

Biological Characterisation and Pathogenicity of a *Pasteurella multocida* Isolate from Sheep in Morocco

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Abstract: In this study, 55 suspected pasteurellosis clinical cases from different provinces of Morocco were investigated. Molecular analysis revealed that 47% of samples were positive for *Pasteurella multocida*, all typed as serogroup A, and 11% positive for *Mannheimia haemolytica*. Eight isolates were recovered from 26 *P. multocida* positive samples, and characterized by biochemical and molecular typing methods. Among these isolates, two strains (S13 and S14) were selected for genes (RNA16S and rpoB) sequence analysis and virulence study in mice, guinea pigs and sheep. Phylogeny study showed similarities of both S14 and S13 isolates with strains from other species. In laboratory animals, the strain S14 was more virulent than S13 and induced severe illness in sheep. The high mortality of infected mice suggests that this model may represent an alternative for testing pathogenicity and vaccine efficacy.

Key words: *Pasteurella multocida*, phylogeny, mice, sheep, pathogenicity.

1. Introduction

Pasteurellosis is caused by bacteria of *Pasteurellaceae* family. It included diseases caused by *Pasteurella multocida* (PM), *Mannheimia haemolytica* (MH) and *Bibersteinia trehalosi* (BT), agram-negative, non-motile, non-spore, and facultative anaerobe coccobacilli. The family has many serotypes with variable pathogenicity and distribution and can adapt to different host species [1-4].

M. haemolytica is an important cause of bacterial respiratory mortality in cattle, sheep and goats. It is distributed worldwide in temperate, subtropical and tropical climates, although the prevalence of serotypes may vary by region and flock. *P. trehalosi* causes serious systemic infections in sheep but also associated with pneumonia in sheep [5, 6]. Infections with *P. multocida* may cause directly or indirectly major economic losses in livestock industry due to their morbidity and mortality. PM often exists as a normal member of upper respiratory tract microbiota of a

variety of animal species. However, environmental conditions, stress, viral or mycoplasma infections, promote bacteria invasion of lung tissue and development of pneumonia. *P. multocida* is responsible for infections in animals, and can be also transmitted to humans [7-10]. PM species are classified into three subspecies: gallicida isolated exclusively from birds, multocida associated with disease in domestic animals and septica, isolated from humans, animals and birds [11]. PM subsp. multocida is classified into five serogroups (A, B, D, E and F) on the basis of capsular antigens expressed on the cell surface and into 16 somatic serovars, based on lipopolysaccharide antigens. Strains of certain serogroups are associated with specific diseases in some animal species [10, 12]. PM serotypes B and E cause haemorrhagic septicaemia, a devastating disease of cattle and buffalo in enzootic areas of Asia and Africa. Types A and D strains are responsible for enzootic pneumonia and shipping fever in cattle, sheep and pigs [13-16].

In Morocco, clinical cases of pasteurellosis have been reported by field veterinarian and lesions observed at the abattoirs, suggesting circulation of

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serotypes of *Pasteurella* among ruminant population. However, only one study was published on isolation and identification of *Pasteurella*, and little was reported on its pathogenicity in small ruminants [17].

In this study, suspected clinical cases of pasteurellosis in different regions of Morocco were investigated to detect by polymerase chain reaction (PCR) different types of *Pasteurella*. Eight isolates of PM were recovered from clinical cases and characterized by biochemical and molecular typing methods. Among them, two isolates were sequenced and used for pathogenicity study on sheep, mice and guinea pigs to set up a model for vaccine testing.

2. Material and Methods

2.1 Clinical Specimens

Lung tissues were obtained from 55 dead animals with lesions compatible with pneumonic disease: 8 goats, 37 cattle and 10 sheep, from different herds and regions of Morocco. Observed lesions include exudative mucopurulent inflammation in the respiratory tract, pleurisy with abundant and fibrinous pleural effusion, pericarditis with gelatinous exudate, and bronchopneumonia with edematous and congested lung.

2.2 Molecular Screening and PM Genotyping

Genomic DNA extraction was performed using the Isolate II genomic DNA Kit (Bioline) according to the

supplier instructions. Molecular detection was performed according to Rayan *et al.* [18] for *M. haemolytica*, to Dassanayake *et al.* [19] for *P. threalosi*, and Townsend *et al.* [20] for *P. multocida*.

Multiplex PCR assay was conducted for molecular characterization of the capsular antigens of PM using specific primer sets for serogroups A, B, D, E, F [19, 20]. The serogroup-specific primer sets were identified according two criteria: (i) primer sets located within unique genes for each of the five serogroups (*hyaD*, *bcbD*, *dcbF*, *ecbJ*, and *fcB*) (Table 1), and (ii) amplicon length sufficient to allow clear size discrimination. The multiplex PCR mixture contained each primer within the six primer sets at a concentration of 3.2 μ M, 1 \times Standard Taq (Mg-free), 2 mM MgCl₂, each with deoxynucleoside triphosphate at a concentration of 0.2 mM, 0.5 U of Taq DNA polymerase. The PCR cycling conditions consisted of an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 60 s, and a final elongation at 72 °C for 5 min. The amplified products were separated by electrophoresis in 1% agarose gels and visualized by ethidium bromide staining. Amplification reactions were performed using Gene Amp 9700 (Applied Biosystem).

2.3 Bacteria Isolation, Culture and Identification

Lung tissue fragments were used to inoculate triptic

Table 1 Sequences of oligonucleotides used in the *P. multocida* multiplex capsular PCR typing assay.

| Serogroup | Gene | Sequence | Amplicon size (bp) |
|-----------|------------------|--|--------------------|
| All | KMT1 | KMT1T7-FWD ATCCGCTATTTACCCAGTGG KMT1SP6-REV GCTGTAAACGAACTCGCCAC | 460 |
| A | <i>hyaD-hyaC</i> | CAPA-FWD TGCCAAAATCGCAGTCAG CAPA-REV TTGCCATCATTGTCAGTG | 1,044 |
| B | <i>bcB</i> | CAPB-FWD CATTTATCCAAGCTCCACC CAPB-REV GCCCGAGAGTTTCAATCC | 760 |
| D | <i>dcbF</i> | CAPD-FWD TTACAAAAGAAAGACTAGGAGCCC CAPD-REV CATCTACCCACTCAACCATATCAG | 657 |
| E | <i>ecbJ</i> | CAPE-FWD TCCGCAGAAAATTATTGACTC CAPE-REV GCTTGCTGCTTGATTTTGTC | 511 |
| F | <i>fcB</i> | CAPF-FWD AATCGGAGAACGCAGAAATCAG CAPF-REV TTCCGCCGTCAATTACTCTG | 851 |

soy agar supplemented with 5% sheep blood plates. Plates were incubated at 37 °C for 24 h under aerobic conditions. Colonies giving gram-negative coccobacilli or short rods with or without bipolar staining on smears were subcultured for identification. Pure *Pasteurella* suspected cultures were subjected to biochemical tests (catalase, oxydase and indole) and analysed by API 20NE (Biomérieux) identification kit, performed according to the supplier instructions. Identified bacteria were seeded in Brain Heart Infusion broth and incubated for 12 h at 37 °C with 100 rpm agitation. The culture optical density was measured at 600 nm wavelength and bacteria concentration determined by serial dilutions. The exponential phase culture was used to determine genotyping and test pathogenicity in animals.

2.4 Sequencing and Phylogenetic Analysis of PM Isolates

Five microliters (5 µL) of the extracted DNA were amplified using One Taq DNA Polymerase (neb) Kit. The reaction was carried out in 50 µL using 16S ribosomal RNA [21] and rpoB [22] gene primer sets (Table 2). The length of the products was 1,400 bp for 16S ribosomal RNA gene and 539 bp for rpoB gene. The resulting PCR products were purified using gel extraction kit (QIAEXII) according to the manufacturer instructions. The purified products were sequenced by GATC Biotech Company. Sequences were aligned and compared with available sequences of PM. A phylogenetic tree was constructed using the neighbor joining method and Kimura two-parameter model in MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets [23].

2.5 Virulence of the PM Isolates in Mice and Guinea Pig

Virulence of two *P. multocida* isolates (S14 and S13) was determined each in ten 5-week-old BALBc mice and six guinea pigs by intra peritoneal inoculation of 0.5 mL of the bacteria suspension containing 1×10^9 CFU/mL. The inoculated mice and guinea pig were observed 3 days post inoculation (pi) for symptoms and mortality. Virulence of the strain (S14) was titrated in 22 mice by intra peritoneal inoculation of 0.5 mL of diluted bacteria suspensions to groups of mice. Mortality and mean time of death for each dilution were recorded to calculate LD₅₀.

2.6 Pathogenicity in Sheep of PM S14 Isolate

Sheep were allowed to acclimate to the laboratory environment for a period of 2 weeks prior to experimental infection with PM. Pathogenicity of the strain (S14) was determined in four 5-month-old healthy sheep. Before infection, nasal, conjunctival and rectal swabs as well as whole blood samples were collected to check the *Pasteurella multocida* status. Two sheep (G1) were inoculated with 2 mL via the oropharyngeal route, and 2 mL by intranasal route using a bacteria suspension of 1.0×10^9 CFU/mL. Two other sheep (G2) were inoculated by the same routes with a lower bacterial concentration (1.0×10^7 CFU/mL). Rectal temperature was monitored daily during 7 days pi. Clinical signs, respiration frequency, feed intake and general behaviour were evaluated throughout this period. Oral and nasal swabs were collected from infected sheep at days 3, 6 and 9 pi. Animals were autopsied 7 days pi, and lungs, mesenteric lymph nodes, liver and spleen were collected for bacteriology and PCR analysis.

Table 2 Sequences of oligonucleotides used for *P. multocida* isolates sequencing.

| Gene | Sequences |
|-------------------|--------------------------------|
| RpoB | GCA GTG AAA GAR TTC TTT GGT TC |
| | GTT GCA TGT TNG NAC CCA T |
| 16S ribosomal RNA | AGA GTT TGA TYM TGG C |
| | GYT ACC TTG TTA CGA CTT |

3. Results

3.1 Molecular Screening and PM Genotyping

The 55 field specimens were submitted for detection of *P. multocida*, *M. haemolytica*, and trehalosi by PCR assay. Results showed 14 PM and 3 MH positive samples from cattle, 5 PM and 3 MH from sheep and 7 PM from goat. All *Pasteurella multocida* strains were typed as serogroup A by the multiplex capsular PCR assay.

3.2 Bacteria Isolation, Culture and Identification

From the 26 specimens identified as *P. multocida*, 8 isolates of PM were recovered from samples of 1 cattle (S9), 5 sheep (S10, S11, S12, S13, S14) and 2 goats (S16, S17). The 8 isolates were positive for catalase, oxidase and indole production, and confirmed as PM by API 20NE and PCR. Two strains S13 and S14, originated from sheep, were selected for their high capacity of growth in limited period of time (12 h).

3.3 Sequencing and Phylogenetic Analysis of PM Isolates

Phylogenetic analysis using ARN 16S gene among the two selected PM strains (S13 and S14) demonstrated that S13 isolate was closely related to PM 30 England strain (99.5%) isolated from bovine

host [24] and to IVRI porcine strain originated from India (99.7%) [25]. The S14 isolate was related to the Chinese PmCQ6 strain isolated from bovine host and evaluated as low virulent strain [26] (Fig. 1). The RpoB gene analysis showed a similarity of 99.38% between the strain S13 and 964 isolated from goose host and originated from Hungary [27]. The strain S14 was related to the Chinese strain PM-L1706 (99.33%) isolated from a chicken host (MG813902) (Fig. 2).

3.4 Virulence of PM Isolates in Mice and Guinea Pigs

Mice infected with the S14 isolate died in a period of 15 h, while those infected with the strain S13 showed 60% of mortality within 24 h. Similarly, in guinea pig the strain S14 registered a higher percentage of mortality (66%) within a shorter period of time (24 h) compared to the S13 isolate (33% of mortality within 44 h) (Table 3). Results of the S14 isolate virulence titration on mice are reported in Table 4, giving a titer of 1.4×10^5 DL₅₀. At this dilution, 50% of inoculated mice died within 67 h.

3.5 Pathogenicity in Sheep of PM S14 Isolate

3.5.1 Clinical Signs

Animals of G1 injected with the bacterial suspension at 1×10^9 CFU/mL, suffered from dyspnea, coughing, nasal discharge and developed hyperthermia (above



Fig. 1 Phylogenetic and molecular evolutionary analysis of 16S ribosomal RNA gene (1.4kb) region conducted using Molecular Evolutionary Genetic Analysis Version 7.0 (MEGA7) with Kimura two-parameter model and Maximum Likelihood Statistical Method. The horizontal lines were proportional to the distance among sequences.

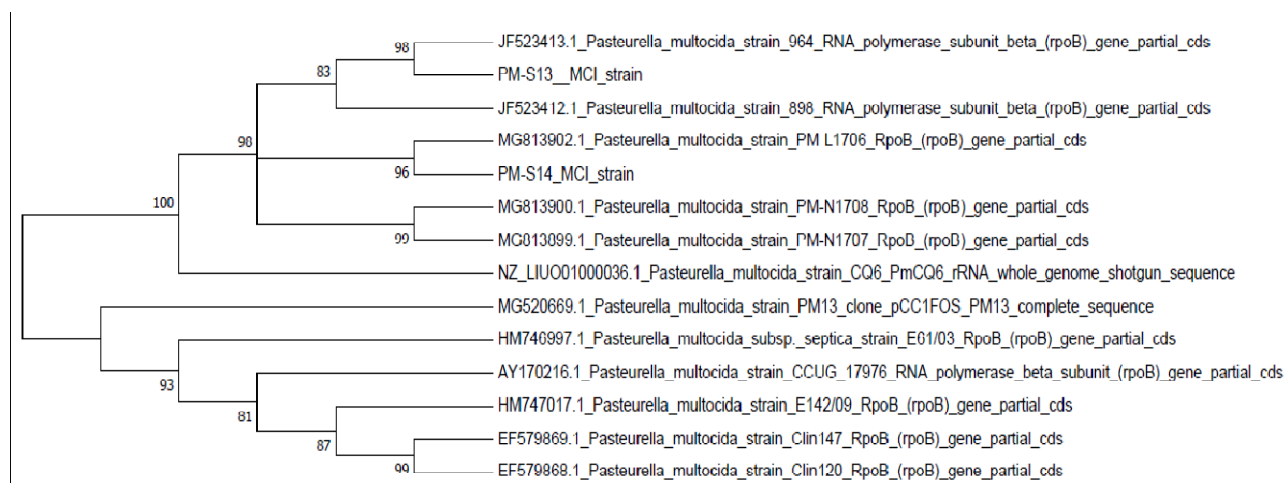


Fig. 2 Phylogenetic and molecular evolutionary analysis of *rpoB* gene (539pb) region conducted using Molecular Evolutionary Genetic Analysis Version 7.0 (MEGA7) with Kimura two-parameter model and Maximum Likelihood Statistical Method. The horizontal lines were proportional to the distance among sequences.

Table 3 Percentage of mortality and time of death in mice ($n = 20$) and guinea pigs ($n = 12$) infected with *P. multocida* (S14, S13) isolates.

| Isolate | Mice | | Guinea pig | |
|---------|---------------|-----------------|---------------|-----------------|
| | Mortality (%) | Lethal time (h) | Mortality (%) | Lethal time (h) |
| S14 | 100 | 15 | 66 | 24 |
| S13 | 60 | 24 | 33 | 44 |

Table 4 Percentage of mortality and time of death in mice ($n = 22$) infected with different concentrations of *P. multocida* S14 culture.

| Bacterial concentration (CFU/mL) | % of mortality | Lethal time (h) |
|----------------------------------|----------------|-----------------|
| 1×10^9 | 100 | 6 |
| 5×10^8 | 100 | 7.5 |
| 2×10^8 | 100 | 9.4 |
| 1×10^8 | 100 | 14 |
| 5×10^7 | 100 | 14 |
| 2×10^7 | 100 | 9.5 |
| 1×10^6 | 100 | 28 |
| 5×10^5 | 100 | 28 |
| 3×10^5 | 100 | 28 |
| 2.5×10^5 | 100 | 28 |
| 2×10^5 | 100 | 58.5 |
| 1.6×10^5 | 100 | 64.5 |
| 1.4×10^5 | 50 | 67 |
| 1.2×10^5 | 0 | - |

39 °C) for 5 days: a peak was noticed between day 1 and day 3 pi (40.5 °C) for animal 17 and between day 1 and day 2 pi (40.2 °C) for animal 18 (Fig. 3). Animals of G2 injected with low concentration of the bacterial suspension, did not show any clinical symptoms, but presented a hyperthermia (above 39 °C) for 1 to 2 days: a peak was registered at day 1 pi

(40 °C) for animal 986 and between day 1 and 2 pi (40.6 °C) for animal 987 (Fig. 3). All infected animals presented an increase in respiratory frequency between day 1 and day 6 pi. A peak was registered at day 1 for animal 17 (50 breath/min) and animal 986 (55 breath/min), followed by animal 18 (60 breath/min) at day 3 pi. The sheep 987 registered

polypnea later at day 6 pi (40 breath/min) (Fig. 4). Control sheep respiratory frequency remains normal at about 20 breath/min.

3.5.2 Post Mortem Lesions

Sheep 18 of G1 inoculated with 1.0×10^9 CFU/mL presented consolidation in caudal lobes of both left and right lungs, with several congestion zones, and intestinal congestion with hypertrophy of mesenteric lymph nodes (Fig. 5). Sheep 17 of G1 showed emphysema and generalized congestion of the right lung. For animals of G2, no lesions were observed at post mortem.

3.5.3 Bacteria Excretion and Tissue Charge

In G1, *Pasteurella* has been detected by PCR in nasal swabs in the two infected animals between D3 and D7 pi. In orotracheal swabs, the genome has been detected only in animal 18, between D6 and D7 pi. In G2 inoculated with 10^7 CFU, no *Pasteurella* has been detected in nasal or orotracheal swabs. At post mortem, PM was detected by PCR and re-isolated from the lung of animal 18 infected by the high dose. In other tissues (spleen, liver, mesenteric and pulmonary lymph nodes), no *Pasteurella* has been detected for both groups G1 and G2.

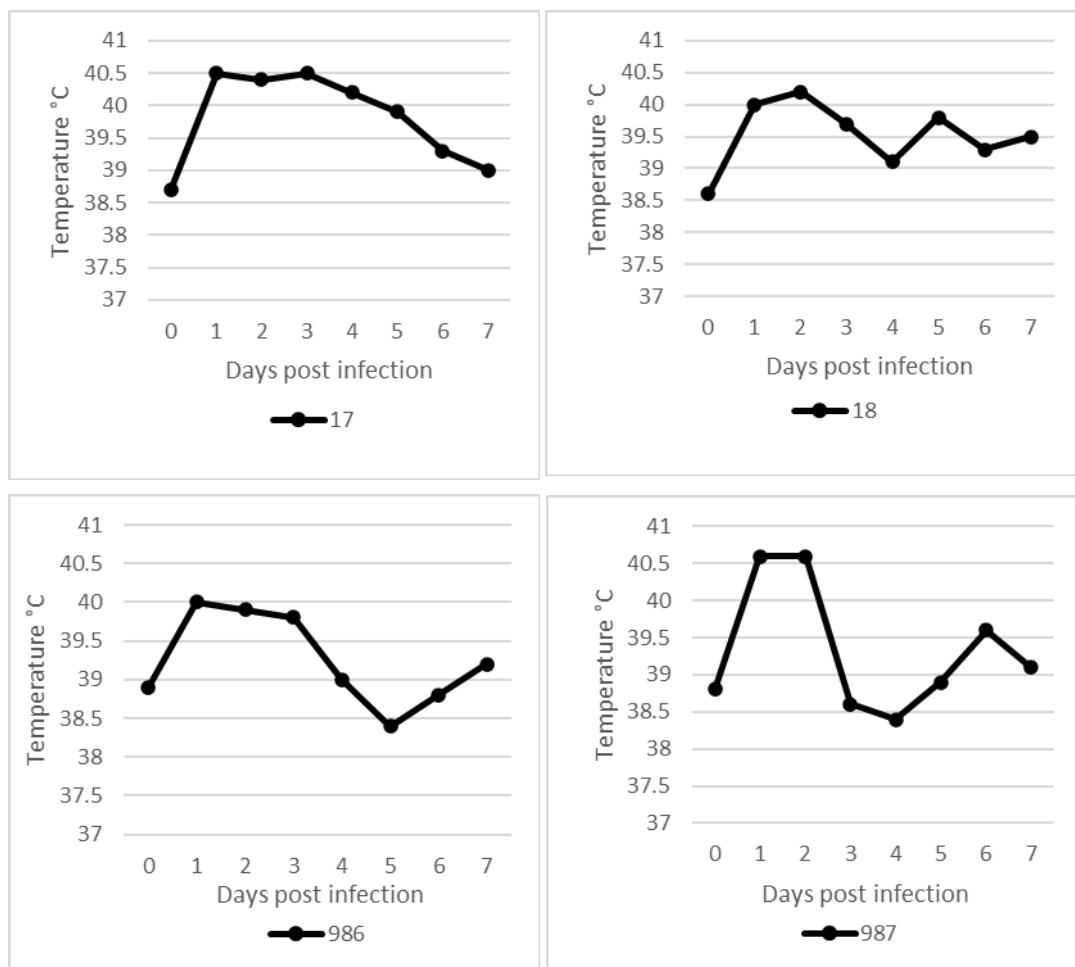


Fig. 3 Rectal temperature of sheep G1 (17 and 18) and G2 (986 and 987) infected with S14 *Pasteurella multocida* isolate.

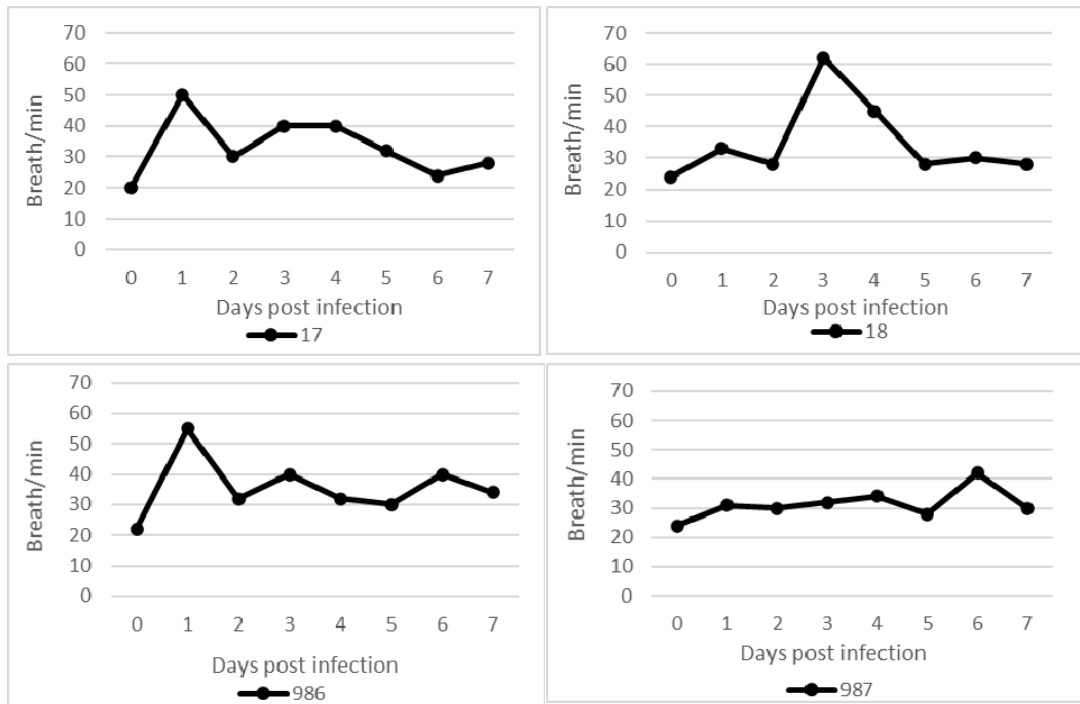


Fig. 4 Breathing rate (breath/min) of sheep G1 (17 and 18) and G2 (986 and 987) infected with S14 *Pasteurella multocida* isolate.

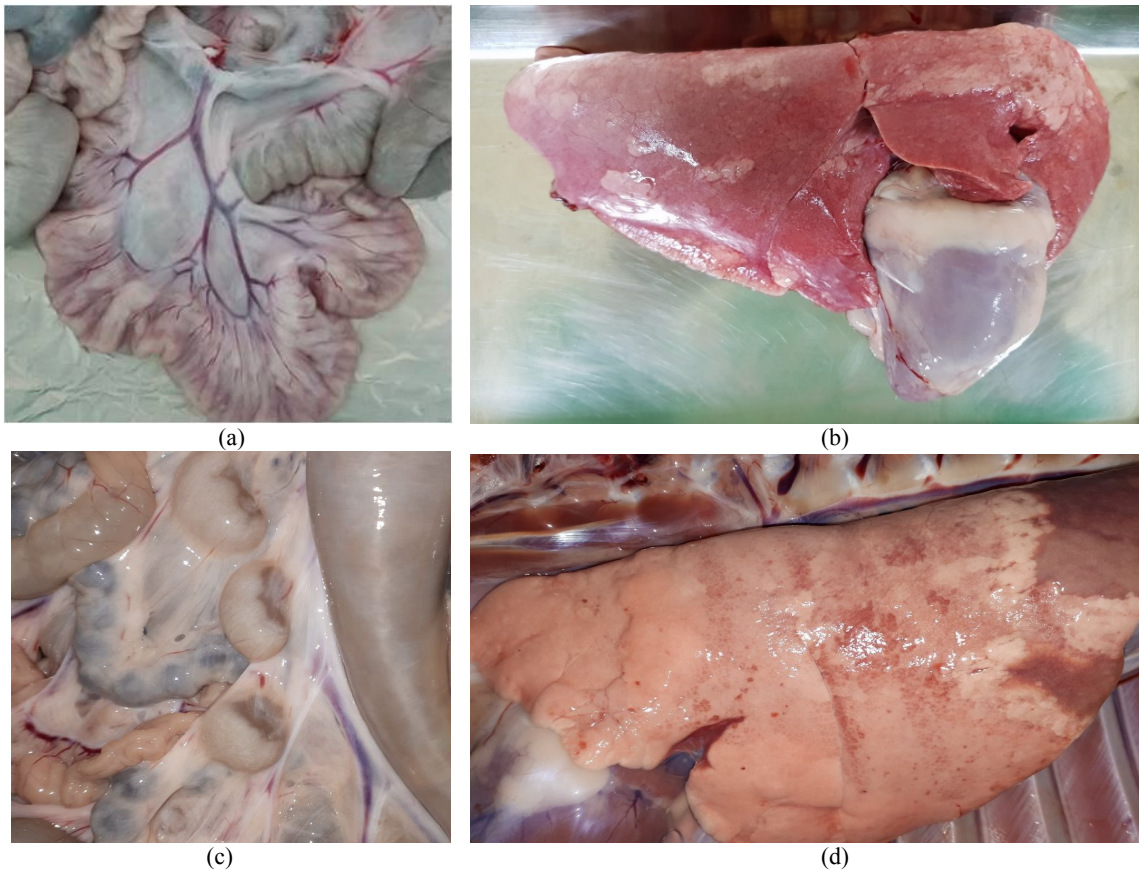


Fig. 5 Macroscopic lesions observed in sheep infected with *Pasteurella multocida* S14 strain: (a) intestinal congestion, (b) and (d) pulmonary congestion and (c) hypertrophy of mesenteric lymph nodes.

4. Discussion

Pneumonia is a major cause of death and economic losses in ruminants. *Pasteurella multocida* has been recognized as a primary or secondary bacterial pathogens associated with severe respiratory infections. In small ruminants, several studies have shown the high prevalence of either PM or MH in sheep from different regions of the world: USA [28, 29], France [30], Mexico [31], India [32, 33], Iran [34], Egypt [35], Ethiopia [36, 37], and Senegal [38]. In Morocco, the unique conducted study showed a higher predominance of *M. haemolytica* compared to *P. multocida* in both sheep and goats [17].

In North Africa, small ruminants' population is around 80 millions heads, contributing to a high percentage in farmers' income; that may be affected by respiratory diseases. Because of lack of data on the causative agents of this pathology, this study aimed to characterize isolates from pathogenic cases observed in the field and assess their virulence with the main objective to develop a specific vaccine to protect small ruminants against this infection.

Among samples collected from dead animals with clinical signs of respiratory diseases, almost half of them (32/55: 58%) showed presence of pasteurellosis: *Pasteurella multocida* (26/55: 47%) and *Mannheimia haemolytica* (6/55: 11%), confirming dominance of PM in the ruminants population. The results in this paper are in accordance with other studies [28, 39] conducted in Senegal and USA showing the higher prevalence of PM in the small ruminant population. This finding is, however, different from what has been reported by Sebbar *et al.* [17], regarding the dominance of MH in Morocco population of ruminants. Also, in Sudan and North Cameroun, *M. haemolytica* is shown to be the most isolated serotype in small ruminants' population with an important antigenic variety [38, 40, 41].

When considering species prevalence, among 55 tested samples, 14/37 were PM positive in cattle (38%)

and 12/18 (67%) in small ruminants with higher percentage in goats. This finding highlights sensitivity of small ruminants to respiratory diseases caused by *P. multocida*. All the 26 positive specimen were typed as serogroup A suggesting the absence of other serotypes in this population. This is in agreement with previous studies [15, 17, 42] showing the high prevalence of this serotype in ruminant population.

The two selected *P. multocida* isolates (S13 and S14), were genetically analyzed using partial 16S rRNA and *rpoB*, the most used genes for *Pasteurella* species identification and phylogeny [43]. Genetic analysis revealed that the two isolates are related to strains from different host species (bovine, porcine and avian) and regions, showing the worldwide distribution of the bacteria and its capability to cross species barrier. This is in accordance with previous phylogenetic studies, suggesting that the transmission of bacteria between different host species, may constitute a factor in the population biology of *P. multocida* [44-47].

In laboratory animals, the virulence of the two strains S14 and S13 was more pronounced in mice compared to guinea pig. The S14 isolate was more virulent than S13 in both mice and guinea pig. Infection study in mice showed that mortality occurs after 6 h at the dose 10^9 compared to lower doses (9 h to 67 h). The same observation has been reported by different authors [17, 48-50] having assessed *P. multocida* pathogenicity in mice. Also, results of this experiment suggest that mice may represent a suitable challenge model for *Pasteurella* vaccine evaluation. This finding was confirmed by other studies [48, 50-52] attempting to find an alternative to animal model for *P. multocida* and haemorrhagic septicaemia in cattle and avian pasteurellosis. In guinea pigs, pathogenicity was reduced compared to mice, but all animals reacted positively to the infection especially with the S14 isolate, suggesting that this animal could also be used to test pathogenicity or vaccine efficacy by challenge.

Healthy sheep infected with S14 appeared to have suffered severe illness and presented lesions similar to natural disease varying only in intensity. This could be attributed to an interaction between a number of factors such as concomitant infections, burden of infections, individual defence mechanism, and immunity level. The results in this paper indicate that the original sample was taken from animals that suffered primarily from *Pasteurella multocida* infection, and that the infectious dose 10^9 CFU is adequate to reproduce the disease in sheep. It can, therefore, be suggested that *P. multocida* is not always associated with viral infections and can be the main causative agent of pneumonia syndrome in sheep. The conducted experiment gives evidence that *Pasteurellosis* can be reproduced in sensitive sheep using a highly pathogen strain at an appropriate dose. This is the first time that experimental infection was conducted in sheep reproducing typical symptoms and lesions. A previous study reported experimental infection of PM in goats using intratracheal route with a lower dose (10^8 CFU) and succeeded in reproducing similar lesions in this species [53].

5. Conclusions

Investigations conducted in ruminant population affected by pneumonia in Morocco showed predominance of PM type A. Small ruminants seem to be more affected than cattle. The isolated strains from sheep are highly pathogen for mice and guinea pig causing mortality in few hours, suggesting that the mouse could be an alternative model for testing pathogenicity and vaccine efficacy. The PM S14 isolate induced characteristic symptoms and lesions in sheep when infected experimentally. This strain could be used as vaccine candidate to protect small ruminant's population against the infection due to *P. multocida*.

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All the authors have seen and approved the content

and have contributed significantly to the work. The authors gratefully acknowledge the support for this study by Multi-Chemical Industry (MCI).

Author Contribution

ZB₁ (Zineb Boumart) carried out the isolation, identification and culture of the isolates; and drafted the manuscript; ZB₂ (Zahra Bamouh) performed the pathogenicity study on mice, guinea pigs and sheep; NS (Noha Semmate) carried out genotyping, sequencing and phylogenetic analysis; KT (Khalid Omari Tadlaouia) participated in the design and the follow up of the study; ME (Mehdi EL Harrak) participated in the design of the study, manuscript drafting and data analysis and interpretation. All authors read and approved the final manuscript.

Conflict of Interest

The authors declare that they have no competing interests.

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