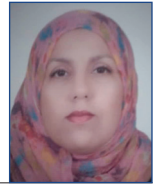


Prevalence and identification of *Corynebacterium pseudotuberculosis* in slaughtered sheep in central Algeria



R. Baazizi*, N. Mimoune*, A. Chahed, D. Baroudi, K. Ramoul, A. S. Abdul-Hussain, N. Ait Issad and D. Khelef

Abstract

Caseous lymphadenitis, also called abscess disease, is an infectious, cosmopolitan disease. The causative agent is a Gram-positive bacillus, *Corynebacterium pseudotuberculosis* that is resistant to antibiotic treatment. Humans become infected with this bacillus, but the disease is considered a neglected zoonosis. The objective of this study was to estimate the prevalence and to identify *Corynebacterium pseudotuberculosis* in sheep slaughtered in central Algeria. For this purpose, 897 animals were examined and samples (pus) were taken from 12 sheep with abscesses to perform bacteriological study. Sex, age, and location of the abscess were noted. The results obtained showed an overall prevalence of 1.33%. The highest rate (50%) was observed

in animals aged between 8 months and 1 year. Males were more affected by abscesses (66.7%) than females (33.3%). As for localisation, 41% of abscesses were found in the pulmonary lymph nodes and 25% in the submandibular region. Infection by *Corynebacterium* was estimated at a rate of 25%, lower than that obtained for *Staphylococcus* (41.7%). Regarding the zoonotic nature of the disease, the bacteria's ability to survive in the external environment, and the high risk of contamination, management measures should be implemented for better disease control and prevention.

Key words: sheep; slaughterhouse; *Corynebacterium pseudotuberculosis*; *Staphylococcus aureus*; prevalence

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Introduction

Caseous lymphadenitis (CL), also known as abscess disease, is an infectious, contagious, inoculable disease of subacute or chronic appearance. It results in abscesses with caseous pus or chronic supuration, localised in the lymph nodes, viscera, skin, udders (Schreuder et al., 1994; Sayed et al., 1995), brain, and spinal cord (Bensid, 2018).

The disease is cosmopolitan (Sharma et al., 2019) and causes considerable losses (Brugère-Picoux, 2019). Although it is an important disease of small ruminants, it remains underestimated (Sharma et al., 2019) because it is not systematically notified. It can also be recognised as an occupational disease (Brugère-Picoux, 2019).

CL is endemic in many countries around the world, the prevalence is high in both intensive (Stapleton et al., 2009) and extensive farming (Windsor, 2011). The breeding of small ruminants occupies an important place in Algeria, with 28 million sheep heads and 7 million goat heads (Baazizi et al., 2019). CL has also been described in wild ruminants.

It is mainly manifested by involvement of the lymph nodes in the head and neck. In later stages, visceral lymph nodes are also affected (Sharma et al., 2019). The pathogen *Corynebacterium pseudotuberculosis* is a Gram-positive bacillus (Baird and Fontaine, 2007; Hussain et al., 2017). This bacterium is intracellular (Muthukumar et al., 2013), immobile, facultatively aerobic or anaerobic, non-encapsulated, and non-spore forming (Alvarez et al., 2017; Ruiz et al., 2020). This bacterium can survive for several months in soil and straw (Muthukumar et al., 2020).

Antibiotic treatment is unsuccessful, perhaps due to the fact that the bacteria are viable inside the shell and its thick capsule, preventing a response to antibiotic therapy (Muthukumar et al., 2020) but also its ability to survive in the envi-

ronment (Prescott, 2002). In addition, the presence of internal abscesses is a major constraint to the action of these antimicrobial agents. The ideal is to stamp out the disease and then repopulate farms after applying the appropriate hygiene measures. Care should be taken to control animals to detect the disease (Muthukumar et al., 2020) and eliminate seropositive animals (Prescott, 2002).

CL is an important animal disease but above all a neglected zoonosis. Post-mortem inspections concerning the discovery of abscess stop at trimming decisions without looking for the cause. This study was conducted with the aim of identifying *Corynebacterium*, an agent of CL in sheep intended for slaughter.

Materials and methods

Study area and period

The study was carried out at a slaughterhouse in Algiers, Algeria during the period from September to December 2019.

Animals

To conduct this study, a total number of 897 animals was examined and samples were taken from 12 sheep bearing abscesses. The data collected concerned the age of animals with abscesses (six sheep aged between 8 months and 1 year, two sheep aged between 2 and 5 years, and four animals aged over 5 years).

Regarding sex, eight males and four females had abscesses. The location of the abscesses was also taken into consideration (Figure 1).

Biological material

For the collection of pus, a longitudinal section was made using a sterile scalpel blade at the level of the abscesses. Pus was poured into sterile vials and taken to the laboratory.



Figure 1. Abscess in the mediastinal lymph node in a slaughtered sheep (a) Submandibular and parotid abscess in a sheep (b).

Media preparation

For the identification of *Corynebacterium pseudotuberculosis*, nutrient agar, BHIB (brain heart infusion broth), Chapman agar powder, Mannitol Salt Agar, and fresh sheep blood were used.

Preparation of agars

In the first step, fresh blood and Chapman agars were prepared. Fresh blood agar allows the reading of haemolysis. This is the orientation criterion for the identification of *Corynebacterium pseudotuberculosis*.

For the preparation of blood agar, the containers first underwent sterilisation; the agar was liquefied in a water bath and then cooled to 45°C. A quantity of 6 mL blood was poured into 118 mL agar to reach a final blood concentration of around 5%. The preparation was then poured into Petri dishes, avoiding the formation of bubbles.

Chapman agar is a selective medium, which allows the growth of halophilic germs, including bacteria of the genus *Staphylococcus*. This medium contains an inhibitor (sodium chloride that inhibits

most Gram+ and Gram- bacteria) that allows the selective isolation of *Staphylococcus*. It also makes it possible to study the fermentation of mannitol by the change in colour (from red to yellow).

For the production of this agar, 111 g dehydrated Chapman medium was diluted in 1 L distilled water, and mixed until the solution was homogeneous. The solution was heated and placed in an autoclave for sterilisation at 121°C and then distributed into Petri dishes.

Bacterial cultures on BHIB

BHIB is a nutrient medium used for the cultivation of a wide variety of microorganisms. To perform this step, pus swabs were dipped into the tubes containing BHIB and incubated at 37°C for 18 to 24 h.

Inoculation on the agars

To carry out this step, a few drops of BHIB were collected and sown on blood agar and on Chapman agar, then incubated for 24 hours at 37°C.

Preparation of slides

The slides were prepared from the bacterial colonies for their Gram staining

in order to visualise and identify the bacteria belonging to the genus *Corynebacterium* and *Staphylococcus* under the microscope. To do this, a drop of distilled water was poured onto the slide, a few colonies were removed using a platinum loop and spread out to obtain a smear and then left to dry for a few minutes. Gram staining was performed to highlight the properties of the bacterial wall and to use these properties to distinguish (by their ability to fix the gentian violet Gram+ or fuschine Gram-) and classify the bacteria. Finally, the slides were examined under an optical microscope.

Catalase test

This test is performed for the identification of Gram+ bacteria. A few drops of 10 volume oxygen peroxide were poured onto a slide. One bacterial colony from each of the agars was brought into contact with oxygen peroxide. The presence of bubbles means a positive result.

Urease test

This test is performed for the identification of *Corynebacterium pseudotuberculosis*. Colonies on blood agar were sown on a urea-tryptophan medium and incubated for 24 hours at 37°C.

Coagulase test

This is also called staphylocoagulase as allows for differentiation among species of the genus *Staphylococcus*. For its production, 0.5 mL culture in BHIB was added to the same quantity (0.5 mL) of oxalated plasma in a sterile haemolysis tube, and incubated for 4 hours at 37°C. The reading was carried out after each hour to note the formation of a clot, signifying a positive reaction.

KIA Test (Kligler Iron Agar Medium)

This medium makes it possible to investigate the utilisation of lactose and fer-

mentation of glucose. Colonies were taken from blood agar and incubated for 24 hours at 37°C.

Statistical analysis

Statistical analysis was carried out with the R software package (version 3.6.2). All statistical comparisons were made by Pearson's chi-square significance test with a confidence interval of 95%. The differences were considered statistically significant with a probability $P \leq 0.05$.

Results and discussion

Prevalence by age group

The age of the slaughtered animals with abscesses varied from 8 months to more than 5 years. The prevalence of abscesses (Figure 2) in animals aged between 8 months and 1 year was 50% (6/12). Other studies have reported a low rate for this age group (0-1 year): 6.3% (Kichou et al., 2016), and 29.2% (Mechaal, 2005). It was also noted that young animals presented more abscesses. This may be due to poor breeding and hygiene conditions, in accordance with previous reports (Al-Gaabary, 2010).

The prevalence in animals between 2 and 5 years was 16.7%, while in animals over 5 years, the rate was 33.3% ($P < 0.05$). These results are lower than those reported by Mechaal (2005), who obtained a high rate in sheep between 2 years and more than 5 years (66.7%). This can be explained by the small sample size compared with other studies, but also because the slaughtered animals were not very old and it is very well known that *C. pseudotuberculosis* significantly affects older animals, given the chronic nature of the infection (Chikhaoui and Khoudja, 2013).

Prevalence by sex

The distribution of the disease by sex revealed a higher rate in males (66.7%;

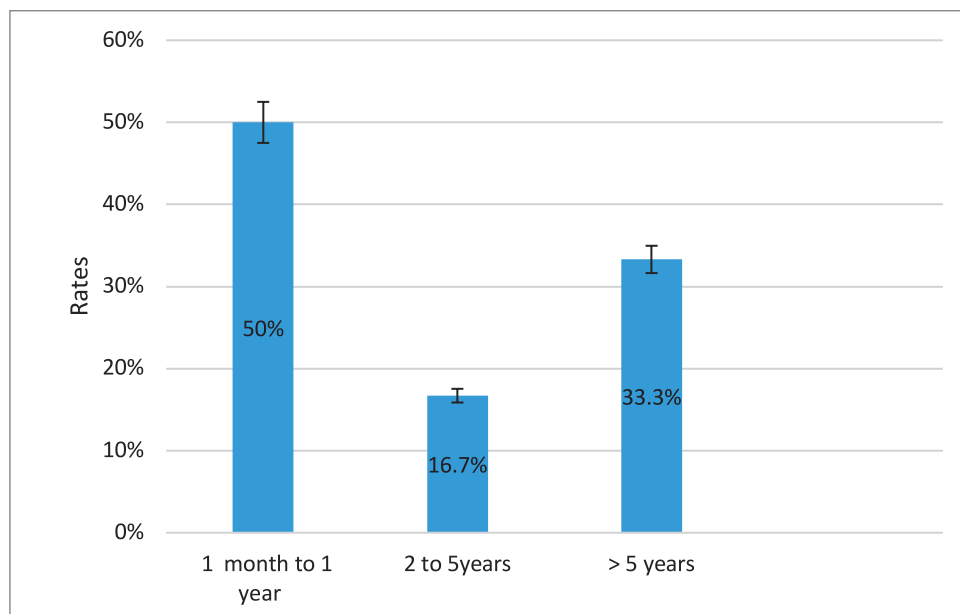


Figure 2. Prevalence of abscesses by age

8/12) than females (33.3%; 4/12) ($P < 0.05$). These results are in agreement with previous reports (Batey, 1986), while Ben Said et al. (2002) reported a similar rates in both sexes (5.4% for males and 4.9% for females). This high value in males is due to their aggressive nature and the consequence of fighting, which causes injury. Castration is also considered a risk factor (Fontaine, 2008). However, more recent studies have suggested that sex has no effect on *C. pseudotuberculosis* infection (Hussain et al., 2017).

Abscess detection can be influenced by gender and anatomic location; a predominance of involvement of the mediastinal lymph nodes was noted with a frequency of 41.7% in elderly females, and this is in agreement with previous studies (Renshaw et al., 1979; Arsenault, 2003). This could be due to the fact that females live longer in herds because they are intended for reproduction and therefore more exposed to various risk factors such as mow-

ing, thorny pastures, and cohabitation with sick animals.

Prevalence by location of abscesses

Of the total of 897 sheep, abscesses were found in 12 sheep. They were observed in the submandibular (3), pre-scapular (3), pulmonary nodes (5), and liver (1) levels. The overall infection rate was 1.33%. This result is lower than that found by Ruiz et al. (2020), who noted 29.5% of animals affected by CL (of 498 animals). The distribution of lesions (Figure 3) in the lymph nodes or organs showed a predominance of involvement of the mediastinal lymph nodes with a frequency of 41.7%. The latter presented a lamellar shape as reported previously (Ruiz et al., 2020). Involvement of internal lymph nodes and organs such as the liver, spleen, and kidneys was observed in sheep as mentioned before (Barral et al., 2019). Only one animal presented a liver abscess (8.3%), in accordance with Zavoshti et al. (2012).

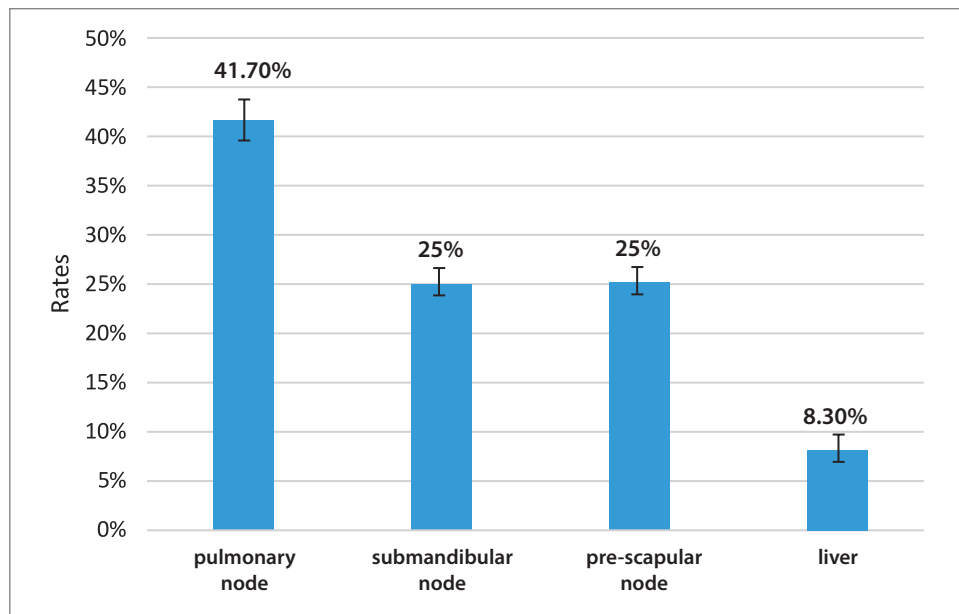


Figure 3. Prevalence of abscesses according to their location

Half (50%) of the abscesses were located externally in the anterior part of the body. These results are in agreement with other reports (Baird, 2008) because CL can only be detected clinically when the superficial lymph nodes are affected (Barral et al., 2019). It can be due to the fact that this region of the body is more exposed to trauma. The bacterium can survive for several months on the ground, and in the straw; it can penetrate through skin lesions, following trauma or when placing identification tags or tattoos; the penetration of the germ can take place via lesions of the mouth and gums (Muthukumar et al., 2020).

The superficial form was recorded with a rate of 25% for the pre-scapular and submaxillary lymph nodes; this rate was higher than that recorded by Ruiz et al. (2020), 6.4%. Concerning the abscesses found on the viscera, the rate in the lungs was 41.7% and in the liver 8.3% ($p < 0.05$), while other authors have reported a rate of

21.5% for combined visceral forms (Ruiz et al., 2020).

Bacteriology results

Macroscopic observation of the cultures

Screening for *Corynebacterium* spp. on blood agar

Three samples (25%, 3/12) were identified. Sample 1 presented a typical macroscopic appearance of the *Corynebacterium* genus, while in samples 8 and 9, the colonies also presented a suspicious appearance of belonging to the *Corynebacterium* genus confirmed by re-isolation (Table 1).

*Detecting *Staphylococcus aureus* on Chapman agar*

Five samples (42%) showed colonies identified as *Staphylococcus aureus* (2, 7, 8, 11, and 12) (Table 1). Four samples (33%) presented colonies corresponding to Staphylococci (4, 6, 9, and 10) but were not identified as *Staphylococcus aureus*

Table 1. Macroscopic observation of cultures

Number	Gender/age	Nature of the sample	<i>Corynebacterium</i> results	<i>Staphylococcus</i> results
1	Male, 2 years	Submandibular lymph node	Presence of macroscopic colonies typical of the genus <i>Corynebacterium</i>	Absence of suspicious <i>Staphylococcus aureus</i> colonies
2	Male, 8 months	Prescapular ganglion	Presence of colonies whose macroscopic appearance does not correspond to the genus <i>Corynebacterium</i>	Presence of pigmented, golden colonies, areoles around the colonies - Mannitol+
3	Male, 2 years	Submaxillary node	Absence of bacterial culture	Absence of bacterial culture
4	Male, 1 year	Mediastinal lymph node	Round, white, shiny colonies surrounded by a zone of haemolysis	Presence of non <i>Staphylococcus aureus</i> (Mannitol-)
5	Male, 1 year	Submaxillary node	Presence of colonies whose macroscopic appearance did not correspond to the genus <i>Corynebacterium</i>	Presence of contaminants
6	Female, > 5 years	Liver	Presence of colonies whose macroscopic appearance does not correspond to the genus <i>Corynebacterium</i>	Presence of non <i>Staphylococcus aureus</i> (Mannitol-)
7	Female, > 5 years	Mediastinal lymph node	Round, white, shiny colonies surrounded by a zone of haemolysis.	Presence of pigmented, golden colonies, aureoles around the colonies - Mannitol+
8	Female, > 5 years	Mediastinal lymph node	Direct isolation Presence of some macroscopically suspicious colonies belonging to the genus <i>Corynebacterium</i>	Presence of pigmented, golden colonies, areoles around the colonies - Mannitol+
9	Female, > 5 years	Mediastinal lymph node	Re-isolation Colonies of typical <i>Corynebacterium</i> morphology.	Presence of non- <i>Staphylococcus aureus</i> (Mannitol -) Absence of suspicious <i>Staphylococcus aureus</i> colonies
10	Male, 1 year	Prescapular node	Direct isolation Presence of some macroscopically suspicious colonies belonging to the genus <i>Corynebacterium</i> .	Absence of suspicious <i>Staphylococcus aureus</i> colonies
11	Male, 1 year	Prescapular node	Re-isolation Colonies of typical <i>Corynebacterium</i> morphology. Round, white, shiny colonies surrounded by a zone of haemolysis	Absence of bacterial culture
12	Male, 1 year	Mediastinal node	Presence of colonies whose macroscopic appearance did not correspond to the genus <i>Corynebacterium</i> . Shiny white round colonies surrounded by a zone of haemolysis.	Presence of pigmented, golden colonies, areoles around the colonies - Mannitol+

(Table 1). One sample revealed co-infection (Number 8) by *Corynebacterium pseudotuberculosis* and *Staphylococcus aureus* (8.3%).

Microscopic observation of cultures (Gram stain)

Corynebacterium suspect strains

Of the four cultures (4, 7, 8, 9) presenting a macroscopic appearance suspicious of belonging to the genus *Corynebacterium*, three samples (7, 8, 9) revealed a microscopic appearance typical of this bacterial genus (Figure 4).

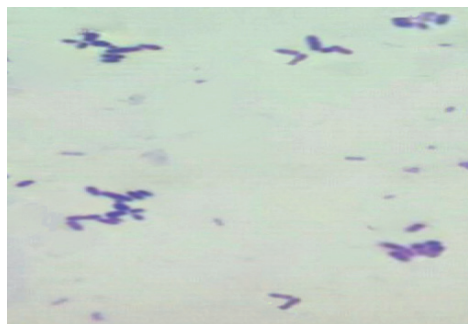


Figure 4. Microscopic appearance of *Corynebacterium*, Gx100.

Strains taken from Chapman agar (Gram stain)

All colonies suspected of belonging to the *Staphylococcus* genus showed a microscopic appearance typical of *Staphylococcus aureus* (Figure 5), (samples: 2, 7, 8, 11, 12).

Results of biochemical tests

On the four colonies suspected of belonging to the genus *Corynebacterium*, biochemical tests made it possible to conclude the results presented below.

Urease test

Two of the four suspected samples macroscopically and microscopically, gave

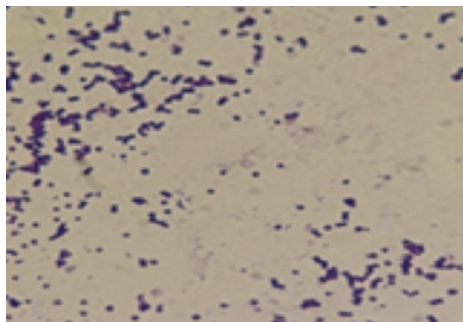


Figure 5. Microscopic appearance of *Staphylococcus*, Gx100

a positive reaction to the urease test. They were characterised by a pink colouring of the colonies for samples 8 and 9, whereas samples 4 and 7 proved to be negative.

KIA Test (Sugar Degradation)

The test (glucose fermentation) proved positive for samples 8 and 9 of the 4 samples, macroscopically and microscopically suspected of belonging to the genus *Corynebacterium pseudotuberculosis*.

Catalase test

The catalase test was carried out on the four strains suspected of belonging to the genus *Corynebacterium* and on the strains taken from Chapman Agar, for the detection of *Staphylococcus aureus* and *Corynebacterium pseudotuberculosis*. The Catalase test was positive for all strains suspected of belonging to the species *Staphylococcus aureus* (samples 2, 7, 8, 11, 12), while two samples (samples 8 and 9) of four suspected of belonging to the genus *Corynebacterium* presented a positive catalase test. This test is specific to both *Staphylococcus aureus* and strains belonging to the genus *Corynebacterium*.

Coagulase test

All strains suspected of belonging to the *Staphylococcus aureus* species presented a positive coagulase test. The application

of biochemical tests aims to confirm they belong to the species *Corynebacterium pseudotuberculosis*, and the overall isolation rate was 16.7% (2/12). This rate is similar to that reported by Mubarak (1999), who showed that out of 117 samples taken from lesions characteristic of CL in sheep, only 20 samples contained the *Corynebacterium pseudotuberculosis* germ (17.1%). On the other hand, our data do not agree with Ben Said et al. (2002), who reported a rate of 53.6%.

Regarding *Staphylococcus aureus*, the germ was found in five samples corresponding to a rate of 41.7%, this is explained by the fact that the samples concerned animals of different ages and in particular young animals between 8 months and 2 years old where infection by Staphylococci was dominant (Baird, 2003; Al-Gaabary, 2009), which causes a pathology that can be confused with caseous lymphadenitis.

Conclusions

This study revealed a prevalence of 1.33% for caseous lymphadenitis. Even if the rate is low, this proves that this disease is present in Algeria. In order to confirm the presence of *Corynebacterium pseudotuberculosis*, other tests can be carried out (genotypic test) on a larger population. CL is a zoonotic disease, and field surveys have shown that veterinarians and abattoir workers do not take special precautions to avoid infections; indeed, the action consists of trimming when an abscess is discovered or an organ is seized at the slaughterhouse. *Corynebacterium pseudotuberculosis*, once established within the host, evades the immune system, therefore chronic infections can last the life of the animal, and the bacteria can infect multiple animals in the herd. In addition, the general condition of infected

animals often remains good and so breeders do not clean up the herds, especially since no national control programme has been put in place. In the short term, the best method to control the infection is to eliminate animals showing clinical signs. In the long term, the implementation of a CL control strategy is recommended because the disease exists despite the low prevalence.

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Prevalencija i identifikacija *Corynebacterium pseudotuberculosis* u zaklanih ovaca u središnjoj regiji Alžira

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Kazeozni limfadenitis (CL), zvan i pseudotuberkuloza, zarazna je kozmopolitiska bolest. Uz-

ročnik je gram-pozitivna bakterija, *Corynebacterium pseudotuberculosis* otporna na liječenje antibioticima.

Ljudi se mogu zaraziti ovom bakterijom, ali se bolest smatra zanemarenom zoonozom. Cilj ove studije bio je procijeniti prevalenciju i identificirati *Corynebacterium pseudotuberculosis* u ovaca zaklanih u središnjoj regiji Alžira. U tu svrhu, 897 životinja je ispitano i uzorci (gnoj) su izuzeti od 12 ovaca s apscesima za potrebe bakteriološke studije. Zabilježeni su spol, dob i lokacija apscesa. Dobiveni rezultati pokazali su sveukupnu prevalenciju od 1,33 %. Najveća stopa (50 %) zamijećena je u životinja u dobi između 8 mjeseci i 1 godine. Mužjaci su imali više apscesa (66,7 %) od

ženki (33,3 %). Što se tiče lokalizacije, 41 % apscesa pronađeno je u plućnim limfnim čvorovima, a 25 % u submandibularnom području. Infekcija bakterijom *Corynebacterium* procijenjena je u stopi od 25 %, što je manje od stope dobivene za *Staphylococcus* (41,7 %). U konačnici, s obzirom na zoonotsku prirodu CL-a, preživljavanje bakterije u vanjskom okruženju te veliki rizik od zaraze, potrebno je implementirati mjere upravljanja za bolju kontrolu i prevenciju bolesti.

Ključne riječi: ovca, klaonica, *Corynebacterium pseudotuberculosis*, *Staphylococcus aureus*, prevalencija