Biological proprieties of Pasteurella multocida isolate from sheep in Morocco

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Abstract

Background In Morocco, observations of pasteurellosis clinical cases reported by field veterinarian and lesions observed in abattoirs, suggest circulation of serotypes of pasteurellosis among small ruminants population. However, little is known on the prevalence of the main serotypes in this region and their pathogenicity. Results We investigated five suspected Pasteurella clinical cases from different provinces of Morocco. We succeeded to isolate 2 strains (S14 and S13) of Pasteurella Multocida A among the five isolates. Identification was achieved by biochemical and molecular biology methods. Phylogeny based on two genes sequence analysis (RNA16S and rpoB) suggested that the two isolates present similarity with others from different species. A pathogenicity study was conducted in mice, guinea pigs and sheep to set up a model for vaccine testing. The strain S14 was more virulent than S13 in laboratory animals and induced severe illness in sheep. Conclusion The high mortality of infected mice suggest that this animal may represent a good alternative for testing pathogenicity and vaccine efficacy.

Introduction

Morocco is a north African country with a large small ruminant population, 24 million, that occupy different biotopes. This population is relatively well supervised by experimented veterinary staff, belonging to private or public sector. Vaccination campaigns are regularly conducted against major small ruminant diseases such as Peste des petits ruminants, Sheep pox, Goat Pox and clostridia. However, observations of pasteurellosis clinical cases reported by field veterinarian and lesions observed in abattoirs, suggest circulation of different serotypes of pasteurellosis among small ruminants population in Morocco. Pasteurellosis in small ruminants has never been reported in the country and no scientific study was published on the identification and/or isolation of Pasteurella and especially there are almost no reports on the pathogenicity of Pasteurella originated from small ruminants.

Pasteurellosis is caused by Pasteurella multocida, Pasteurella haemolytica (reclassified in 1999 as Mannheimia haemolytica) and Pasteurella trehalosi. Pasteurella and Mannheimia organisms are gram-negative, non motile, coccobacillary, non-sporing, fermentative and facultative anaerobe of the Pasteurellaceae family. This family tends to inhabit the mucosal surfaces of the gastro intestinal, respiratory and genital tract of mammals. Pasteurella serotypes have different levels of virulence, host-species adaptability with possible inter-species transmissibility, antigenicity, immunogenicity, drug resistance and a lack of inter-serotype cross-reactivity [1, 2].

M. haemolytica and B trehalosi are distributed worldwide, and diseases caused by them are common in sheep and goats of all ages, although the prevalence of serotypes may vary by region and flock. M haemolytica is the most commonly isolated bacteria in clinical cases, followed closely by P trehalosi, P multocida is seen less frequently. P. multocida is a normal member of upper respiratory tract microbiota in a wide variety of species. However, stress caused by environmental factors (extreme cold), viral infections, and immunosuppression promote bacteria invasion of lung tissue and development of pneumonia. P. multocida is responsible for a wide range of infections in both domestic and wild animals,

causing bronchopneumonia and hemorrhagic septicemia in bovines, atrophic rhinitis in swine, fowl cholera in birds and human infections following animal bites [3–6]. Infections with P. multocida lead to great economic losses in the farming industry due to their severe morbidity and mortality. The species multocida is divided into three subspecies, namely P. multocida subsp. gallicida, P. multocida subsp. multocida and P. multocida subsp. septica, mainly on the basis of the ability to ferment dulcitol and sorbitol [7].

P. multocida is classified into five capsular serogroups (A, B, D, E and F) on the basis of capsular antigens and into 16 somatic serotypes, based on lipopolysaccharide and likely to be associated with specific types of disease [6, 8]. P. multocida serotypes B and E cause haemorrhagic septicaemia of cattle, buffalo, goat, camel and deer; serotype D causes atrophic rhinitis of pigs and rabbits; serotypes A and D cause enzootic pneumonia and shipping fever of cattle, sheep and pigs; and serotypes A and F cause avian cholera of all bird species [9–11]. Besides poultry, P. multocida in this serogroup has also been isolated from rabbits [12].

In this study, we investigated five suspected Pasteurella clinical cases in different regions of Morocco. We studied biological characteristic and pathogenesis in laboratory animals. We conducted molecular sequencing of the isolates and a challenge study on sheep and mice to set up a model for vaccine testing. The resulting pathogenesis in sheep was evaluated by following disease symptoms, lesions and bacterial charge in different tissues. This is the first time a Pasteurella strain was isolated in the small ruminant population of Morocco and characterized by sequencing and pathogenicity in sheep and laboratory animals.

Material And Methods

Clinical cases and bacterial isolates

Lung tissues were obtained from dead animals with lesions compatible with pneumonic disease: 1 goat (S11), 1 calve (S12) and 3 sheep (S13, S14 and S15), from distinct herd and regions of Morocco: Skhour Rhamna, Oujda, Berkan and Casablanca respectively.

Culture and biochemical identification

Lung tissue fragments were used to inoculate triptic soy agar supplemented with 5% sheep blood plates. Plates were then 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. A 24-hour pure Pasteurella suspected culture was also subjected to biochemical tests (catalase, oxydase and indole) and analysed by API 20NE (Biomerieux, France) biochemical identification kit, performed according to the supplier instructions.

Inoculum preparation

The organism was seeded in BHI (Brain Heart Infusion) broth and incubated at 37°C with shaking at 100rpm. The log phase cultures, used for injection animals, were prepared in BHI broth. The optical density (OD) of the culture was measured using 600nm wavelength light source. Bacterial concentration was determined by quantification via serial dilution.

Molecular detection and genotyping

Suspected colonies of Pasteurella were inoculated in vials containing 2 ml BHI broth and were grown for 24 h at 37°C for genomic DNA extraction that was performed using the Isolate II genomic DNA Kit (Bioline) according to the supplier instructions. Molecular detection of P. multocida, M. haemolytica and P. threalosi was carried out using specific primer for each species: M. haemolytica (FWD: 5'-GGCTATTATAGCTAACAAGCGGTC-3' and REV: 5'-GTTTGTAAGATATCCCATTT -3') [13], P. threalosi (FWD: 5'-GCCTGCGGACAAACGTGTTG-3'and REV: 5'-TTTCAACAG AACCAAAATCACGAATG-3') [14] and P. multocida (FWD 5'-GCTGTAAACGAACTCGCCAC-3' and REV 5'-ATCCGCTATTTACCCAGTGG-3'[15]. The mix for each PCR was prepared using 1x Standard Taq (Mg-free) buffer from NEB, 1.5 mM MgCl2, 0.2 mM dNTP, 0.4µM of each specific primer of P. multocida, M. haemolytica and P. threalosi, 2 units of Taq polymerase and 10 µg of DNA template to have a final volume of 25 µl. Polymerase chain reaction was performed with initial denaturation temperature of 95°C for 4min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 30 sec and final extension of 72°C for 5 min.

The amplified product was separated by electrophoresis on 1% agarose gel (Sigma Aldrich CHEMIE GmbH, Germany) in 1x TBE (89 mM Tris base, 89 mM Acid borique; 2 mM EDTA [pH 8.0]) buffer at room temperature using gradients of 10V/cm. For gel analysis, 10 µl of the product was loaded in each gel plot. 1Kb DNA Ladder from NEB was used to determine the fragment sizes. Finally, results were visualized after staining the gel in 0.5mg/ml ethidium bromide.

Following PCR analysis, a multiplex PCR assay was conducted for molecular characterization of the capsular antigens of P. multocida using primer sets specific for serogroups A, B, D, E, F [15, 16]. The serogroup-specific primer sets were identified according to the following criteria: (i) primer sets located within genes established as unique for each of the five serogroups (hyaD, bcbD, dcbF, ecbJ, and fcbD) (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 0.4µM, 1x Standard Taq (Mg-free), 2 mM MgCl2, each deoxynucleoside triphosphate at a concentration of 0.2mM, 2U 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 58°C for 30s, 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.

All amplification reactions were performed using Gene Amp 9700 PCR system (Applied Biosystem).

Sequencing and phylogenetic analysis

DNA was extracted from fresh bacterial cultures, using ISOLATE II Genomic DNA Kit (Bioline), and resuspended in 100µl of nuclease free distilled water. Five microliters of the extracted DNA were amplified using OneTaq DNA Polymerase (neb) Kit. The reaction was carried out in 50 µl using 16S ribosomal RNA [17] and rpoB [18] gene primer sets (table 2) under the following conditions: denaturation for 5 min at 95°C and 35 cycles of amplification as follows: denaturation at 94°C for 30s, annealing at 54°C for 30 s, extension at 72°C for 40 s, and final extension at 72°C for 5 min. The length of the products was 1400 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 then sequenced by GATC Biotech Company.

The sequences were aligned and compared with publicly available sequences representing genetic diversity among *Pasteurella multocida* strains of avian, bovine, ovine and porcine origin. A phylogenetic tree was constructed using the neighbour–joining method and Kimura two–parameter model in MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets [19].

Pathogenicity of the isolates in laboratory animals (mice and guinea pig)

In experiment 1, pathogenicity of the five P. multocida isolates was determined in ten 5 weeks old BALBc mice per strain (n=20) and six guinea pigs (n=12) by intra peritoneal inoculation of 0.5 ml of bacterial suspensions ($1x10^9$ cells/ml) grown in nutrient broth. The inoculated mice and guinea pig were observed for up to 3 days post inoculation for mortality.

In experiment 2, pathogenicity of the strain (S14) was determined in 22 mice by intra peritoneal inoculation of 0.5 ml of bacterial suspension ($1x10^9$ cells/ml). Mortality and time of death associated with each dilution (1/2, 1/5, 1/10, 1/20, 1/50, 1/1000, 1/2000, 1/3000, 1/4000, 1/5000, 1/6000, 1/7000 and 1/8000) were recorded to calculate $LD_{50.}$

Pathogenicity in sheep

Pathogenicity of the strain (S14) was determined in four 5 months-old healthy sheep. Before infection, nasal, conjunctival and rectal swabs as well as whole blood samples were collected to check the Pasteurella multocida status in sheep. Two sheep (G1) were inoculated with 4 mL subcutaneously, 2 ml orally and 2 ml by intranasal route of bacterial suspension ($1.0\ 10^9$ CFU/ml) and two other sheep (G2) inoculated with lower concentration ($1.0\ 10^7$ CFU/ml). Rectal temperatures were monitored daily during

the 7-day post infection (p.i). Clinical signs such as respiratory rate, respiratory symptoms, feed intake and general behaviour were evaluated throughout the study. Oral and nasal swabs were collected from infected sheep at days 3, 6 and 9 pi. At day 7 pi, animals were autopsied and their lungs, mesenteric lymph nodes, liver and spleen were collected for bacteriological and PCR analysis.

Results

Clinical cases

Lung tissues were obtained from dead animals: 3 sheep, 1 calve and 1 goat, from distinct herd and regions of Morocco. At necropsy, animals had lesions compatible with pneumonic disease including: pleurisy with abundant and fibrinous pleural effusion, pericarditis with gelatinous exudate on the pericardium, bronchopneumonia with increased volume of the lungs which are edematous and haemorrhagic and exudative mucopurulent inflammation in the respiratory tract.

Culture and biochemical identification

From the 5 field specimens isolated from sheep, calve and goat(S11, S12, S13, S14, S15), P. multocida was recovered from only two tissue lung samples of sheep (2/5). All isolates with positive reactions for catalase, oxidase, and indole production, were confirmed as P. multocida by API 20NE.

Molecular detection and genotyping of Pasteurella

The 5 field specimens isolated were used for detection of *P.* multocida, threalosi and *M. haemolytica* by PCR assay. From these specimens PCR showed 2 positive results (S13 and S14) and only *P. multocida* was detected. Both Pasteurella multocida strains were typed as serogroup A by the multiplex capsular PCR assay.

Sequencing and phylogenetic analysis

Phylogenetic analysis using ARN 16S gene among the two P. multocida strains (S13 and S14) demonstrated that S13 isolate was closely related to both an England strain (PM30) (99.5%) [20] isolated from bovine host and to a porcine isolate (IVRI) originated from India (99.7 %) [21]. The S14 isolate was related to the Chinese PmCQ6 strain isolated from bovine host and evaluated as low virulent strain [22].

The RpoB gene analysis showed a similarity (99.38%) between the strain S13 and 964 isolated from goose host and originated from Hungary [23]. The strain S14 was related to the Chinese strain PM-L1706 (99.33%) isolated from a chicken host (MG813902).

Determination of virulence *P. multocida* isolates in laboratory animals (mice and guinea pig)

In experiment 1, the pathogenicity of 2 isolates was estimated by infecting 2 groups of 10 mice and 6 guinea pigs with 0.5 mL of a log phase culture containing infective doses of 1.10^9 CFU. Inoculated animals were observed for 3 days post inoculation for mortality. All mice infected with the strain S14 died in a short period of 15h, while those infected with the strain S13 showed 60% of mortality within 24h. Similarly, in guinea pig the strain S14 registered a higher percentage of mortality with a short time period (66%, 24h) compared to the strain S13 (33%, 44h). However, it seems that the virulence of these two strains is more pronounced in mice than in guinea pig (table 4).

In experiment 2, virulence of the strain S14 was determined in a group of 22 mice based on mortality and time of death associated with different culture dilutions (table 4). All mice died at tested dilutions with a time period that ranged from 6h to 67h.

Pathogenicity in sheep

Clinical signs

Sheep were allowed to acclimate to the laboratory environment for a period of 2 weeks prior to experimental infection with Pasteurella multocida. During that time, all experimental animals were healthy and free of disease, with normal rectal temperatures. Animals of G1 (17 and 18) injected with the bacterial suspension, suffered from dyspnea, coughing, nasal discharge and developed a long hyperthermia (above 39°C) for 6 days: a peak was noticed between day 1 and day 3 p.i (40.5°C) for animal 17 and at day 2 pi (40.2°C) for animal 18 (figure 3). Animals of G2 did not show any clinical symptoms but presented a hyperthermia (above 39°C) for 4 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 (figure 3).

Respiratory symptoms

Both groups of animals presented an important 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 986 (55 breath/min), day 3 for animal 18 (60 breath/min) and day 6 for animal 987 (40 breath/min) (figure 4).

Post mortem lesions

At post mortem at day 7 pi, sheep of G1 inoculated with $1.0\ 10^9$ CFU presented lesions in both left and right lung, hypertrophy of mesenteric lymph nodes and intestinal congestion (figure 5). For G2 animals,

no lesions were observed at post mortem.

Bacteria excretion and tissue charge

In G1 inoculated with 1.10^9 CFU, Pasteurella has been detected by PCR in nasal swabs and lung in both animals 17 and 18 between d3 and d7 pi. In orotracheal swabs, the genome has been detected only in animal 18, between d6 and d7 p.i. In G2 inoculated with 10^7 CFU, no Pasteurella has been detected in nasal and orotracheal swabs. In the other organs (spleen, liver, mesenteric and pulmonary lymph nodes), no pasteurella has been detected for both groups G1 and G2.

Discussion

To date, no scientific studies were published on the isolation and identification of Pasteurella in Morocco. This bacterium is usually associated with viral diseases such as PPR which is endemic in North Africa. In this region, the prevalence of different serotypes still unknown, however, field veterinarian reports on clinical cases and lesions observed in abattoirs, suggest circulation of Pasteurella or Manheimia among ruminant populations.

No vaccination compaigns against pastereurella are conducted in small ruminant population. Recently, two vaccines against pasteurellosis combined to clostridia have been introduced in the market with satisfactory results in the field, and a clear difference was observed between vaccinated and unvaccinated flocks regarding zootechnique performance and health general status.

The present study aimed to determine circulating Pasteurella strains in small ruminants and assess their pathogenicity with a main objective to develop a specific vaccine that could match to the field needs.

In our study we collected five samples from dead animals in the field with clinical symptoms similar to Pasteurella. Only two of the 5 strains were identified as Pasteurella multocida A suggesting that this serotype is dominant in the field, but other agents maybe incriminated since 3 isolates need to be identified. Indeed, studies previously conducted in other African countries, showed the predominance of Pasteurella multocida in sheep from Senegal [24], however, in Sudan and north Cameroun, M. Heamolytica was the most isolated serotype in small ruminants population with an important antigenic variety [25–27].

The two Pasteurella isolates fulfilled biochemical and molecular criteria of identification. These 2 strains, were genetically analysed using partial 16S rRNA and rpoB gene sequence comparison, the most used genes for Pasteurella species identification and phylogeny [28]. Phylogenetic study revealed that both P. multocida A ovine isolates (S14 and S13) are related to strains from different host species (bovine, porcine and avian), suggesting that this type is not specific to one animal species and may cause haemorrhagic septicaemia in pigs, cattle and avian hosts; which is in accordance with other previous

phylogenetic studies [29–31]. Thus, the transmission of bacteria between different host species, may constitute a factor in the population biology of P. multocida [32].

Pathogenicity on laboratory animals showed high sensitivity of mice to Pasteurella infection and the higher virulence of the strain S14 compared to S13 in both mice and guinea pig, that showed different mortality time depending on the strain and the inoculated dose. This suggest that the S14 strain may have caused severe clinical symptoms and important mortalities in the field. Furthermore, kinetic infection study with S14 strain in mice showed that mortality occurs rapidly after 6h using the dose 109 and there was marginally later onset with lower doses (9h to 67h), which is in accordance with different studies having assessed P. multocida pathogenicity in mice [33–35]. Also, results of this experiment suggest that mice may constitute a suitable challenge model for Pasteurella vaccine evaluation. This results were confirmed by other studies attempting to find an alternative animal model for P. multocida and haemorrhagic septicaemia in cattle and avian pasteurellosis [33, 35–37], but more investigations are needed to develop this model.

Regarding guinea pigs, pathogenicity was reduced compared to mice, but all animals reacted positively to the infection by both S14 et S13 isolates with an advantage of S14. This species could also be used to test vaccine potency by challenge.

Clinically, sheep infected with S14 appeared to have suffered the most severe illness and presented lesions similar with the natural disease varying only in intensity. The infectious dose, 10^9 seems to be adequate to reproduce the disease in sheep. However, observed symptoms were much more severe in one sheep than in another that showed only lung lesions and mild symptoms. This could be attributed to an interaction between a number of factors such as burden of infections, individual defence mechanism, and immunity level.

Conclusion

Our study suggests that multocida A is frequent in the country with high incidence and economical losses. However, more investigations and survey studies are required to conclude on the predominance of Pasteurella serotypes in small ruminants population. To control the disease, vaccination remains the most efficient tool, particularly with vaccines based on local strains.

Declarations

Ethics approval and consent to participate

Animal experiments were carried out in accordance with the international guidelines for care and handling of experimental animals, as the protocol has been submitted and approved by the Internal Ethic Committee “The internal ethic committee for animal experiment, MCI santé animale”.

Consent for publication

Not applicable

Availability of data and material

All data generated or analysed during this study are included in this published article

Competing interest

The authors declare that they have no competing interests

Funding

No funding was obtained for this study

Authors' contributions

ZB_1 carried out the isolation, identification and culture of the isolates; and drafted the manuscript, ZB_2 and LR performed the pathogenicity study on mice, guinea pigs and sheep. MJ carried out genotyping, sequencing and phylogenetic analysis, KT participated in the design and the follow up of the study, ME participated in the design of the study, manuscript drafting and data analysis and interpretation. All authors read and approved the final manuscript.

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Tables

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	460
		KMT1SP6-REV GCTGTAAACGAACTCGCCAC	
A	hyaD-hyaC	CAPA-FWD TGCCAAAATCGCAGTCAG	1,044
		CAPA-REV TTGCCATCATTGTCAGTG	
B	bcbD	CAPB-FWD CATTTATCCAAGCTCCACC	760
		CAPB-REV GCCCGAGAGTTTCAATCC	
D	dcbF	CAPD-FWD TTACAAAAGAAAGACTAGGAGCCC	657
		CAPD-REV CATCTACCCACTCAACCATATCAG	
E	ecbJ	CAPE-FWD TCCGCAGAAAATTATTGACTC	511
		CAPE-REV GCTTGCTGCTTGATTTTGTC	
F	fcbD	CAPF-FWD AATCGGAGAACGCAGAAATCAG	851
		CAPF-REV TTCCGCCGTCAATTACTCTG	

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

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 dilutions of P. multocida S14 culture.

Dilutions	% of mortality	Lethal time (h)
Pur	100	6
1/2	100	7.5
1/5	100	9.4
1/10	100	14
1/20	100	14
1/50	100	9.5
1/1000	100	28
1/2000	100	28
1/3000	100	28
1/4000	100	28
1/5000	100	58.5
1/6000	100	64.5
1/7000	50	67
1/8000	0	-

Figures

Figure 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.

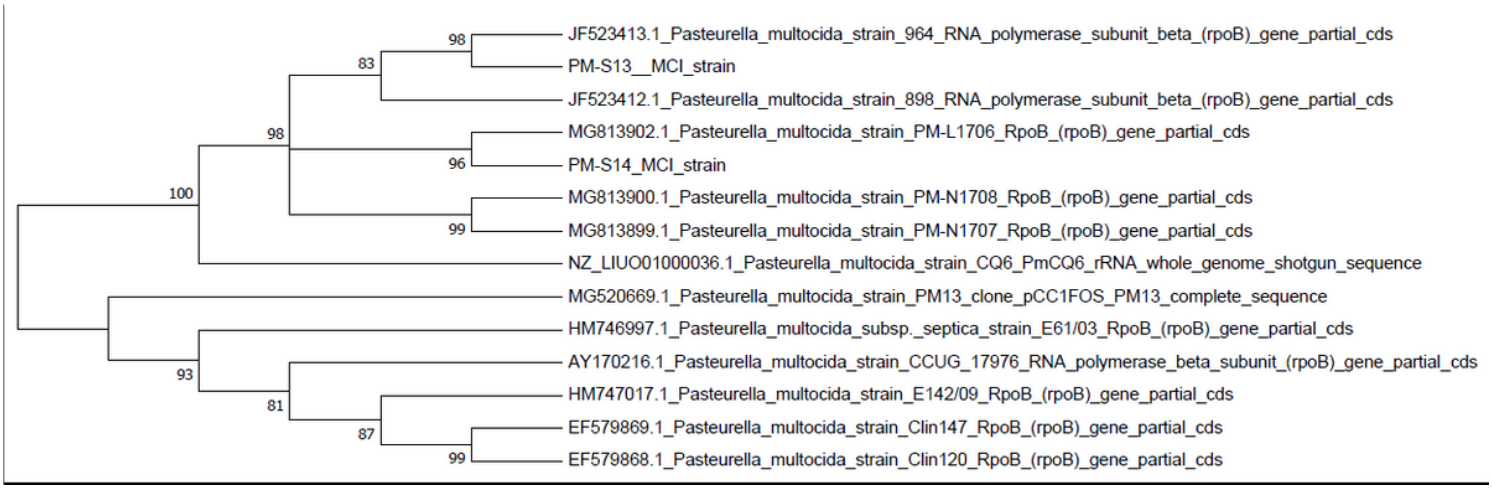

Figure 2

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.

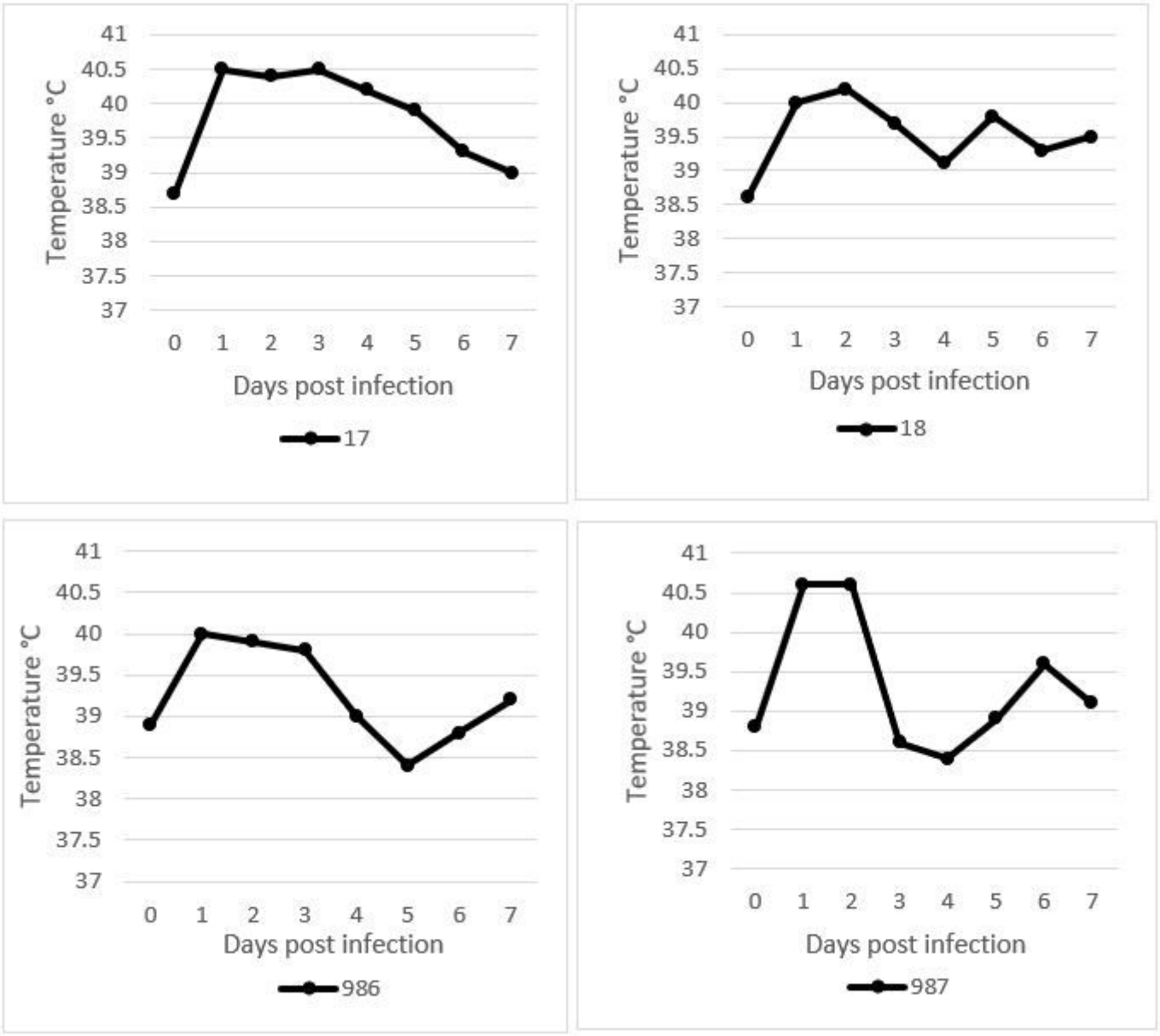

Figure 3

Rectal temperature of infected sheep G1 (17 and 18) and G2 (986 and 987) with Pasteurella multocida strain (S14).

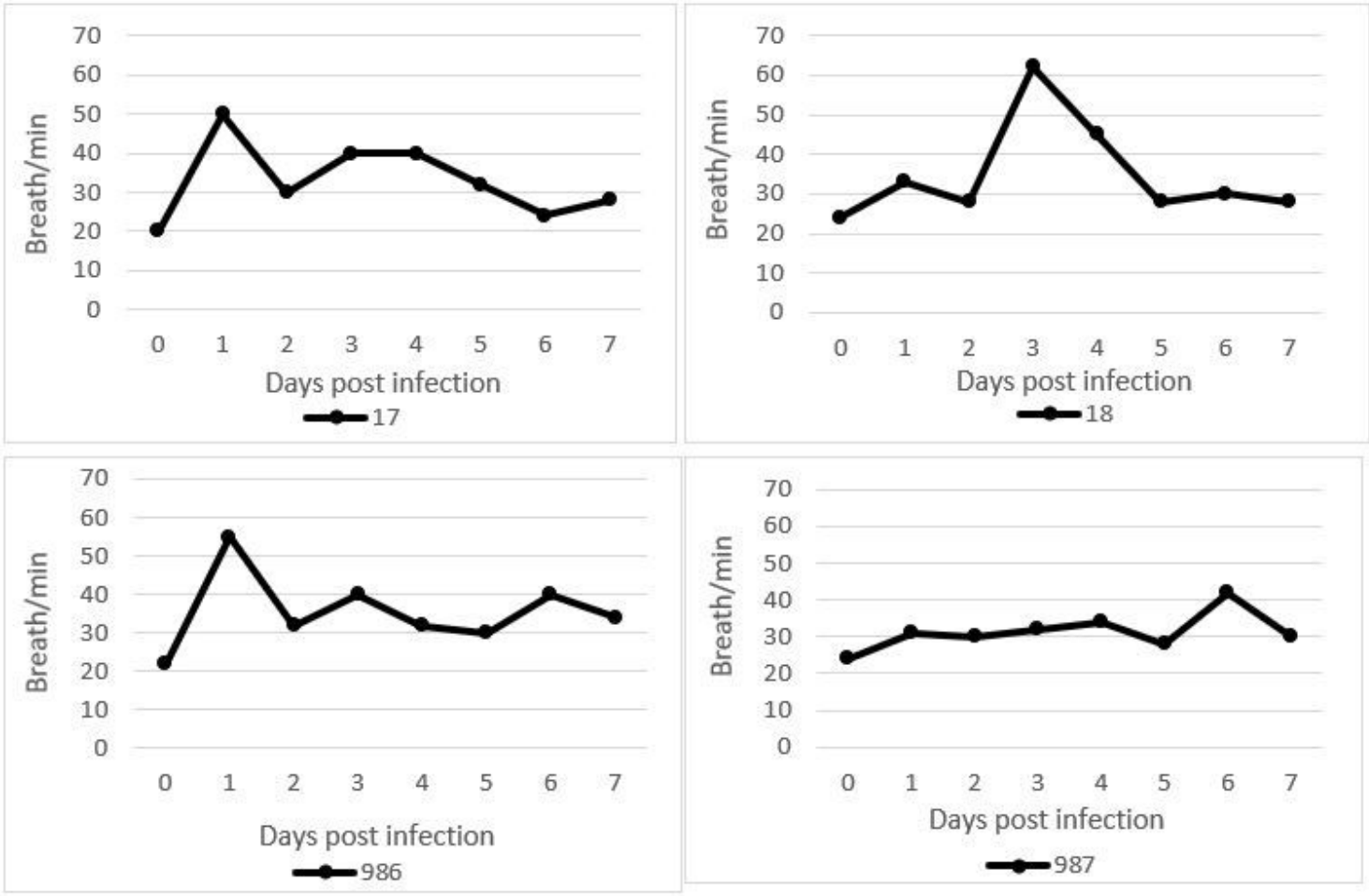

Figure 4

Breathing rate of infected sheep G1 and G2 with Pasteurella multocida strain (S14)

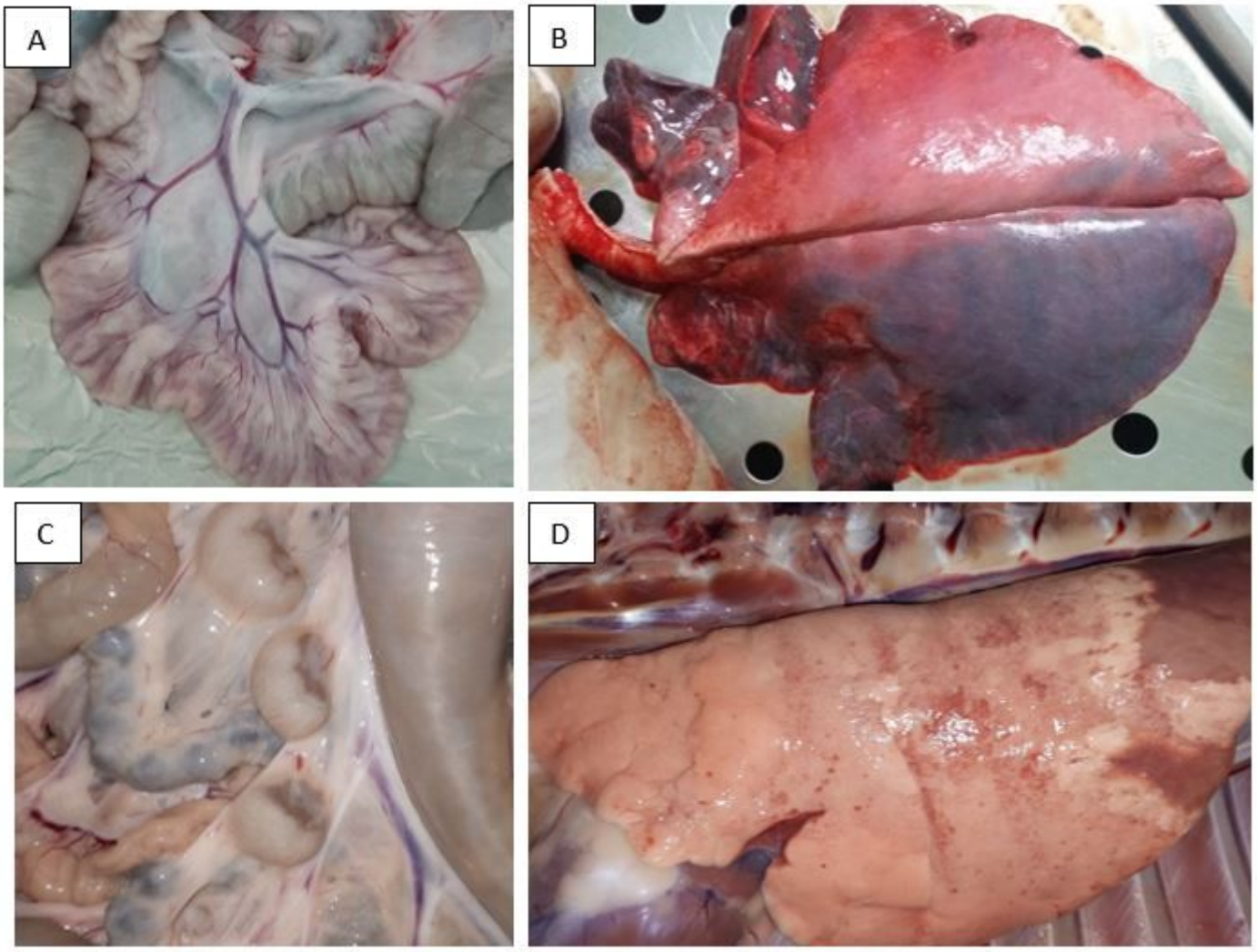

Figure 5

Macroscopic lesions observed in infected sheep with Pasteurella multocida S14 strain: (A) intestinal congestion, (B) and (D) pulmonary congestion and (C) hypertrophy of mesenteric lymph nodes.