycoides kit, a fluorophore FAM signal for the fusA gene (indicative of the Mycoides cluster) was detected in sample G5-98, with a cycle threshold (Ct) value of 30.5.

It is important to note that this assay is currently utilized as a research tool at APHA, and due to the inability to isolate any mycoplasmas, this finding cannot be substantiated. Among the additional six DNA samples (samples 1, 3, 4, 5, 6, and 7) submitted alongside the culture and serum samples, only sample 6 exhibited a DGGE-PCR profile resembling that of *M. ovipneumoniae*. However, specific PCR assays for M. ovipneumoniae and M. conjunctivae returned negative results. The DGGE-PCR profile from sample 6 was subsequently sent for sequencing, which identified *Acholeplasma laidlawii* as the organism present.

Discussion

The findings from the serological, cultural, and molecular analyses of caprine samples provide critical insights into the prevalence and detection of Mycoplasma species, particularly *M. capricolum* subsp. *capripneumoniae* (Mccp), in goats. The serological results indicated that only a small proportion of the samples tested positive for CCPP antibodies, with only two out of 23 samples yielding positive results in the IDEXX ELISA. This low seroprevalence aligns with previous studies that have reported varying prevalence rates of CCPP in different regions, suggesting that the disease may not be as widespread in the sampled population as previously thought. [10, 11].

However, the positive results from the immunoblotting test (IBT) for the same samples indicate that there may still be a significant presence of the pathogen, albeit at lower levels or in a non-recent exposure state, as suggested by the negative results in the CapriLAT test. The negative results from the CapriLAT test for the two samples that were positive in both the ELISA and IBT tests could imply a waning IgM response, which is often indicative of either a past infection or a non-recent exposure to the pathogen. This observation is consistent with findings from other studies that have noted the complexities of interpreting serological results, particularly in the context of cross-reactivity among related Mycoplasma species [12, 13].

The presence of sample C513 reacting positively across multiple serological tests raises concerns about potential cross-reactivity, particularly between the CFT and *M. ovipneumoniae* ELISA, which utilize crude antigens and may lack specificity for closely related organisms within the M. mycoides cluster [14, 15]. Cultural analysis of the samples revealed no signs of mycoplasma-like growth on Eaton media, which is concerning given the serological findings. The absence of growth could be attributed to several factors, including the possibility that the samples contained non-viable organisms or that the mycoplasmas were present in low quantities that were undetectable by culture methods. This aligns with findings from other studies that have reported challenges in isolating Mycoplasma species from clinical samples, particularly when the organisms are present in low numbers or when contamination occurs [16, 17].

The molecular analysis, specifically the PCR results, further corroborated the difficulties in detecting Mycoplasma species in the tested samples. None of the samples tested positive for the Mycoides Cluster endpoint PCR, and while some bands were observed in the DGGE-PCR analysis, none matched the expected Mycoplasma species. This lack of positive identification through PCR is concerning, as it suggests that the presence of Mycoplasma species may be lower than anticipated or that the molecular assays used may require optimization for better sensitivity and specificity [18, 19].

The detection of a FAM signal for the fusA gene in one sample indicates potential Mycoplasma presence; however, without successful isolation, the significance of this finding remains uncertain. Interestingly, among the additional DNA samples submitted, only one sample exhibited a DGGE-PCR profile resembling *M. ovipneumoniae*, but specific PCR assays for this pathogen returned negative results. This discrepancy highlights the complexities of molecular diagnostics in identifying Mycoplasma species, where the presence of non-target organisms, such as *Acholeplasma laidlawii*, may complicate interpretations [20, 19]. In conclusion, the results of this study underscore the challenges associated with diagnosing Mycoplasma infections in caprine populations.

The low seroprevalence of CCPP, combined with negative culture results and inconclusive molecular findings, suggests that further investigations are warranted to better understand the epidemiology of Mycoplasma species in goats. Future studies should focus on refining diagnostic techniques, including the development of more specific serological assays and optimizing molecular methods to enhance the detection of Mycoplasma species in clinical samples. Additionally, understanding the potential for cross-reactivity among serological tests will be crucial in accurately assessing the prevalence and impact of these pathogens in goat populations.

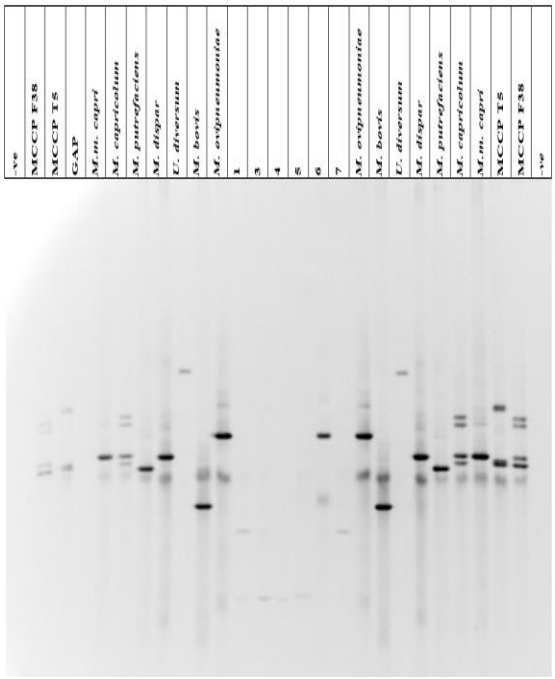

Figure 1. Resultsample #6 is close but not exactly matching *M. ovipneumoniae.* Sent for PCR product sequencing which confirmed the product was

Acholeplasma laidlawii.A. laidlawii is normally only placed on the bovine DGGE gels..

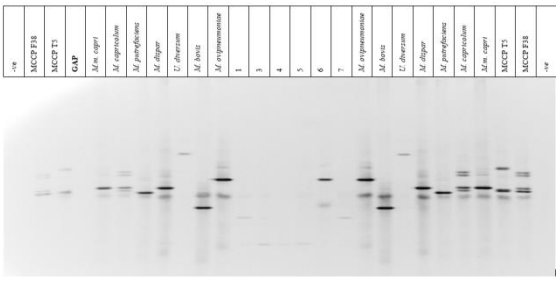

Figure 2. Result sample #6 is close but not exactly matching *M.ovipneumoniae.* Sent for PCR product sequencing which confirmed the product was *Acholeplasma laidlawii.A. laidlawii* is normally only placed on the bovine DGGE gels.

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Competing Interests

The authors declare that there are no competing interests.

References

[1] Mousa, W., Zaghawa, A., Elsify, A., Nayel, M., Ibrahim, Z., Alkheraije, K., … & Salama, A. (2021). Clinical, histopathological, and molecular characterization of mycoplasma species in sheep and goats in egypt. Veterinary World, 2561-2567. https://doi.org/10.14202/vetworld.2021.2561-2567

[2] Amores, J., Corrales, J., Gómez-Martín, Á., Sánchez, A., Contreras, A., & Fe, C. (2010). Comparison of culture and pcr to detect mycoplasma agalactiae and mycoplasma mycoides subsp. capri in ear swabs taken from goats. Veterinary Microbiology, 140(1-2), 105-108. https://doi.org/10.1016/j.vetmic.2009.06.036

[3] Settypalli, T., Lamien, C., Spergser, J., Lelenta, M., Wade, A., Gelaye, E., … & Diallo, A. (2016). One-step multiplex rt-qpcr assay for the detection of peste des petits ruminants virus, capripoxvirus, pasteurella multocida and mycoplasma capricolum subspecies (ssp.) capripneumoniae. Plos One, 11(4), e0153688. https://doi.org/10.1371/journal.pone.0153688

[4] Pereyre, S., Tardy, F., Renaudin, H., Cauvin, E., Machado, L., Tricot, A., … & Bebear, C. (2013). Identification and subtyping of clinically relevant human and ruminant mycoplasmas by use of matrix-assisted laser desorption ionization–time of flight mass spectrometry. Journal of Clinical Microbiology, 51(10), 3314-3323. https://doi.org/10.1128/jcm.01573-13

[5] Spergser, J., Heß, C., Loncaric, I., & Ramírez, A. (2019). Matrix-assisted laser desorption ionization–time of flight mass spectrometry is a superior diagnostic tool for the identification and differentiation of mycoplasmas isolated from animals. Journal of Clinical Microbiology, 57(9). https://doi.org/10.1128/jcm.00316-19

[6] Amosun, E. and Emikpe, B. (2020). Pathogenicity of <i>mycoplasma bovis</i> isolates from some states in nigeria inoculated intramammarily into lactating new zealand white rabbits. Sokoto Journal of Veterinary Sciences, 17(4), 80-83. https://doi.org/10.4314/sokjvs.v17i4.9

[7] Imandar, M., Pourbakhsh, S., Jamshidian, M., & Salehi, T. (2018). Isolation, identification and molecular characterization of mycoplasma bovis in mastitic dairy cattle by pcr and culture methods. Journal of the Hellenic Veterinary Medical Society, 69(1), 815. https://doi.org/10.12681/jhvms.16433

[8] Saeed, Z. and AL-Ghizawi, G. (2014). The role of mycoplasmas in oro-dental diseases. Basrah Journal of Surgery, 20(2), 23-28. https://doi.org/10.33762/bsurg.2014.98487

[9] Soehnlen, M., Kunze, M., Karunathilake, K., Henwood, B., Kariyawasam, S., Wolfgang, D., … & Jayarao, B. (2011). In vitro antimicrobial inhibition of mycoplasma bovis isolates submitted to the pennsylvania animal diagnostic laboratory using flow cytometry and a broth microdilution method. Journal of Veterinary Diagnostic Investigation, 23(3), 547-551. https://doi.org/10.1177/1040638711404155

[10] Ahaduzzaman, M. (2020). Contagious caprine pleuropneumonia (ccpp): a systematic review and meta-analysis of the prevalence in sheep and goats. Transboundary and Emerging Diseases, 68(3), 1332-1344. https://doi.org/10.1111/tbed.13794

[11]Selim, A., Megahed, A., Kandeel, S., Alanazi, A., & Al-Mohammed, H. (2021). Determination of seroprevalence of contagious caprine pleuropneumonia and associated risk factors in goats and sheep using classification and regression tree. Animals, 11(4), 1165. https://doi.org/10.3390/ani11041165

[12]Allen, A., Goupil, B., & Valentine, B. (2013). A retrospective study of brain lesions in goats submitted to three veterinary diagnostic laboratories. Journal of Veterinary Diagnostic Investigation, 25(4), 482-489. https://doi.org/10.1177/1040638713493627

[13] Baziki, J., Charles, B., Nwankpa, N., Chitsungo, E., Boukary, C., Maina, N., … & Koffi, Y. (2019). Development and evaluation of epitope-blocking elisa for detection of antibodies against contagious caprine pleuropneumonia in goat sera. Veterinary Sciences, 6(4), 82. https://doi.org/10.3390/vetsci6040082

[14] El-Deeb, W., Fayez, M., Elsohaby, I., Salem, M., Alhaider, A., & Kandeel, M. (2020). Investigation of acute-phase proteins and cytokines response in goats with contagious caprine pleuropneumonia with special reference to their diagnostic accuracy.

Peerj, 8, e10394. https://doi.org/10.7717/peerj.10394

[15] Swai, E., Kaaya, J., & Noah, E. (2013). Antibody response to mycoplasma capricolum subsp. capripneumoniae bacterium in small holder dairy goats in tanzania. Tropical Animal Health and Production, 45(7), 1603-1608. https://doi.org/10.1007/s11250-013-0405-4

[16] Yatoo, M., Parray, O., Bhat, R., Nazir, Q., Haq, A., Malik, H., … & Dhama, K. (2019). Novel candidates for vaccine development against mycoplasma capricolum subspecies capripneumoniae (mccp)—current knowledge and future prospects. Vaccines, 7(3), 71. https://doi.org/10.3390/vaccines7030071

[17] Semmate, N., Zouagui, Z., Elkarhat, Z., Bamouh, Z., Fellahi, S., Tligui, N., … & Harrak, M. (2022). Molecular characterization and pathogenicity of mycoplasma capricolum subsp. capricpolum from goats in morocco. Animal Diseases, 2(1). https://doi.org/10.1186/s44149-022-00042

[18] Jones, B., Rich, K., Mariner, J., Anderson, J., Jeggo, M., THEVASAGAYAM, S., … & Roeder, P. (2016). The economic impact of eradicating peste des petits ruminants: a benefit-cost analysis. Plos One, 11(2),