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(RESEARCH ARTICLE)

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Isolation and identification of mycoplasma strains in the inner ear of cattle and small ruminants in Mali

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Abstract

Mycoplasmas are microorganisms characterized by the absence of a cell wall and affecting animals and humans. In domestic ruminants, the role of inner ear swabbing was determined in the isolation of mycoplasmas in cattle and small ruminants in Mali. From 250 asymptomatic carriers, inner ear swabs were taken from 126 cattle and 124 small ruminants in the regions of Kayes, Koulikoro, Sikasso, Ségou and the District of Bamako. Microbiological analysis by isolation on Hyflick medium revealed 38 strains of mycoplasma including 23 in cattle (6 strains from Kayes, 3 strains from Sikasso, 1 strain from Ségou, 13 strain from Bamako) and 15 strains from small ruminants (1 strain from Sikasso, 2 from Ségou, 12 from Bamako). Growth inhibition and PCR tests (FusA, Polc) made it possible to identify in cattle 8 strains of *Mycoplasma bovis*, 13 strains of *Mycoplasma yeatsii* and 2 strains of *Mycoplasma alkalescens* and in small ruminants, 5 strains of *Mycoplasma mycoides capri*, 3 strains of *Mycoplasma yeatsii* and 7 strains of *Mycoplasma agalactiae*. Overall, the prevalence rate of mycoplasmas from inner ear swabs was 42.11% (16/38) of *Mycoplasma yeatsii,* 21.05% (8/38) of *Mycoplasma bovis*, 18.42% (7 /38) of *Mycoplasma agalactiae*, 13.16% (5/38) of *Mycoplasma mycoides capri* and 5.26% (2/38) of *Mycoplasma alkalescens*. The results of this study confirmed that the ear canal of cattle and small ruminants could be suitable sources for the detection of pathogenic strains such as *Mycoplasma bovis*, *Mycoplasma agalactiae*, *Mycoplasma mycoides subsp. capri* in asymptomatic herds of cattle and small ruminants.

Keywords: Identification; *Mycoplasma bovis*; *Mycoplasma agalactiae*; *Mycoplasma mycoides capri*; *Mycoplasma alcalensces*; *Mycoplasma yeattsi*

1. Introduction

Mycoplasmas are microorganisms without cell walls with a short-lived persistence in the environment. Each mycoplasma species of importance to veterinary or human health has a tropism generally related to the primary pathology and clinical signs. The signs of pneumonia, mastitis and arthritis are the consequences of infections with mycoplasmas with pulmonary tropism such as *Mycoplasma mycoides mycoides* (responsible for contagious bovine pleuropneumonia), mammary and joint tropism such as *Mycoplasma bovis*, *Mycoplasma agalactiae* and *Mycoplasma mycoides capri*. However, the occurrence of mycoplasma species in tissues or organs other than its preferred sites is not uncommon [1]. Several strains of mycoplasmas in co-infection can be isolated in the auditory canal of animals, in particular *Mycoplasma agalactiae*, *Mycoplasma capricolum*, *Mycoplasma mycoides* subsp*. capri, Mycoplasma mycoides* subsp*. mycoides* LC and *Mycoplasma putrefaciens* and in the mites *Psoroptes cuniculi* and *Raillietla caprae* present in the external auditory canal of domestic animals [2]but also in wild animals[3]. *Mycoplasma ovipneumoniae* and *Mycoplasma bovis* can occasionally cause otitis media and internal[4, 5]. Indeed, the carriage of *Mycoplasma mycoides, Mycoplasma capricolum* and *Mycoplasma agalactiae* in the auditory canal of ruminants is important in the persistence and re-emergence of contagious agalactia in goats. However, *Mycoplasma mycoides Large Colony* can also be recovered from

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the ear canals of healthy goats in herds showing no clinical signs of mycoplasmosis [6]. Other species such as *Mycoplasma bovis, Mycoplasma bovigenitium, Mycoplasma alkalescens, Mycoplasma dispar, Mycoplasma conjunctivae* and *Mycoplasma capricolum* are capable of infecting cattle and causing mastitis, arthritis, respiratory disease, reproductive system disorders and infectious keratoconjunctivitis [7]. The objective of the present study was to evaluate the presence of mycoplasmas in the internal auditory canal of domestic ruminants and to estimate the association between ear isolates (highly pathogenic or non-pathogenic) and the status of herds vis- against mycoplasma infections.

2. Material and methods

2.1. Transport media and growth medium

Swab specimen transport medium was prepared with basal medium consisting of PPLO (800 ml), horse serum (200 ml), and antibiotic such as penicillin G or ampicillin (4 ml). This transport medium was distributed in sterile Eppendorf tubes due to 1ml/tube in which each swab containing the sample is deposited.

The culture medium used was the modified Hyflick medium composed of:

Ingredients	Quantity (ml and g)
Distilled water	250 ml
Glucose	10 g
Sodium pyruvate	20 g
Ampicillin	2 g
Horse serum	500 ml
Fresh yeast extract	250 ml

2.2. Ear canal swab collection method

Earwax was collected from the deep part of the external auditory canal of each ear with a swab. This method has the advantage of being well tolerated by the animals compared to the use of a curette which has the advantage of collecting more cerumen but has the disadvantage of being often poorly tolerated by the animal. To collect a sample from the ear canal, you must:

- Distribute the transport medium in sterile cryotubes for 1ml,
- Immerse the tip in the transport medium until the cotton is soaked,
- Hold the ear in a position perpendicular to the head (case of live animal) to highlight the auditory canal,
- In the case of animal heads, hold to one side then incise the bottom of the ear until the auditory canal is visible before introducing the swab to collect the earwax,
- Gently push the cotton end into the animal's ear canal, then twist it on itself to collect the sample, then rotate in the same direction to remove the swab,
- Use scissors to cut the rod of the swab to the size of the cryotube to be able to screw the lid tightly. The cryotube should contain the entire tip and part of the stem,
- Label the cryotube and enter the necessary information (identification number and type of sample, nature of the sample) making sure that the identification number mentioned on the tube corresponds to that of the information sheets on the sample,
- Place the cryotube under ice to the laboratory where it is incubated for 24 hours before culturing the sample.

2.3. Method for culturing ear swab specimens

Ear swab samples were inoculated into broth and modified Hayflick Medium agar[8]: PPLO (BD/Lot 6244621); Sodium pyruvate (Sigma/Lot 115K07251); D-(+) Glucose (SIGMA / Batch BCBC0777); Fresh Yeast Extract (LCV-ELF/Lot19005); Horse Serum (Gibco/Lot 1750660) 45%; Ampicillin (Sigma/Lot BCBR6229V). Growth on solid medium was assessed by observation of mycoplasma colonies using a binocular magnifying glass.

The following isolation protocol was used

- After 24 hours of incubation, the swab was cultured by:
- 1/10th dilutions on eight (8) tubes (10-1–10-8) due to 1ml of sample in 9ml of growth medium (broth),
- Prepare 8 boxes of agar per sample, i.e. one box per dilution (-1 to -8),
- Place 100µl per dilution on each agar (100µl of the -1 dilution on the agar box labeled -1). This method has the advantage of not only having the status of the sample (positive or negative) but also the titer of the positive culture,
- Incubate the broths (with slow agitation) and the inoculated agar at 37°C in a humid atmosphere at 5% CO2,
- Broths and agar are examined daily for signs of bacterial growth (broth) and the characteristic morphology of "fried egg" Mycoplasma colonies,
- Purification cloning followed by isolate harvesting was carried out on liquid medium for identification.

2.4. Method of identification of mycoplasma isolates

The identification of *Mycoplasma agalactiae* isolates was made by the growth inhibition test with discs impregnated with *Mycoplasma agalactiae* reference serum and by PCR with specific primers using the Bioingentech VetPCR *M.agactaiae* Detection Kit (Ref : VET-0006-96D). The identification of the mycoplasma strains of the mycoides group was carried out by PCR Fus A (Mycoplasma mycoides subsp. capri) and the other strains of mycoplasma (*Mycoplasma bovis, Mycoplasma yeatsii, Mycoplasma alkalescens*) by PCR PolC 16S.

3. Results

The overall carriage rate of mycoplasma from the inner ears of domestic ruminants was 15.20%, including 18.257% in cattle and 12.10% in small ruminants. As the survey was conducted in 4 regions (Kayes, Koulikoro, Sikasso, Ségou) and the District of Bamako in Mali, the highest mycoplasma carriage rates were recorded in cattle in the Kayes region (100% in Nioro, 75% in Kenieba), in Sikasso (28.57% in Kadiolo, 25% in Bougouni) and 27.08% in the District of Bamako. Compared to small ruminants, the carriage rate of 100% was observed in Sikasso (circle of Kadiolo), in Ségou (33.33% in Bla) and in Bamako (22.22%) (Table 1).

Table 1 Microbiological prevalence rate by site

Regions	Circles/District	Cattle			Small ruminants		
		Number	Strain	Prev.(%)	Number	Strain	Prev.(%)
Bamako	Bamako	48	13	27.08	54	12	22.22
Kayes	Nioro	3	3	100.00	7	0	0.00
	Yelimane	5	0	0.00	5	0	0.00
	Kayes	5	0	0.00	5	0	0.00
	Kenieba	4	3	75.00	6	0	0.00
Koulikoro	Bangamba	8	0	0.00	2	0	0.00
	Nara	2	0	0.00	0	0	0.00
	Katy	5	0	0.00	5	0	0.00
	Kangaba	5	0	0.00	5	0	0.00
Segou	Blah	4	0	0.00	6	2	33.33
	San	5	0	0.00	4	0	0.00
	Tominian	6	1	16.67	4	0	0.00
	Segou	5	0	0.00	5	0	0.00
Sikasso	Bougouni	4	1	25.00	6	0	0.00
	Kadiolo	7	2	28.57	1	1	100.00

	Sikasso	5	0	0.00	5	0	0.00
	Koutiala	5	0	0.00	4	0	0.00
Total		126	23	18.25	124	15	12.10

Prev. = prevalence

The study identified 23 strains of mycoplasma in cattle, including 13 strains of *Mycoplasma yeatsii* (56.5%), 8 strains of *Mycoplasma bovis* (34.8%) and 2 strains of *Mycoplasma alkalescens* (8.7%). Compared to small ruminants swabbed, 7 strains of *Mycoplasma agalactiae* (46.7%) were identified, 5 strains of *Mycoplasma mycoides subsp. capri* (33.33%) and 3 strains of *Mycoplasma yeatsii* (20%) (Table 2, Fig**ure** 1-3).

Table 2 Identification of ear canal mycoplasma strains

strains of Mycoplasmas	**Cattle**		**Small ruminants**		**Overall**	
	Number	**Prev.(%)**	**Number**	**Prev.(%)**	**Number**	**Prev.(%)**
Mycoplasma bovis (Mbovis)	8	34.8	0	0.0	8	21.05
Mycoplasma mycoides capri (Mmc)	0	0.0	5	33.3	5	13.16
Mycoplasma yeatsii (Myeat)	13	56.5	3	20.0	16	42.11
Mycoplasma alkalescens (M.alk)	2	8.7	0	0.0	2	5.26
Mycoplasma agalactiae (M.agal)	0	0.0	7	46.7	7	18.42
Total	23	100.0	15	100.0	38	100.00

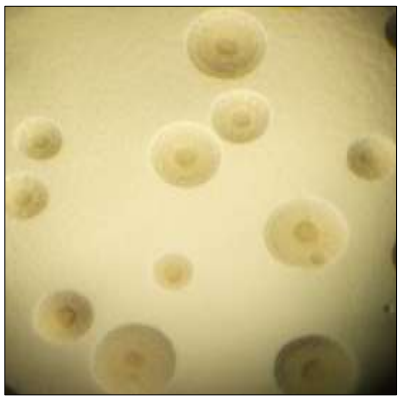

Figure 1 *Mycoplasma agalactiae* colonies

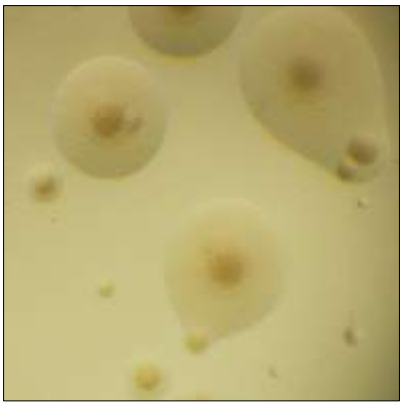

Figure 2 *Mycoplasma mycoides capri* colonies

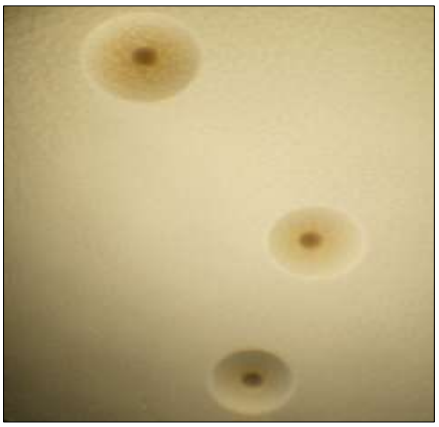

Figure 3 *Mycoplasma bovis* colonies

Compared to the sampling sites, *Mycoplasma bovis* was isolated in Bamako, Nioro, Kenieba, Tominia and Kadiolo; *Mycoplasma mycoides* subsp*. capri* isolated in Bamako, Bla and Kadiolo; all strains of *Mycoplasma agalactiae* were tested in Bamako on small ruminants. As for *Mycoplasma yeatsii*, it was largely isolated in Bamako then in Nioro and Kadiolo; *Mycoplasma alkalescens* has been isolated in Bamako and Bougouni (Table III).

Table 3 Distribution of ear mycoplasma strains by circle

Circles/District	***Mycoplasma bovis***	***Mycoplasma mycoides capri***	***Mycoplasma agalactiae***	***Mycoplasma yeatsii***	***Mycoplasma alkalescens***
Bamako	1	2	7	14	1
Nioro	2	0	0	1	0
Kenieba	3	0	0	0	0
Blah	0	2	0	0	0
Tominian	1	0	0	0	0
Bougouni	0	0	0	0	1
Kadiolo	1	1	0	1	0
Total	8	5	7	16	2

4. Discussion

The isolation of several species of mycoplasma (*Mycoplasma bovis, Mycoplasma mycoides capri, Mycoplasma agalactiae, Mycoplasma yeattsi* and *Mycoplasma alkalescens*) in the inner ear of cattle and small ruminants has been confirmed by several authors where the external auditory canal of these animals allowed the isolation of several strains of mycoplasma. In experimental goats, *Mycoplasma mycoides subsp. mycoides* LC was cultured from ear canal and *Mycoplasma bovoculi* in Holstein cattle [9]. In small ruminants, several species of mycoplasmas, pathogenic or not, have been identified [10]. To determine whether the presence in the external auditory canal of the mites *Raillietia caprae* and mycoplasma was associated. *Mycoplasma cottewii* and *Mycoplasma yeatsii* were the only mycoplasmas isolated from uninfected goats and were also the predominant isolates (29 of 34) from infested goats and/or mites [11]. A correlation has been noted between parasitic otitis associated with *Psoroptes sp.* mites and the presence of mycoplasmas in the ear of goats[12]. A prevalence rate of 73.7% was obtained in goats for the strains *Mycoplasma arginini*, *Mycoplasma mycoides subsp. mycoides* and *Mycoplasma mycoides* subsp*. capri* [13]. On the other hand, in cattle the prevalence rate of auricular carriage was 80% (48/60) including 12.5% (6/48) of *Mycoplasma alcalenses*, 2.1% (1/48) of *Mycoplasma arginini*, 8.35% (4/48) *Mycoplasma bovirhinis*, 2.1% (1/48) *Mycoplasma bovis*, 25.0% (12/48) *Mycoplasma conjunctivae*, 14.6% (7/48) *Mycoplasma mycoides* subsp. *mycoides* LC and 10.4% (5/48) of *Mycoplasma capricolum* [14]. Carriage often involves multiple strains and/or species in the same ear canal. It should be checked whether these isolates, resulting from epidemics, have specific molecular markers (pathogenicity factors) [15]. Clinically normal goats may harbor several species of mycoplasma in the external auditory canal[16]. Different studies have documented the

presence of several species of Mycoplasma in the external auditory canal of goats, including highly pathogenic species (*Mycoplasma mycoides mycoides* Large Colony, *Mycoplasma capricolum capricolum, Mycoplasma putrefaciens* and *Mycoplasma agalactiae*) and non-pathogenic species (*Mycoplasma yeattsi* and *Mycoplasma capricolum*) [17–19]. Other Mycoplasma strains have been isolated from the goat ear canal *Mycoplasma auris*, *Mycoplasma cottewii* and *Mycoplasma yeatsii* [20]. *Mycoplasma auris* was part of the *Mycoplasma hominis* group of the hominis group. *Mycoplasma cottewii* and *Mycoplasma yeatsii* are very closely related with only four nucleotide differences, and they grouped together with *Mycoplasma putrefaciens* in the group *Mycoplasma mycoides* [21]. In animal experiment, some researchers have demonstrated a difference in the antigenic profile of the strains of ear swabs and those used to reproduce the disease [22]. As part of the control of mycoplasma infections, some artificial insemination centers have adopted measures for systematic swabbing of the auditory canal of experimental animals [23] where *Mycoplasma capricolum capricolum* ~~(Mcc)~~, the cause of contagious agalactia, was isolated from the ear canal of sheep [24]. In addition to ruminants, the auditory canal of other animal species is a reserve of mycoplasmas causing polyps in cats [25]. Compared to *Mycoplasma mycoides capri*, during an experiment, the septicemic strains and the carrier strains could not be distinguished by their genetic heritage or by their pathogenic capacity under experimental conditions [26], on the other hand this strain could be found in saprophyte form in the auditory canal of animals [27]. The inner ear swab collection technique described in this study could be used as a routine procedure for the diagnosis of mycoplasma infections in asymptomatic cattle and small ruminants.

5. Conclusion

The results of this study showed that the auditory canal of asymptomatic bovine ruminants and small ruminants can early harbor mycoplasmas independently of any infection or infestation by mites. These strains of mycoplasma can be pathogenic during experimental reproduction and maintain animal mycoplasmosis by inter-species transmission.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

This research work on animal mycoplasmas was conducted by Dr Amadou SERY without any conflict of interest along with his laboratory colleagues. Each member of the team played their full role in the successful conduct of this study. Colleagues Mamadou KONE, Bekaye SACKO, Abdoul Kader BOUARE took care of sample collection and laboratory analysis. Dr Cheick Abou Kounta SIDIBE and Dr Mamadou NIANG were responsible with me for the design and implementation of the activities of this research.

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