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5 Epidemiological Study on Bovine Parainfluenza 3 Virus in Sheep: 6 Seroprevalence, Risk Factors and Distribution in Two Regions of Algeria

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Abstract

BACKGROUND: Respiratory viral diseases cause significant economic losses in ruminants, where the bovine Parainfluenza 3 virus is a member. There is no available data regarding the epidemiological situation of the virus in Algeria.

OBJECTIVES: The present study aims to determine the seroprevalence and identify the associated risk factors of bovine Parainfluenza 3 virus (BPI3V) in sheep from two different climatic regions of Algeria.

METHODS: A total of 108 serum samples were collected from sheep at different ages and tested for antibodies against BPI3V using an indirect enzyme-linked immunosorbent assay (ELISA). Additionally, RT-PCR tests were performed on nasal swabs to detect the viral genome.

RESULTS: At the animal level out of 108 sera tested, 82 (75.93%, 95% CI [66.75-83.63]) showed antibodies against BPI3V. At the herd level all 23 herds tested (100%) had at least one animal with BPI3V antibodies.

Our results showed no association between the presence of antibodies to BPI3V and the region (*P*=0.72). however, at the herd level, risk factors such as flock size and favorable factors like climate change, feed deficit, postpartum stress, and dust were identified. At the animal level, a highly significant association was found between BPI3V seroprevalence and the age of the animals (*P*<0.0001). Specifically, the sheep group over 3 years was more susceptible than other age groups. Furthermore, a significant difference in BPI3V seroprevalence based on sex was observed (*P*<0.003). All collected nasal swabs were negative for BPI3V genome detection using real-time PCR.

CONCLUSION: This study represents the first serological survey on BPI3V in Algeria and confirms its circulation in sheep from two regions. The high serum prevalence of BPI3V observed in the study

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population emphasizes the importance of addressing this viral disease to mitigate economic losses in ruminants.

KEYWORDS: Algeria, Bovine parainfluenza 3 virus, ELISA, Risk factors, RT-PCR

Introduction

The small ruminant respiratory complex is one of the major causes of morbidity and mortality in sheep flocks. It results from exposure to adverse weather conditions, animal movement, overcrowding, and stress which increase the susceptibility of animals to viral and bacterial infections (Scott, 2011). The prevalence of PI3 virus has been documented in various countries, including Egypt 2.2% (Gafer *et al*., 2009), Iran 95% (Shoukri *et al*., 2013), Turkey 10.53% (Ceribasi *et al*., 2014), and India 3.69% (Kamdi *et al*., 2020).

Respiratory viral infections have a severe economic impact on ruminants. BPI3V, along with other viruses such as bovine respiratory syncytial virus (BRSV), bovine herpes virus-1 (BHV-1), and small ruminant morbillivirus (SRMV), in conjunction with bacteria and mycoplasma (Mashhour *et al*., 2020; Ashrafi *et al*., 2022), contribute to the respiratory disease complex in ruminants, leading to severe illness (Ellis, 2010; Rasooli *et al*., 2023).The most important risk factors for ruminant respiratory disease are low environmental temperature and high humidity, increased animal density, stress, dust, poor ventilation, and parasites (Goodwin-Ray *et al*., 2008; Scott, 2011).

BPI3V is an enveloped, non-segmented, negative-sense, single-stranded RNA virus that belongs to the genus *Respirovirus* in the family *Paramyxoviridae* (Ellis, 2010; Newcommer *et al*., 2017). This virus is widely distributed and causes respiratory tract infection in cattles (Alcan *et al*., 2000), sheep(Gafer *et al*., 2009), goats (Eberle *et al*., 2015), camels (Ma *et al*., 2021). moreover, PI3V can be transmitted between different species (Brako *et al*., 1984). In

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73 areas where cattle are infected with PI3V, a similar rate of infection is expected in small
74 ruminants (Yesilbag and Gungor, 2009). BPI3V infection seems to predispose the host to
75 secondary bacterial infections (Murphy *et al*., 1999) due to its immunosuppressive effects
76 (Ellis, 2010), especially under stressful conditions (Haanes *et al*., 1997). The prevalence of
77 this viral pathogen has been reported in several countries (Solis-Calderon *et al*., 2007;
78 Betancur *et al*., 2017). However, there is a lack of documentation regarding the epidemiology
79 of respiratory diseases caused by BPI3V in small ruminants in Algeria. Therefore, our study
80 aimed to conduct a preliminary serological analysis and genome detection of the BPI3V on
81 sheep, as well as identify the risk factors associated with BPI3V seropositivity in two regions
82 of Algeria.
83

84 Materials and Methods

85 Ethical statement
86 The ethical statement is not required for this study since it does not involve any harm to the sheep. It
87 is important to note that no sheep were harmed during the sampling process.
88 The sheep included in this study are owned by private sheep farmers who were fully informed about
89 the objectives of the research. The sampling procedures were carried out by certified veterinarians
90 with the explicit consent of the sheep owners.
91 All methods employed in this study adhered to the regulations set by Algeria regarding the handling
92 and treatment of domestic animals, specifically Law 08-88 of 26th January 1988 on the activities of
93 veterinary medicine and the protection of animal health.

94 Study areas

95 This study was carried out during the winter and spring seasons of the year 2018 in two
96 different climatic regions of Algeria (Batna and Boumerdes). Batna is located in the eastern

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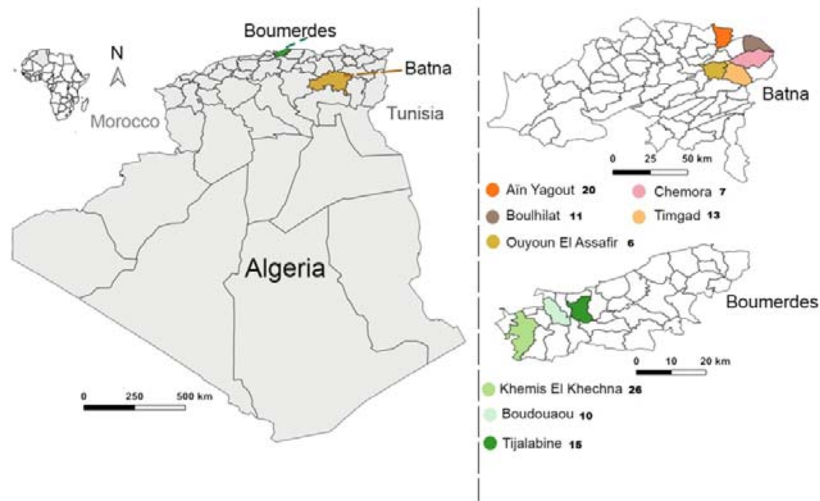
97 part of Algeria, belonging to the Aurès mountain range (4°7′ N, 35°36′ E). It has a semi-arid
98 climate with an annual rainfall of 496 mm. The average temperature is 4°C in January and
99 35°C in July. Winter nights experience temperatures below freezing with frequent frosts,
100 while summer temperatures can reach up to 45°C in the shade. The region of Batna has a
101 sheep population potential of 1.137.361, including 638.423 ewes (DSA Batna, 2019).
102 Boumerdes is located on the central coast of Algeria (36°46′ North, 3°28′ East) and has a
103 humid climate characterized by two distinct seasons: mild, rainy winters and hot, humid
104 summers, with an average annual rainfall of 672 mm. The sheep population potential in
105 Boumerdes is 33942, including 13470 ewes (DSA Boumerdes, 2020).
106 The samples were taken from sheep farms located in 5 districts of Batna and 3 districts of
107 Boumerdes (Figure 1).



108

109 **Figure 1:** The geographical location of districts, in Batna and Boumerdes regions, Algeria.

110

111 **Study population and sample size determination**

112 The cross-sectional study focused on herds with history of respiratory disease to detect the
113 circulation of BPI3V. A total of 23 flocks were included in the study, with 16 out of 23 being

114 mixed flocks (promiscuity of sheep with cattle and goats), while 7/23 were sheep flocks. The
115 flocks consisted of 1127 crossbreed sheep, with an average flock size of 49 (10-150).). From
116 these flocks, 108/1127 (9.58%) sheep with respiratory signs (sero-mucosal/mucopurulent
117 discharge, cough, tachypnea, dyspnea, and, fever) or without were selected for this study, of
118 which 80 were females and 28 males, aged between 4 months and 6 years (mean age: 2.8
119 years). It is important to note that the sheep included in this study had not been vaccinated
120 against PI3V.

121 The majority of the sheep sampled were females accounting for 80 out of the 108 (74.07%).
122 This imbalance in gender distribution can be attributed to the fact that the studied farms
123 primarily focused on breeding programs, where lambs were raised until they reached 3
124 months of age.

125 The sample size for this study was determined using a table for estimating prevalence in a large
126 population with desired fixed-width confidence limits, following the sampling method provided by
127 Thrusfield (2005). Based on an expected prevalence of 10% (as reported by Saeed et al., 2016 in
128 Sudan), a desired absolute precision of 5%, and a confidence level of 95%, the table indicated a
129 sample size of 99 (Thrusfield, 2005). However, the sample size was increased to 108 as the remaining
130 reactions in the second kit were used to repeat suspected reactions

131

132 **Sample and data collection**

133 Blood samples from individual sheep were collected by directly puncturing the jugular vein
134 using vacuum tubes (5 mL Vacutainer®). The blood samples were identified and transported
135 to the laboratory and were left at room temperature until a clot has formed. After, sera were
136 obtained by centrifugation at 3000 rpm for 10 min, transferred to sterile 1.5 ml tubes

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137 (Eppendorf), and stored at -20 C° until the examination. The samples were processed in the
138 laboratory of the Biotechnology Research Centre in Constantine, Algeria.
139 Nasal swabs were taken from sheep showing clinical signs of pneumonia and placed in a viral
140 transport medium (VTM) (Xpert®). Samples were identified and transported on ice to the
141 laboratory and stored at -80°C until analysis. Molecular analysis was performed at the
142 laboratory of Pasteur Institute in Algeria for influenza and other respiratory viruses, Sidi-Fredj
143 Unit.
144 Data at both the animal and herd levels were collected concurrently with the serosurvey
145 through interviews with willing farmers. To facilitate this process, a semi-structured
146 questionnaire was prepared. The questionnaire primarily focused on gathering information
147 related to animal biodata, such as age and sex. Additionally, it included questions regarding
148 the region of study, including climate and season, as well as herd management data, such as
149 hygiene practices and herd size. These later factors were categorized as follows: age (<1 year,
150 [1-3] years, >3 years), sex (male, female), study area (Boumerdes, Batna), hygiene level
151 (dirty, fair, and clean), season (winter, spring), promiscuity with other animals (yes, no), the
152 introduction of new animals into the herd (yes, no), transport (yes, no), herd size (10-50,
153 51-100, >100-150), favorable factors (climate change, feed deficit, postpartum stress and,
154 dust).
155

156 **Serological analysis**

157 A commercial indirect ELISA kit developed by Bio- X Diagnostics, Jemelle Belgium (BIO K
158 239/2), was used to detect antibodies against BPI3V in sheep. The test was performed
159 according to the manufacturer's instructions. Serum samples were diluted in PBS (1:100),
160 volumes of 100 µl were dispensed into each well and incubated for one hour at 21°C +/- 3°C,

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then rinsed 3 times with wash buffer. A solution of bovine immunoglobulin peroxidase conjugate was dispensed into each well and incubated for a further hour at 21°C+/-3°C. After the second incubation, the plate was washed again and chromogen (tetramethylbenzidine) was added to each well of the plate and incubated for 10 min in the dark at room temperature. Supposing specific immunoglobulin is present in the test sera. In that case, the conjugate remains bound to the microwell containing the viral antigen, and the enzyme catalyzes the transformation of the colorless chromogen into a pigmented compound. The resulting blue color's intensity is proportional to the title of the specific antibody in the sample. To halt the reaction, 50 µl of stop solution (phosphoric acid) was added.

Finally, the optical density (OD) was measured at 450 nm by the EnSpire® multimode plate reader.

(Indirect ELISA, Bio- X Diagnostics, Jemelle Belgium (BIO K 239/2))

Molecular analysis

Nucleic acid extraction: Viral RNAs were extracted with the QIAamp® Viral RNA Mini Kit (Qiagen) using 140 µl of each sample according to the manufacturer's instructions. Nucleic acids were eluted in a final volume of 60 µl and stored at -80 °C until examination.

Real-time polymerase chain reaction (RT-PCR): 86 samples were tested by RT-PCR for bovine Parainfluenza 3 virus RNA using the ViroReal® bovine Parainfluenza 3 virus kit (DVEV02811). All reactions were performed in a total volume of 20 µl containing: 10 µl of sample eluate and 10 µl of Master Mix which consisted of 2 µl of nuclease-free water, 5 µl of RNA reaction mix, 1 µl of bovine PI3 assay mix, 1 µl of IPC RNA assay mix and 1 µl of IPC RNA target diluted at 1:500. Reactions were performed using the ABI PRISM ® 7500 one-step reverse transcription real-time PCR thermocycler (Applied Biosystems). Samples were amplified in 47 cycles starting with the first step, which is the synthesis of the DNA strand

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185 complementary to the viral RNA by reverse transcriptase at 50 C° for 15 min, followed by a
186 denaturation step at 95 C° for 25 seconds, and a final elongation step at 60 C° for 1 min.
187 (ViroReal® bovine Parainfluenza 3 virus kit (DVEV02811)).

188 **Statistical analysis**

189 The apparent prevalence (AP) was obtained by dividing the number of positive animals by the
190 number of animals tested.

191 Univariable statistical analyses of the present study were performed using R Statistical
192 Software (version 4.0.2). An initial explanatory analysis was performed using the Chi-square
193 test and Fisher's exact test to assess the independence between risk factors and BPI3V
194 seropositivity. Variables with a *P*-value less than 0.2 were deemed statistically significant and
195 selected for multivariable analysis using a regression model. A binary logistic regression
196 model was applied to measure the association between BPI3V seropositivity and risk factors
197 using IBM Spss®, version 22.0 (Armonk, NY, USA). The variables were considered as risk
198 factors if the odds ratio >1 and the $P \leq 0.05$.
199

200 ## Results

201 **Serological study of BPI3V**

202 **Seropositivity:** At the time of sampling, all the investigated herds had a history of respiratory
203 diseases. Serological results are summarized in Table 1.

204 At the individual animal level, out of the 108 sheep sera tested, 82 (75.93%, 95% CI [66.75%-
205 83.63%]) were positive for BPI3V, while 26 (24.07%) were negative.

206 At the herd level, all 23 out of 23 farms (100%) had at least one animal with antibodies
207 against bovine parainfluenza virus type 3.

208 **Risk factors of parainflunza virus 3**

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209 **At the individual animal level:** Animal-related factors, such as age and sex were significant
210 in the explanatory analysis. The highest seroprevalence was observed in older sheep (>3
211 years) in both study regions (93%), However, the lowest seropositivity rate was recorded in
212 lambs (<1 year) (53%). Seropositivity for BPI3V was significantly higher in females
213 compared to males (*P*<0.003; Table 1), with rates of 84% and 54% respectively. Age was
214 included in the binary logistic regression model and was identified as a risk factor for BPI3V
215 infection. Adult sheep (>3 years) had a higher susceptibility to developing antibodies against
216 BPI3V compared to young sheep (<1 year) (*P*<0.0001; Table 1). Sheep aged over three years
217 exhibited a higher predisposition to developing antibodies against BPI3V in comparison to
218 sheep under one year of age (OR=6.94; 95% IC [1.16-41.44]; Table 2).
219 **At the herd level:** Among herd-related factors, only flock size and favorable factors were
220 significant in univariable analysis and were subjected to multivariable analysis (binary
221 regression). The presence of BPI3V antibodies was significantly higher in sheep with feed
222 deficit (17/19) 89% and those exposed to climate change (43/50) 86% (*P*<0.005; Table1).
223 There were significant differences in BPI3V seroprevalence based on flock size, with a lower
224 chance of having a seropositive animal in larger flocks compared to smaller flocks (*P*<0.003;
225 Table 1), with rates of 61% and 72% respectively.
226 However, other factors like hygiene, transportation, and introducing new animals were not
227 significant. Although, it was expected that promiscuity with other animals specifically cattle,
228 on the farm would be a significant risk factor for BPI3V seropositivity, but this factor was not
229 significant (*P*<0.94).
230 At the region level: The seroprevalence of BPIV3 was similar between sheep from the Batna and
231 Boumerdes regions, at 74% and 78% respectively. The explanatory analysis revealed no association

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between the presence of antibodies to BPI3V and the different climatic regions studied (P<0.72, Table 1).

Molecular study of BPI3V

All nasal swabs collected in this study were passed through real-time PCR to detect the genome of BPI3V. Of the 86 examined swabs, no sample was found positive for the bovine Parainfluenza 3 virus, as shown in Figure 2.

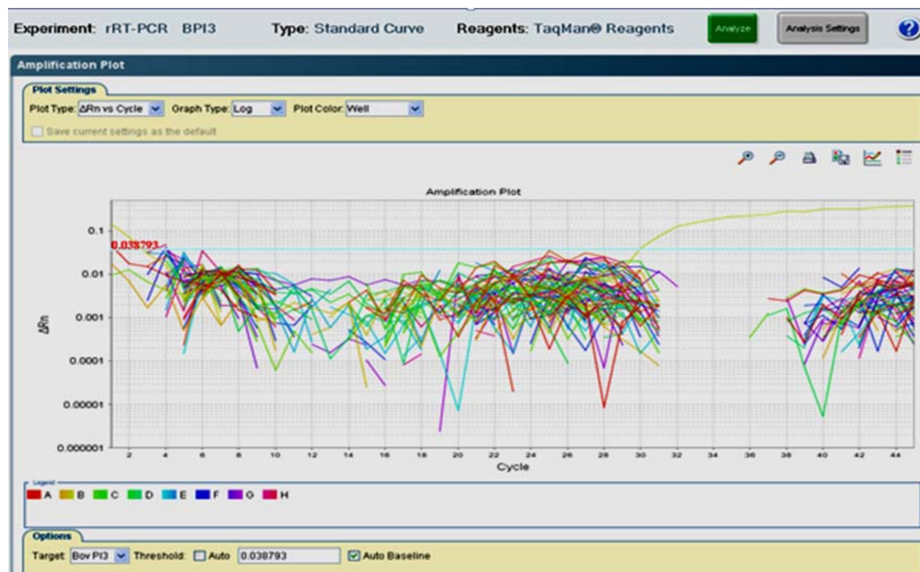

Figure 2: The logarithmic amplification curves of the samples tested and the positive control.

Discussion

The prevalence of the PI3 virus in naturally infected flocks around the world is assessed using PCR, RT-iiPCR, culture, virus isolation, electron microscopy, DFAT, IFAT, and IP techniques (Tiwari *et al*., 2016; Jarikre and Emikpe, 2017; Emikpe *et al*., 2019; Ma *et al*., 2021; Ren *et al*., 2023).

Several serological surveys conducted in many countries reported a wide distribution of the PI3 virus in sheep (Cabello *et al*., 2006; Gafer *et al*., 2009; Saeed *et al*., 2016). This study represents the first seroepidemiological survey of BPI3V in sheep flocks in two different

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248 climatic regions of Algeria. Our study confirmed the circulation of the BPI3V, with a
249 seroprevalence of 75.93% (82/108).
250 A higher prevalence was reported in Brazil with rates of 82% (Aline *et al*., 2018) and 52.5%
251 (Franco *et al*., 2020). However, several studies have reported lower prevalence rates: 16.7%
252 in Grenada (Tiwari *et al*., 2016), 11.73% in Japan (Giangaspero *et al*., 2013), 8.8% in Turkey
253 (Yesilbarg and Gungor, 2009), 62.2% in Iran (Hazrati *et al.*,1976) and in Sudan, BPI3V
254 antigen was detected in 9.8% of sheep lung samples (Saeed *et al*., 2016).
255 The variation in prevalence estimates in sheep from different countries can be explained by
256 diverse factors such as differences in geographical region, husbandry methods and
257 management conditions, flock size, type of farming, age of animals, disease management,
258 disease control programs, type of samples taken and laboratory diagnostic methods (Mainar-
259 Jaime *et al*., 2001; Hussain *et al*., 2019). After 6 to 8 weeks, the levels of mucosal antibodies
260 against the virus decrease significantly, while serum antibodies remain present for a period of
261 3 to 5 months (Makoschey and Berge., 2021). It's important to note that antibody detection in
262 serum does not indicate recent illness, whereas detection of viral antigens requires samples in
263 the acute phase of the disease.
264 Many risk factors predispose to respiratory disease complex. These include herd-related
265 factors such as temperature changes, animal transportation, feed changes, high stocking
266 density and the introduction of an animal into the herd, etc. Animal-related factors such as the
267 age of the animal and its immune status also play a role (Figueroa-Chavez *et al*., 2012). In this
268 study, there were no significant differences between the prevalence of antibodies against
269 BPI3V in the two regions studied despite the difference in environmental conditions. In
270 contrast, a significant difference was found between animals from the uplands, Mexico City,
271 and the tropic, Veracruz, Mexico (Contreras-Luna *et al*., 2017). Regarding the age of the

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272 animals, the prevalence of BPI3V was higher in adult sheep (>3 years) compared to those in
273 other age groups (<1 year and [1-3] years) (Table 1). We reported a seroprevalence of 93% in
274 adult sheep, which is higher than the prevalence reported in Peru (50%) (Cabello and Rivera,
275 2006) and Mexico (81.4%) (Contreras-Luna *et al*., 2017). In contrast to our study, the results
276 obtained in Mexico showed no effect of age on the seroprevalence of BPI3V in sheep
277 (Contreras-Luna *et al*., 2017). Adults tend to have a higher seroprevalence of BPI3V, which
278 may be attributed to multiple previous infections at this age (Noori *et al*., 2018). The risk of
279 disease is higher in the medium flock (91%) than in the large flock (61%). This finding
280 contradicts the result reported in Mexico by Solis-Calderon *et al*. (2007). they indicated that
281 BPI3V seropositivity was higher in large herds and suggested that exposure to other animals
282 should be higher in extensive herds.
283 A significant association was found between BPI3V seroprevalence and promoting factor.
284 The respiratory system of animals can be compromised by environmental factors such as
285 inadequate feeding, early weaning, extreme temperatures (both low and high), lack of rest,
286 and stress of transportation. Additionally, dust particles can act as irritants and increase the
287 susceptibility of animals to respiratory diseases (Callan and Garry, 2002). Climate plays a
288 major role in modulating the virulence of the pathogen and reducing host defense, thereby
289 increasing susceptibility (Rahal *et al*., 2014). As mentioned earlier, adults were found to be
290 more susceptible than young animals. Since the majority of the sampled females were elderly
291 and the males were young, the seroprevalence of BPIV3 was higher in females compared to
292 males. This can be attributed to the higher number of females sampled (as the selected farms
293 were focused on breeding strategies), but it is also believed that animals raised for a longer
294 period have a higher likelihood of contracting the disease.

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295 Despite the presence of respiratory disease symptoms and antibodies to BPI3V in the tested
296 subjects, no BPI3V genome was detected in nasal swabs by the RT-PCR. This finding is
297 consistent with that of Gaeta *et al*. (2017), who reported no viral genome was found in
298 tracheobronchial swabs of cattle with the respiratory disease, regardless of positive serology.
299 However, several other studies have reported the detection of BPI3V genome in nasal swabs,
300 although in a limited number of cases. For instance, the viral genome was detected in 8 out of
301 119 samples tested in Serbia using RT-PCR (Veljovic *et al*., 2016), 11 out of 89 samples
302 tested in Japan using RT-qPCR (Goto *et al*., 2023), 2 out of 127 nasal swabs tested in Turkey
303 using RT-PCR (Timurkan *et al*., 2019), and 69.3% of sheep were found positive in Mexico
304 (Contreras-Luna *et al*. 2017). Additionally, in China 16.59% of samples were detected as
305 BPI3V-positive using RT-iiPCR (Ren *et al*. 2023).
306 The absence of a viral genome in our investigation could be attributed to two possible factors.
307 Firstly, it is possible that other pathogens were responsible for the respiratory infections
308 observed in the subjects. Secondly, the timing of sample collection may have been
309 inappropriate, as it is difficult to determine the exact time when the animals contracted the
310 infection. According to Grubor *et al*. (2004) and Ackermann (2014), PI3V and RSV typically
311 disappear from the respiratory tract within 17- and 14-days post-infection in young
312 experimentally infected lambs, respectively. Furthermore, the presence of antibodies to
313 BPI3V and the lack of viral antigen detection could indicate that the animals have
314 experienced a regressive infection and have developed specific immune responses (Gaeta *et*
315 *al*., 2017).
316

317 **Conclusion**

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This study represents the first serological survey conducted on BPI3V, confirming its presence in sheep populations across two regions of Algeria. The seroprevalence was detected in 75.93% of sampled sheep. Our findings indicate that age and sex significantly influence the seroprevalence of BPI3V, while herd-level factors such as favorable conditions and flock size may also contribute as significant risk factors. However, the region of study does not affect the seroprevalence of BPI3V. These results provide valuable insights for future large-scale epidemiological studies, which can aid in the development of effective prevention and control programs for respiratory diseases in sheep. It is worth noting that the BPI3V genome was not detected in any of the swabs using the RT-PCR test. Therefore, further in-depth investigations are recommended to explore the role of this virus in initiating respiratory diseases, as well as investigating potential concurrent infections.

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Conflict of interest

The authors declared that there is no conflict of interest.

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