Prevalence of *Salmonella enterica* subsp. *diarizonae* serotype 61:k:1:5:(7) in nasal secretions and stool of sheep flocks with and without cases of chronic proliferative rhinitis in Spain

Luis Figueras[a,b]; Luis Miguel Ferrer[a]; José María González[a,b]; Juan Pedro Bueso[c]; Juan José Ramos[a]; Inés Rubira[b]; Erika Burian[d]; Delia Lacasta[a*]

[a] Animal Pathology Department. Instituto Agroalimentario de Aragón-IA2 (Universidad de Zaragoza-CITA). Veterinary Faculty of Zaragoza. C/Miguel Servet 177. 50013 Zaragoza. Spain.

[b] Gabinete Técnico Veterinario S.L. C/ Isla conejera sn. 50013 Zaragoza. Spain.

[c] Laboratorio Agroambiental de Aragón. Montañana. Spain.

[d] Clínica Veterinaria Coso. C/ Coso 144. 50002 Zaragoza. Spain.

[*]Corresponding author: Delia Lacasta. dlacasta@unizar.es

Abstract

Salmonella enterica subsp. *diarizonae* serotype 61: k: 1,5, (7) (SED) is a microorganism well adapted to sheep~~,~~; however, it has also been described producing chronic proliferative rhinitis (CPR) in ~~these animals~~ovine. CPR causes a proliferative inflammation of the ventral nasal turbinates that may totally obstruct the nasal cavity.

The main objective of the present ~~survey~~study was to investigate the prevalence of SED in nostrils and stool of ~~asymptomatic adult~~ sheep without CPR clinical signs in commercial sheep farms of Spain with and without previous clinical cases of ~~chronic proliferative rhinitis in Spain~~CPR.

Five samplings were performed in 10 commercial sheep farms ~~during~~for one year ~~(5/5).~~. Samples from nostrils and ~~feces~~faeces were taken from four ~~asymptomatic chronic proliferative rhinitis~~ animals without CPR visible clinical signs that belonged to four different age ranges at each farm visit.

The prevalence of positive animals was 45.3~~%~~%, and the number of positive samples in nostrils was higher than in ~~feces~~faeces (38.5% vs 22.5%). Only on one farm was no positive result obtained in the entire study. In almost all positive farms, sheep belonging to the youngest age ranges accounted for more than 50% of positive isolates. Finally,

farms with a previous diagnosis of CPR were 1.784 times more likely to have an animal with positive isolation than farms without a previous diagnosis. This could suggest that the infection pressure in the farm might ~~favor~~favour the occurrence of clinical cases of the disease. However, further studies will be necessary to unravel why this saprophytic bacterium is able to cross the epithelial barrier causing severe rhinitis in certain animals.

Keywords: Sheep; *Salmonella enterica* subsp. *diarizonae* serotype 61: k: 1.5, (7); chronic proliferative rhinitis; prevalence.

Introduction

Salmonella enterica subsp. *diarizonae* serotype 61: k: 1.5, (7) (SED) is considered a microorganism adapted to sheep, these animals behaving normally as asymptomatic carriers (~~Harvey et al., 1966; Greenfield et al., 1973;~~ Zweifel et al., 2004; Sörén et al., 2015~~). This serotype has occasionally been isolated in other warm-blood species, such as cattle, horses, pigs and wild boars (Hall and Rowe, 1992; Davies et al., 2001; Alvseike and Skjerve, 2002; Zottola et al., 2013), and, punctually, in man (Hall and Rowe, 1992; Giner-Lamia et al., 2019~~).

~~There are several~~Several works carried out in abattoirs ~~that show~~reported the presence of SED in healthy sheep. Thus, in the United Kingdom (Sojka et al., 1983; Hall and Rowe, 1992; Davies et al., 2001) and Norway (Alvseike ~~et al., 2000; Alvseike~~ and Skjerve, 2002; Sandberg et al., 2002~~)~~), the presence of this microorganism is widely described. ~~Likewise~~Similarly, it has also been isolated from sheep in other countries such as Switzerland (Zweifel et al., 2004; Bonke et al., 2012; Stokar-Regenscheit et al., 2017), Iceland (Hjartardottir et al., 2002), Sweden (Sörén et al., 2015), Canada (Pritchard, 1990) or the United States (Dargatz et al., 2015; Wolf and Schefers; 2017). However, in all these

studies, only intestinal content was analyzed, and the percentages of isolation found were generally low. Only one study investigates the presence of SED in the respiratory tract, specifically in tonsils, and they find 43% of positive animals in this location (Bonke et al., 2012).

According to the results obtained from all these studies, *Salmonella enterica* subsp. *diarizonae* serotype 61: k: 1.5, (7) can be ~~consider~~considered a saprophyte microorganism of sheep~~,~~; however, it has also been described producing disease in these animals. Thus, chronic proliferative rhinitis (CPR) is an upper respiratory tract ~~condition~~disorder of sheep associated with SED that was described for the first time in the USA in 1992 (Meehan et al., 1992) and later reported in Spain (Lacasta et al., 2012), Switzerland (Stokar-Regenscheit et al., 2017) and the USA again (Wolf and Schefers, 2017). ~~This~~CPR is a slow and progressive disease that causes severe inflammation of the ventral nasal turbinates with very poor prognosis for the untreated affected animals (Rubira et al., 2019). It has been stated that the inflammatory response occurs when the bacterium penetrates the nasal epithelial cells causing a massive inflammatory reaction (Lacasta et al., 2012). ~~Histopathological evaluation reveals the presence of numerous gram-negative bacilli within many epithelial cells and immunohistochemistry confirms the presence of bacteria of genus *Salmonella* inside proliferating epithelial cells and macrophages of the nasal mucosa in the affected animals (Lacasta et al., 2012; Stokar-Regenscheit et al., 2017; Wolf and Schefers, 2017).~~

Furthermore, SED has also been related to ~~diarrhea~~diarrhoea in lambs (Long et al., 1978~~;~~ ~~Harp et al., 1981~~; Davies et al., 2001; Alvseike and Skjerve, 2002; Chatzopoulos et al., 2016), abortions and stillbirths in sheep (Long et al., 1978; ~~Greenfield et al., 1973;~~ Davies et al., 2001), ~~abortion in goats (Schnydrig et al., 2018)~~ and also producing orchitis and epididymitis in rams (Ferreras et al., 2007; Celeghini et al., 2013). Infections with SED

in humans have also been reported, although its zoonotic potential is generally considered low (Sörén et al., 2015; Giner-Lamia et al., 2019).

The importance of this bacterium as a pathogen capable of producing CPR in sheep seems to have been increased in recent years, with the number of reports of CPR in international publications and conferences growing. According to the knowledge of the authors, so far, no prevalence studies have been carried out to ~~analyze~~analyse the presence of these bacteria in the upper respiratory tract of ~~asymptomatic~~ sheep without clinical signs of CPR. The main objective of the present ~~survey~~study was to investigate the prevalence of *Salmonella enterica* subsp. *diarizonae* serotype 61: k: 1,5, (7) in nostrils and stool of ~~asymptomatic~~ adult sheep without visible clinical signs of CPR in commercial sheep farms of Spain with and without previous clinical cases of ~~chronic proliferative rhinitis in Spain~~CPR diagnosed.

Material and methods

~~This study was~~All procedures were carried out under Project Licence PI 22/11 approved by the ~~Ethical~~Ethics Committee for Animal ~~Experimentation of~~Experiments from the University of Zaragoza~~, Spain,~~. The care and use of animals were performed accordingly with the ~~number PI 22/11~~Spanish Policy for Animal Protection RD53/2013, which meets the European Union Directive 2010/63 on the protection of animals used for experimental and other scientific purposes.

Ten commercial sheep farms located in Aragon, a northeast region of Spain, were selected for the ~~survey~~study. In five of these farms, CPR had never been detected before and, in the other five, animals with ~~chronic proliferative rhinitis~~CPR had been previously diagnosed, and~~, in~~ pathological and microbiological studies had confirmed the ~~other five,~~

~~this disease had never been detected before~~process. All the farms were meat flocks, and seven of them were reared in a semi-intensive production system and the other three in an intensive system with permanent stabling. The management of all the farms in semi-intensive production system was very similar, with stabling of the ewes at the end of gestation and during lactation until weaning, when lambs were 45 days old. The three farms reared in an intensive system remained stabled without grazing, fed on cereals and fodder. The health programme was similar in all the farms, and it was carried out by the same veterinary group. The number of sheep in the farms ranged from 358 to 3,200 animals (Table 1). The farms were identified correlatively from 1 to 10 for the study.

Five complete samplings were performed ~~in~~on each farm ~~during~~for one year. Samples from nostrils, right and left, and ~~feces~~faeces were taken from four ~~asymptomatic chronic proliferative rhinitis~~ animals without CPR visible clinical signs that belonged to four different age ranges (0-2 years, 2-4 years, 4-6 years and more than ~~6~~six years) at each farm visit. ~~Sampling~~Samplings of the nostrils ~~was~~were performed using a sterile swab with culture medium (Deltalab-Eurotubo®Sterile) of both nostrils separately, identifying whether it was right or left. The ~~feces~~faeces sample was taken directly from the rectum of the animals with a sterile glove, trying to obtain as much as possible and never less than 10 grams. Each sample was identified with the tag number or individual identification of the animal, the age range to which it belonged, the date of sampling and the farm. Once the sampling was done, the samples were immediately sent to the Agroambiental Laboratory of the Government of Aragon in refrigeration for culture and identification.

Further to the five complete samplings performed in each farm (50 samplings), nine additional samplings were carried out on farms 2, 5 and 10, in which only samples from nostrils were taken. In these farms, the number of nostril samples was increased because

the greater collaboration by the farmers allowed us to expand the number of samples. Therefore, in total, we worked with 50 complete samplings (200 ~~feces~~faeces samples and 400 nostril samples) and ~~9~~nine additional nostril samplings (72 samples collected from 36 sheep), which made a total of 472 nostril samples (236 animals) and 200 ~~feces~~faeces samples (Table 1).

Samples were cultured into Buffered Peptone Water (APT) Xylose Lysine Deoxycholate Agar (XLD) and Salmonella Shigella Agar (SS), and incubated at 37◦C for 24–48h. Colonies resembling Salmonella were chosen and identified by a conventional biochemical test: ~~Tripe~~Triple Sugar Iron agar (TSI), Lysine ~~decarboxilase~~decarboxylase, Ornithine ~~decarboxilase~~decarboxylase, Arginine decarboxylase dihydrolase, Christensen'sUrea, Voges-Proskauer and the Beta-galactosidase test. Selected colonies were sent for serotyping to the Central Veterinary Laboratory (Algete, Madrid, Spain).

The statistical study was carried out at two levels according to the unit of study used: farm and animal. Although the microbiological analyzes were performed separately from the right and left nostril, the statistical studies were performed from both sides together. All the variables used in this work were of a qualitative type and for their study association tests between variables, such as Chi-square, were used, using Fisher correction, if necessary, and calculating the relative risks whenever possible. These association tests were performed for the presence/absence of SED in nostrils, ~~feces~~faeces or ~~in~~ either. In all cases, a statistical significance $p<0.05$ was required to validate the hypothesis. ~~To~~In order to calculate the estimated prevalence, both individual and collective~~,~~ calculations were carried out assuming a 95% confidence interval. ~~For~~To calculate the ~~calculation of~~ collective prevalence within each farm, 20 sheep were assumed as the value of the sample for all farms except farm 2 and 10 with 28 animals, and farm 5 with 40 sheep. In all cases, it was assumed that the diagnosis was right. The Winepi software

(http://www.winepi.net/) was used, assuming as a study population of 3,478 sheep farms located in Aragon in 2013 and a global census for the same year and the same region of 1,576,218 sheep.

Results

The data collected were statistically analyzed at two levels: animals and farm, and thus the results will be exposed.

Animals

The prevalence of positive animals in nostrils or ~~feces~~faeces was 45.3% (107/236). When analyzing only the 50 complete samplings (~~feces~~faeces and nostrils), the number of positive samples in nostrils was higher than in ~~feces~~faeces: 22.0% (44/200) were positive only in nostrils, 6.0% (12/200) were positive only in ~~feces~~faeces, and 16.5% (33/200) were positive in nostrils and ~~feces~~faeces simultaneously. In total, 38.5% (77/200) showed positive isolation in nostrils compared to 22.5% (45/200) in ~~feces~~faeces.

~~Concerning positive isolations related to the age of the animals~~In addition, 24.3% (26/107) belonged to the group from 0-2 years, 30.8% (33/107) to the group of sheep from 2-4 years old, 23.4% (25/107) to the group from 4-6 years old and 21.5% (23/107) to the group older than ~~6~~six years. No significant differences were found between groups ($p>0.005$).

~~In order to~~To evaluate the ~~possible~~ influence of the ~~inclement weather at the time of taking the sample,~~climate when collecting the samples, these were grouped according to the season of the year. The highest proportion of positive samples in one of the two locations, nostrils or ~~feces~~faeces, was obtained in summer (55.8%), although the statistical analysis

did not show significant differences (p> 0.05). ~~Regarding positive isolations only in nostrils, the results were very similar, also being in summer when more samples were positive (48.1%). The lowest proportion of nostril isolates was obtained in winter and autumn, with 37.5% of positive samples in both cases.~~ However, when the results of the positive animals in ~~feces~~faeces were analyzed~~, despite being similar to the previous results and presenting the highest proportion of positive samples in summer~~, it was obtained a significant reduction in the proportion of positive samples during the autumn, resulting statistically significant (p <0.05); which established a 2.882, 2.597 and 3.774 times higher risk of finding SED in ~~feces~~faeces samples during winter, spring and summer, respectively, than in autumn.

Farms

Nine ~~farms out~~ of the ten analyzed farms had at least one positive isolation of *Salmonella enterica* subsps. *diarizonae* serotype 61:k:1:5:(7) in one of the locations (nostrils or ~~feces~~faeces). Only farm number 8 did not present, at any time and in any location, isolation of SED (Table 1). In addition, all positive farms had ~~isolates~~SED isolations in both locations, nostrils and ~~feces, with the exception of~~faeces, except farm number 3, that only had positive isolates in the nostrils and in a ~~smaller~~lower proportion (4/20) than the other eight positive flocks of the study. All farms, ~~with the exception of~~except flock number 1, had a higher percentage of isolations in the nostrils. Interestingly, farm number 1, which presented the highest number of isolates in ~~feces~~faeces, was the one with the highest number of total positive isolates (22/40), ~~9~~nine being positive in nostrils and 13 in ~~feces~~faeces samples. However, the second farm with more total positive isolates, farm number 6, presented the majority of the isolations in nostrils (14/40), as ~~happen~~happened in the rest of the positive flocks, although in a smaller proportion (Table 1).

In almost all positive farms, sheep belonging to the youngest age ranges (0-2 and 2-4 years) accounted for more than 50% of positive isolates. Only farm 10 had the highest number of isolates in the group of sheep aged 4-6 years.

The estimated prevalence for each farm was calculated taking into account the population under study and the number of animals analyzed: 20 sheep for farms 1, 3, 4, 6, 7, 8 and 9; 28 animals for farms 2 and 10, and 40 sheep for farm 5. In all cases, it was assumed that the diagnosis was accurate, with a 95% confidence interval. Thus, farm number 1 had an estimated prevalence of 70.0% (50.01%, 89.99%) and farm number 2 had 57.1% (39.08%, 75.12%). The flock number 6 was in which the highest estimated prevalence was found at 80.0% (62.56%, 97.44%), while farm number 8 was the only one with a prevalence of 0.0% (Table 1).

The collective and individual prevalence in the Aragon region (Spain) was also calculated with the data of the year of ~~completion of~~ the study (2013). In this year, 3,478 sheep farms were active in Aragon, and there was a total census of 1,576,218 sheep. Prevalence calculations were carried out assuming a 95% confidence interval. With these data, an estimated collective prevalence of 90.0% (71.4%, 100.0%) and an individual prevalence of 45.3% (38.9%, 51.6%) were obtained.

Finally, the presence of SED was analyzed according to whether the farms had presented cases of chronic proliferative rhinitis or not. This comparison showed significant differences ($p = 0.036$). Farms with a previous diagnosis of CPR were 1.784 times more likely to have an animal with positive isolation than farms without a previous diagnosis. However, the analysis of the location of the bacteria, either in nostrils or ~~feces~~faeces, did not show differences.

Interestingly, in farm number 8, in which CPR had not been diagnosed prior to this study and which was negative for the presence of SED in all samples taken, *Salmonella* spp. isolations were found in some of the animals. Specifically, *Salmonella enterica* subsp. *enterica* serotype ~~Abortuovis~~Abortusovis, *Salmonella enterica* subsp. *enterica* serotype Anatum, *Salmonella enterica* subsp. *enterica* serotype Mbandaka *and Salmonella enterica* subsp. *enterica* serotype Agona. On the contrary, this fact was not observed in any of the other nine farms in the study in which only *Salmonella enterica* subsp. *diarizonae* serotype 61: k: 1.5, (7) was isolated.

Discussion

At present, and despite SED studies ~~of *Salmonella enterica* subsp. *diarizonae* serotype 61:k:1,5,(7) isolates~~carried out in ~~sheep at~~ abattoirs ~~reported~~ in several countries, there are very few prevalence ~~surveys~~studies conducted in flocks. Only Norway, Switzerland and Sweden reported flock-based studies (Alvseike and Skjerve, 2002; Sandberg et al., 2002; Bonke et al., 2012; Sörén et al., 2015), however, in all of them only intestinal content was analyzed. Despite the association of SED with upper respiratory tract diseases in sheep (Meehan et al., 1992; Lacasta et al., 2012; Stokar-Regenscheit et al., 2017; Wolf and Schefers, 2017), none of the works reviewed in the literature, neither in flocks nor in abattoirs, took samples of the respiratory system, only Bonke et al. (2012) analyzed tonsils at abattoirs, with 43% of positive isolations. Surprisingly, the results of our study show that SED was more frequently isolated from nostril samples ~~of asymptomatic sheep~~ than from stool (38.5% vs 22.5%).

In Sweden, as in Norway and Finland, the prevalence of Salmonella in food~~-~~producing animals is very low due to a control program initiated more than 50 years ago. However,

in a prevalence study carried out in Sweden in 2012 over 237 sheep flocks, an overall prevalence of SED of 17.6% around all the country was obtained. As scientific opinions and ~~an~~ evaluation of on-farm control measures performed concluded that the impact of sheep associated *S. enterica* subsp. *diarizonae* on human health was very low, Swedish authorities decided to make an exemption for *S. enterica* subsp. *diarizonae* in sheep in the current Salmonella control measures (Sörén et al., 2015).

In a study carried out in Norway in 50 flocks, the prevalence was 14% (Sandberg et al., 2002) and in a subsequent work also performed in Norway on 133 farms, the herd prevalence was found to range from 10 to 45% (Alvseike and Skjerve, 2002). Unexpectedly, in our ~~survey, 9~~study, nine of the ~~10~~ten analyzed farms were positive (extrapolated collective prevalence of 90%) with an individual prevalence of 45.3%, although there was also ~~great~~high variability among farms, ranging from 0.0% to 80.0%. Therefore, it ~~can~~could be concluded that SED ~~is~~seems to be endemic in Spain, with a very high prevalence compared to what has been reported in other countries (Alvseike and Skjerve, 2002; Davies et al., 2001; Sandberg et al., 2002; Sörén et al., 2015). However, based on the results obtained in the present ~~survey~~study, one would think that perhaps if in those studies carried out in northern Europe, samples had been taken from the nostrils in addition to ~~feces, perhaps~~faeces, the prevalence found would have been higher.

In other ~~surveys~~studies performed in abattoirs, the prevalence found was even lower, thus, in Britain, Milnes et al. (2008) found a prevalence of 1.0%, in Iceland, 2.0% (Hjartardottir et al., 2002), in Ethiopia, 3.0 to 17.6% (~~Chandra et al., 2006;~~ Woldemariam et al., 2005), in the United States, 26.9% (Dargatz et al., 2015) and in Switzerland from 11 to 43% (Bonke et al., 2012; Zweifel et al., 2004). Nevertheless, sheep ~~studied~~analyzed in abattoir ~~surveys~~studies are generally elderly culling animals, which biases the results even more since in our study we observed that the oldest (older than ~~6~~six years) are the age range

with the lowest percentage of isolation. This partially contrasts with the results obtained by Bonke et al. (2012) and Sandberg et al. (2002), that related the age to the presence of SED in sheep, and both concluded that SED is most often isolated from animals older than ~~2~~two years.

In our study, the animals that showed positive results in nostrils had a high possibility of being positive also in ~~feces~~faeces. This differs from the results reported by Bonke et al. (2012), that despite obtaining a high prevalence in tonsils, these same animals were negative in ~~feces~~faeces. The authors justified the poor results of isolation in intestinal content with the small amount of sample analyzed and the poor conservation.

In addition, no ~~large~~substantial differences in SED isolates were found according to the season of the year, showing only significant differences in the lowest presence of positive isolates in ~~feces~~faeces during autumn. Other published studies reported the highest percentage of isolations during spring, associating this with seasonality and lambing (Davies et al., 2001; ~~Davison et al., 2005;~~ Hjartardottir et al., 2002). Likewise, it has been described that there is ~~a~~ greater isolation of the bacteria in animals in the last stage of pregnancy (Bonke et al., 2012). In other studies, the increase in the number of isolates is associated with ~~stress~~stressful situations such as vaccinations (Lacasta et al., 2012), the transfer of animals to pastures in autumn (Hjartardottir et al., 2002) or related to management, production or immune system status (Long et al., 1978; ~~Hannam et al., 1986;~~ Pritchard, 1990). By contrast, in the ~~survey~~study of Sandberg et al. (2002), stress is dismissed as a risk factor.

Lastly, ~~significant differences were~~a significantly higher percentage of isolates of SED was found in our ~~survey~~study in the ~~isolates of those~~ flocks with previous cases of CPR ~~and~~than in those in which the disease had never been diagnosed~~, with a higher percentage of isolates of SED in the farms with previous cases of rhinitis.~~. This could suggest that

the infection pressure in the farm might ~~favor~~favour the occurrence of clinical cases of the disease, since, as concluded in the experimental infection carried out by Lacasta et al. (2017), the simple presence of the bacteria in the nasal secretions is not enough to trigger clinical signs of the disease and other factors, yet to be discovered, are necessary for SED to pass through the epithelial cells of the nostrils and elicit the inflammatory reaction. Further studies will be necessary to unravel why this saprophytic bacterium of the high respiratory tract is able to cross the epithelial barrier causing severe inflammation in ~~certain~~some animals.

Funding

This study was supported by the Aragón Government and the European Social Fund (Construyendo Aragón 2016-20).

Acknowledgements

Authors would like to acknowledge veterinary colleagues and sheep farmers who have collaborated in the development of this work.

Conflict of interest statement

The authors have nothing to disclose.

References

~~1. Alvseike, O., Nerbrink, E., Skjerve, E., Nesbakken, T., 2000. Growth of *Salmonella choleraesuis* subspecies *diarizonae* serovar 61:k:1,5,(7) in broth and fresh mutton. Int. J. Food Microbiol. 57, 159-167.~~

~~2.~~1. Alvseike, O., Skjerve, E., 2002. Prevalence of a *Salmonella* subspecies *diarizonae* in Norwegian sheep herds. Prev. Vet. Med. 52, 277-285.

~~3.~~2. Bonke, R., Wacheck, S., Bumann, C., Thum, C., Stueber, E., Koenig, M., Stephan, R., Fredriksson- Ahomaa, M., 2012. High prevalence of *Salmonella enterica* subsp *diarizonae* in tonsils of sheep at slaughter. Food Res. Int. 45, 880-884.

~~4.~~3. Celeghini, E.C.C., Gregory, L., Pinheiro, E.S., Piva, F.M., Carneiro, P.A.B., Parapinski-Santos, B., Bianchi, M., Benesi, F.J., 2013. Orchiepididymitis in ram by *Salmonella enterica* sub *diarizonae*: first case in South America. Arq. Bras. Med. Vet. Zootec. 65, 139-144.

~~5. Chandra, M., Singh, B.R., Shankar, H., Agarwal, M., Agrawal, R.K., Sharma, G., Babu, N., 2006. Study on prevalence of Salmonella infection in goats. Small Rumin. Res. 65, 24-30.~~

~~6.~~4. Chatzopoulos, D.C., Sarrou, S., Vasileiou, N.G.C., Ioannidi, K.S., Peteinaki, E., Valiakos, G., Tsokana, C.N., Papadopoulos, E., Spyrou, V., Mavrogianni, V.S., Giannakopoulos, A., Sbiraki, A., Lacasta, D., Bueso, J.P., Athanasiou, L.V., Billinis, C., Fthenakis, G.C., 2016. Dissemination of intestinal pathogens between lambs and puppies in sheep farms. Small Rumin. Res. 141, 5-10.

~~7.~~5. Dargatz, D.A., Marshall, K.L., Fedorka-Cray, P.J., Erdman, M.M., Kopral, C.A., 2015. Salmonella prevalence and antimicrobial susceptibility from the National Animal Health Monitoring System Sheep 2011 Study. Foodborne Pathog. Dis. 12, 953-957.

~~8.~~6. Davies, R.H., Evans, S.J., Preece, B.E., Chappell, S., Kidd, S., Jones, Y.E., 2001. Increase in *Salmonella enterica* subspecies *diarizonae* serovar 61:k:1,5,(7) in sheep. Vet. Rec. 149, 555-557.

~~9. Davison, H.C., Smith, R.P., Pascoe, S.J.S., Sayers, A.R., Davies, R.H., Weaver, J.P., Kidd, S.A., Dalziel, R.W., Evans, S.J., 2005. Prevalence, incidence and geographical distribution of serovars of Salmonella on dairy farms in England and Wales. Vet. Rec. 157, 703-U702.~~

~~10.~~7. Ferreras, M.d.C., Muñoz, M., Perez, V., Benavides, J., García-Pariente, C., Fuertes, M., Aduriz, G., García-Marin, J.F., 2007. Unilateral orchitis and epididymitis caused by *Salmonella enterica* subspecies diarizonae infection in a ram. J. Vet. Diagn. Invest. 19, 194-197.

~~11.~~8. Giner-Lamia, J., Vinuesa, P., Betancor, L., Silva, C., Bisio, J., Soleto, L., Chabalgoity, J.A., Puente, J.L., García-del Portillo, F. 2019. Genome analysis of *Salmonella enterica* subsp.

diarizonae isolates from invasive human infections reveals enrichment of virulence-related functions in lineage ST1256. BMC Genomics 20:99.

~~12. Greenfield, Greenway, J.A., Bigland, C.H., 1973. Arizona infections in sheep associated with gastroenteritis and abortion. Vet. Rec. 92, 400-401.~~

~~13.~~9. Hall, M.L.M., Rowe, B., 1992. *Salmonella arizona* in the united-kingdom from 1966 to 1990. Epidemiol. Infect. 108, 59-65.

~~14. Hannam, D.A.R., Wray, C., Harbourne, J.F., 1986. Experimental *Salmonella arizonae* infection of sheep. Br. Vet. J. 142, 458-466.~~

~~15. Harp, J. A., Myers, L.L., Rich, J.E., Gates, N.L., 1981. Role of *Salmonella arizonae* and other infective agents in enteric disease of lambs. Am. J. Vet. Res. 42, 596-599.~~

~~16. Harvey, R.W.S., Price, T.H., Dixon, J.M.S., 1966. *Salmonella* of subgenus 3 (arizona) isolated from abattoirs in England and Wales. J. Hyg. Cambridge 64, 271-&~~

~~17.~~10. Hjartardottir, S., Gunnarsson, E., Sigvaldadottir, J., 2002. Salmonella in sheep in Iceland. Acta Vet. Scand. 43, 43-48.

~~18.~~11. Lacasta, D., Ferrer, L.M., Ramos, J.J., Bueso, J.P., Boborbia, M., de Arcaute, M.R., Figueras, L., Gonzalez-Sainz, J.M., De las Heras, M., 2012. Chronic proliferative rhinitis associated with *Salmonella enterica* subspecies *diarizonae* in sheep in Spain. J. Comp. Pathol. 146, 72-72.

~~19.~~12. Lacasta, D., Figueras, L., Bueso, J.P., De las Heras, M., Ramos, J.J., Ferrer, L.M., González, J.M., ~~Ruíz~~Ruiz de Arcaute, M., Ortín, A., Marteles, D., Navarro, T., Fernández, A., 2017. Experimental infection with *Salmonella enterica* subsp. *diarizonae* serotype 61:k:1,5,(7) in sheep: Study of cell mediated immune response. Small Rumin. Res. 149, 28-33.

~~20.~~13. Long, J.R., Finley, G.G., Clark, M.H., Rehmtulla, A.J., 1978. Ovine fetal infection due to *Salmonella arizonae*. Can. Vet. J. 19, 260-263.

~~21.~~14. Meehan, J.T., Brogden, K.A., Courtney, C., Cutlip, R.C., Lehmkuhl, H.D., 1992. Chronic proliferative rhinitis associated with *Salmonella arizonae* in sheep. Vet. Pathol. 29, 556-559.

~~22.~~15. Milnes, A.S., Stewart, I., Clifton-Hadley, F.A., Davies, R.H., Newell, D.G., Sayers, A.R., Cheasty, T., Cassar, C., Ridley, A., Cook, A.J.C., Evans, S.J., Teale, C.J., Smith, R.P., McNnally, A., Toszeghy, M., Futter, R., Kay, A., Paiba, G.A., 2008. Intestinal carriage of verocytotoxigenic

Escherichia coli O157, *Salmonella, thermophilic Campylobacter* and *Yersinia enterocolitica*, in cattle, sheep and pigs at slaughter in Great Britain during 2003. Epidemiol. Infect. 136, 739-751.

~~23.~~16. Pritchard, J., 1990. Salmonella arizonae in sheep. Can. Vet. J. 31, 42-42.

~~24.~~17. Rubira, I., Figueras, L., De las Heras, M., Bueso, J.P., Castells, E., Climent, M., Lacasta, D., 2019. Chronic proliferative rhinitis in sheep: An update. Small Rumin. Res., 179, 21-25.

~~25.~~18. Sandberg, M., Alvseike, O., Skjerve, E., 2002. The prevalence and dynamics of *Salmonella enterica* IIIb 61:k:1,5,(7) in sheep flocks in Norway. Prev. Vet. Med. 52, 267-275.

~~26. Schnydrig, P., Overesch, G., Regli, W., Bee, A., Rodriguez-Camposa, S., 2018. *Salmonella enterica* subspecies *diarizonae* serovar 61:(k):1,5,(7) as cause of caprine abortion. Small Rumin. Res. 166, 78-82.~~

~~27.~~19. Sojka, W.J., Wray, C., Shreeve, J.E., Bell, J.C., 1983. The incidence of Salmonella infection in sheep in England and Wales, 1975 To 1981. Br. Vet. J. 139, 386-392.

~~28.~~20. Sörén, K., Lindblad, M., Jernberg, C., Eriksson, E., Melin, L., Wahlstrom, H., Lundh, M., 2015. Changes in the risk management of *Salmonella enterica* subspecies *diarizonae* serovar 61:(k):1,5,(7) in Swedish sheep herds and sheep meat due to the results of a prevalence study 2012. Acta Vet. Scand. 3; 57:6.

~~29.~~21. Stokar-Regenscheit, N., Overesch, G., Giezendanner, R., Roos, S., Gurtner, C., 2017. *Salmonella enterica* subsp. *diarizonae* serotype 61:k:1,5,(7) associated with chronic proliferative rhinitis and high nasal colonization rates in a flock of Texel sheep in Switzerland. Prev. Vet. Med. 145, 78–82.

~~30.~~22. Woldemariam, E., Molla, B., Alemayehu, D., Muckle, A., 2005. Prevalence and distribution of Salmonella in apparently healthy slaughtered sheep and goats in Debre Zeit, Ethiopia. Small Rumin. Res. 58, 19-24.

~~31.~~23. Wolf, C., Schefers, J., 2017. Challenges posed by a flock problem of *Salmonella diarizonae* induced proliferative rhinitis. 9th International Sheep Veterinary Congress. Harrogate. United Kingdom.

~~32. Zottola, T., Montagnaro, S., Magnapera, C., Sasso, S., De Martino, L., Bragagnolo, A., D'Arnici, L., Condoleo, R., Pisanelli, G., Iovane, G., Pagnini, U., 2013. Prevalence and antimicrobial susceptibility of Salmonella in European wild boar (Sus scrofa); Latium Region - Italy. Comp. Immunol. Microbiol. Infect. Dis. 36, 161-168.~~

~~33.~~24. Zweifel, C., Zychowska, M.A., Stephan, R., 2004. Prevalence and characteristics of Shiga toxin- producing *Escherichia coli*, *Salmonella* spp. and *Campylobacter* spp. isolated from slaughtered sheep in Switzerland. Int. J. Food Microbiol. 92, 45-53.

Table 1: Animal census per farm, number of analyzed sheep, positive animals per samples taken from nostrils and ~~feces~~faeces and the total number of positive animals on each farm. Although the nostril samples were taken and analyzed individually from the right and left side, for the statistical study, both were analyzed together. Finally, estimated prevalence and confident interval 95% taking into account the population under study and the number of animals analyzed. In red, farms with CPR cases, in black, negative farms.

FARMS	Census	animals (n)	Pos. nostrils	Pos. feces	An. positive	Prevalence	CI 95%
Farm 1	2261	20	9/20	13/20	14	70.0%	50.01%, 89.99%
Farm 2	838	28	14/28	5/20	16	57.1%	39.08%, 75.12%
Farm 3	1492	20	4/20	0/20	4	20.0%	2.59%, 37.41%
Farm 4	732	20	9/20	4/20	9	45.0%	23.50%, 66.50%
Farm 5	1199	40	15/40	3/20	16	40.0%	25.07%, 54.93%
Farm 6	1906	20	14/20	6/20	16	80.0%	62.56%, 97.44%
Farm 7	1167	20	10/20	3/20	10	50.0%	28.25%, 71.72%
Farm 8	3200	20	0/20	0/20	0	0.0%	0.00%, 0.00%
Farm 9	358	20	9/20	7/20	11	55.0%	33.81%, 76.19%
Farm 10	1697	28	11/28	3/20	11	39.3%	21.36%, 57.24%
All farms	**14850**	**236**	**95/236**	**44/200**	**107**	**45.3%**	**39.00%, 51.60%**

Highlights

- A prevalence of 45.3% of isolations of *Salmonella enterica* subsp. *diarizonae* was found.
- The number of positive samples in nostrils was higher than in faeces.
- Ninety per cent of the analyzed farms had positive animals.
- Farms with a previous diagnosis of CPR were more likely to have a positive animal.

Prevalence of *Salmonella enterica* subsp. *diarizonae* serotype 61:k:1:5:(7) in nasal secretions and stool of sheep flocks with and without cases of chronic proliferative rhinitis in Spain

Luis Figueras[a,b]; Luis Miguel Ferrer[a]; José María González[a,b]; Juan Pedro Bueso[c]; Juan José Ramos[a]; Inés Rubira[b]; Erika Burian[d]; Delia Lacasta[a*]

[a] Animal Pathology Department. Instituto Agroalimentario de Aragón-IA2 (Universidad de Zaragoza-CITA). Veterinary Faculty of Zaragoza. C/Miguel Servet 177. 50013 Zaragoza. Spain.

[b] Gabinete Técnico Veterinario S.L. C/ Isla conejera sn. 50013 Zaragoza. Spain.

[c] Laboratorio Agroambiental de Aragón. Montañana. Spain.

[d] Clínica Veterinaria Coso. C/ Coso 144. 50002 Zaragoza. Spain.

[*]Corresponding author: Delia Lacasta. dlacasta@unizar.es

Abstract

Salmonella enterica subsp. *diarizonae* serotype 61: k: 1,5, (7) (SED) is a microorganism well adapted to sheep; however, it has also been described producing chronic proliferative rhinitis (CPR) in ovine. CPR causes a proliferative inflammation of the ventral nasal turbinates that may totally obstruct the nasal cavity.

The main objective of the present study was to investigate the prevalence of SED in nostrils and stool of sheep without CPR clinical signs in commercial sheep farms of Spain with and without previous clinical cases of CPR.

Five samplings were performed in 10 commercial sheep farms for one year. Samples from nostrils and faeces were taken from four animals without CPR visible clinical signs that belonged to four different age ranges at each farm visit.

The prevalence of positive animals was 45.3%, and the number of positive samples in nostrils was higher than in faeces (38.5% vs 22.5%). Only on one farm was no positive result obtained in the entire study. In almost all positive farms, sheep belonging to the youngest age ranges accounted for more than 50% of positive isolates. Finally, farms with a previous diagnosis of CPR were 1.784 times more likely to have an animal with positive isolation than farms without a previous diagnosis. This could suggest that the infection

pressure in the farm might favour the occurrence of clinical cases of the disease. However, further studies will be necessary to unravel why this saprophytic bacterium is able to cross the epithelial barrier causing severe rhinitis in certain animals.

Keywords: Sheep; *Salmonella enterica* subsp. *diarizonae* serotype 61: k: 1.5, (7); chronic proliferative rhinitis; prevalence.

Introduction

Salmonella enterica subsp. *diarizonae* serotype 61: k: 1.5, (7) (SED) is considered a microorganism adapted to sheep, these animals behaving normally as asymptomatic carriers (Zweifel et al., 2004; Sörén et al., 2015).

Several works carried out in abattoirs reported the presence of SED in healthy sheep. Thus, in the United Kingdom (Sojka et al., 1983; Hall and Rowe, 1992; Davies et al., 2001) and Norway (Alvseike and Skjerve, 2002; Sandberg et al., 2002), the presence of this microorganism is widely described. Similarly, it has also been isolated from sheep in other countries such as Switzerland (Zweifel et al., 2004; Bonke et al., 2012; Stokar-Regenscheit et al., 2017), Iceland (Hjartardottir et al., 2002), Sweden (Sörén et al., 2015), Canada (Pritchard, 1990) or the United States (Dargatz et al., 2015; Wolf and Schefers; 2017). However, in all these studies, only intestinal content was analyzed, and the percentages of isolation found were generally low. Only one study investigates the presence of SED in the respiratory tract, specifically in tonsils, and they find 43% of positive animals in this location (Bonke et al., 2012).

According to the results obtained from all these studies, *Salmonella enterica* subsp. *diarizonae* serotype 61: k: 1.5, (7) can be considered a saprophyte microorganism of sheep; however, it has also been described producing disease in these animals. Thus,

chronic proliferative rhinitis (CPR) is an upper respiratory tract disorder of sheep associated with SED that was described for the first time in the USA in 1992 (Meehan et al., 1992) and later reported in Spain (Lacasta et al., 2012), Switzerland (Stokar-Regenscheit et al., 2017) and the USA again (Wolf and Schefers, 2017). CPR is a slow and progressive disease that causes severe inflammation of the ventral nasal turbinates with very poor prognosis for the untreated affected animals (Rubira et al., 2019). It has been stated that the inflammatory response occurs when the bacterium penetrates the nasal epithelial cells causing a massive inflammatory reaction (Lacasta et al., 2012).

Furthermore, SED has also been related to diarrhoea in lambs (Long et al., 1978; Davies et al., 2001; Alvseike and Skjerve, 2002; Chatzopoulos et al., 2016), abortions and stillbirths in sheep (Long et al., 1978; Davies et al., 2001), and also producing orchitis and epididymitis in rams (Ferreras et al., 2007; Celeghini et al., 2013). Infections with SED in humans have also been reported, although its zoonotic potential is generally considered low (Sörén et al., 2015; Giner-Lamia et al., 2019).

The importance of this bacterium as a pathogen capable of producing CPR in sheep seems to have been increased in recent years, with the number of reports of CPR in international publications and conferences growing. According to the knowledge of the authors, so far, no prevalence studies have been carried out to analyse the presence of these bacteria in the upper respiratory tract of sheep without clinical signs of CPR. The main objective of the present study was to investigate the prevalence of *Salmonella enterica* subsp. *diarizonae* serotype 61: k: 1,5, (7) in nostrils and stool of adult sheep without visible clinical signs of CPR in commercial sheep farms of Spain with and without previous clinical cases of CPR diagnosed.

Material and methods

All procedures were carried out under Project Licence PI 22/11 approved by the Ethics Committee for Animal Experiments from the University of Zaragoza. The care and use of animals were performed accordingly with the Spanish Policy for Animal Protection RD53/2013, which meets the European Union Directive 2010/63 on the protection of animals used for experimental and other scientific purposes.

Ten commercial sheep farms located in Aragon, a northeast region of Spain, were selected for the study. In five of these farms, CPR had never been detected before and, in the other five, animals with CPR had been previously diagnosed, and pathological and microbiological studies had confirmed the process. All the farms were meat flocks, and seven of them were reared in a semi-intensive production system and the other three in an intensive system with permanent stabling. The management of all the farms in semi-intensive production system was very similar, with stabling of the ewes at the end of gestation and during lactation until weaning, when lambs were 45 days old. The three farms reared in an intensive system remained stabled without grazing, fed on cereals and fodder. The health programme was similar in all the farms, and it was carried out by the same veterinary group. The number of sheep in the farms ranged from 358 to 3,200 animals (Table 1). The farms were identified correlatively from 1 to 10 for the study.

Five complete samplings were performed on each farm for one year. Samples from nostrils, right and left, and faeces were taken from four animals without CPR visible clinical signs that belonged to four different age ranges (0-2 years, 2-4 years, 4-6 years and more than six years) at each farm visit. Samplings of the nostrils were performed using a sterile swab with culture medium (Deltalab-Eurotubo®Sterile) of both nostrils separately, identifying whether it was right or left. The faeces sample was taken directly from the rectum of the animals with a sterile glove, trying to obtain as much as possible

and never less than 10 grams. Each sample was identified with the tag number or individual identification of the animal, the age range to which it belonged, the date of sampling and the farm. Once the sampling was done, the samples were immediately sent to the Agroambiental Laboratory of the Government of Aragon in refrigeration for culture and identification.

Further to the five complete samplings performed in each farm (50 samplings), nine additional samplings were carried out on farms 2, 5 and 10, in which only samples from nostrils were taken. In these farms, the number of nostril samples was increased because the greater collaboration by the farmers allowed us to expand the number of samples. Therefore, in total, we worked with 50 complete samplings (200 faeces samples and 400 nostril samples) and nine additional nostril samplings (72 samples collected from 36 sheep), which made a total of 472 nostril samples (236 animals) and 200 faeces samples (Table 1).

Samples were cultured into Buffered Peptone Water (APT) Xylose Lysine Deoxycholate Agar (XLD) and Salmonella Shigella Agar (SS), and incubated at 37◦C for 24–48h. Colonies resembling Salmonella were chosen and identified by a conventional biochemical test: Triple Sugar Iron agar (TSI), Lysine decarboxylase, Ornithine decarboxylase, Arginine decarboxylase dihydrolase, Christensen'sUrea, Voges-Proskauer and the Beta-galactosidase test. Selected colonies were sent for serotyping to the Central Veterinary Laboratory (Algete, Madrid, Spain).

The statistical study was carried out at two levels according to the unit of study used: farm and animal. Although the microbiological analyzes were performed separately from the right and left nostril, the statistical studies were performed from both sides together. All the variables used in this work were of a qualitative type and for their study association tests between variables, such as Chi-square, were used, using Fisher correction, if

necessary, and calculating the relative risks whenever possible. These association tests were performed for the presence/absence of SED in nostrils, faeces or either. In all cases, a statistical significance $p<0.05$ was required to validate the hypothesis. In order to calculate the estimated prevalence, both individual and collective calculations were carried out assuming a 95% confidence interval. To calculate the collective prevalence within each farm, 20 sheep were assumed as the value of the sample for all farms except farm 2 and 10 with 28 animals, and farm 5 with 40 sheep. In all cases, it was assumed that the diagnosis was right. The Winepi software (http://www.winepi.net/) was used, assuming as a study population of 3,478 sheep farms located in Aragon in 2013 and a global census for the same year and the same region of 1,576,218 sheep.

Results

The data collected were statistically analyzed at two levels: animals and farm, and thus the results will be exposed.

Animals

The prevalence of positive animals in nostrils or faeces was 45.3% (107/236). When analyzing only the 50 complete samplings (faeces and nostrils), the number of positive samples in nostrils was higher than in faeces: 22.0% (44/200) were positive only in nostrils, 6.0% (12/200) were positive only in faeces, and 16.5% (33/200) were positive in nostrils and faeces simultaneously. In total, 38.5% (77/200) showed positive isolation in nostrils compared to 22.5% (45/200) in faeces.

In addition, 24.3% (26/107) belonged to the group from 0-2 years, 30.8% (33/107) to the group of sheep from 2-4 years old, 23.4% (25/107) to the group from 4-6 years old and

21.5% (23/107) to the group older than six years. No significant differences were found between groups (p>0.005).

To evaluate the influence of the climate when collecting the samples, these were grouped according to the season of the year. The highest proportion of positive samples in one of the two locations, nostrils or faeces, was obtained in summer (55.8%), although the statistical analysis did not show significant differences (p> 0.05). However, when the results of the positive animals in faeces were analyzed, it was obtained a significant reduction in the proportion of positive samples during the autumn, resulting statistically significant (p <0.05); which established a 2.882, 2.597 and 3.774 times higher risk of finding SED in faeces samples during winter, spring and summer, respectively, than in autumn.

Farms

Nine of the ten analyzed farms had at least one positive isolation of *Salmonella enterica* subsps. *diarizonae* serotype 61:k:1:5:(7) in one of the locations (nostrils or faeces). Only farm number 8 did not present, at any time and in any location, isolation of SED (Table 1). In addition, all positive farms had SED isolations in both locations, nostrils and faeces, except farm number 3, that only had positive isolates in the nostrils and in a lower proportion (4/20) than the other eight positive flocks of the study. All farms, except flock number 1, had a higher percentage of isolations in the nostrils. Interestingly, farm number 1, which presented the highest number of isolates in faeces, was the one with the highest number of total positive isolates (22/40), nine being positive in nostrils and 13 in faeces samples. However, the second farm with more total positive isolates, farm number 6, presented the majority of the isolations in nostrils (14/40), as happened in the rest of the positive flocks, although in a smaller proportion (Table 1).

In almost all positive farms, sheep belonging to the youngest age ranges (0-2 and 2-4 years) accounted for more than 50% of positive isolates. Only farm 10 had the highest number of isolates in the group of sheep aged 4-6 years.

The estimated prevalence for each farm was calculated taking into account the population under study and the number of animals analyzed: 20 sheep for farms 1, 3, 4, 6, 7, 8 and 9; 28 animals for farms 2 and 10, and 40 sheep for farm 5. In all cases, it was assumed that the diagnosis was accurate, with a 95% confidence interval. Thus, farm number 1 had an estimated prevalence of 70.0% (50.01%, 89.99%) and farm number 2 had 57.1% (39.08%, 75.12%). The flock number 6 was in which the highest estimated prevalence was found at 80.0% (62.56%, 97.44%), while farm number 8 was the only one with a prevalence of 0.0% (Table 1).

The collective and individual prevalence in the Aragon region (Spain) was also calculated with the data of the year of the study (2013). In this year, 3,478 sheep farms were active in Aragon, and there was a total census of 1,576,218 sheep. Prevalence calculations were carried out assuming a 95% confidence interval. With these data, an estimated collective prevalence of 90.0% (71.4%, 100.0%) and an individual prevalence of 45.3% (38.9%, 51.6%) were obtained.

Finally, the presence of SED was analyzed according to whether the farms had presented cases of chronic proliferative rhinitis or not. This comparison showed significant differences ($p = 0.036$). Farms with a previous diagnosis of CPR were 1.784 times more likely to have an animal with positive isolation than farms without a previous diagnosis. However, the analysis of the location of the bacteria, either in nostrils or faeces, did not show differences.

Interestingly, in farm number 8, in which CPR had not been diagnosed prior to this study and which was negative for the presence of SED in all samples taken, *Salmonella* spp. isolations were found in some of the animals. Specifically, *Salmonella enterica* subsp. *enterica* serotype Abortusovis*, Salmonella enterica* subsp. *enterica* serotype Anatum*, Salmonella enterica* subsp. *enterica* serotype Mbandaka *and Salmonella enterica* subsp. *enterica* serotype Agona. On the contrary, this fact was not observed in any of the other nine farms in the study in which only *Salmonella enterica* subsp. *diarizonae* serotype 61: k: 1.5, (7) was isolated.

Discussion

At present, and despite SED studies carried out in abattoirs in several countries, there are very few prevalence studies conducted in flocks. Only Norway, Switzerland and Sweden reported flock-based studies (Alvseike and Skjerve, 2002; Sandberg et al., 2002; Bonke et al., 2012; Sörén et al., 2015), however, in all of them only intestinal content was analyzed. Despite the association of SED with upper respiratory tract diseases in sheep (Meehan et al., 1992; Lacasta et al., 2012; Stokar-Regenscheit et al., 2017; Wolf and Schefers, 2017), none of the works reviewed in the literature, neither in flocks nor in abattoirs, took samples of the respiratory system, only Bonke et al. (2012) analyzed tonsils at abattoirs, with 43% of positive isolations. Surprisingly, the results of our study show that SED was more frequently isolated from nostril samples than from stool (38.5% vs 22.5%).

In Sweden, as in Norway and Finland, the prevalence of Salmonella in food-producing animals is very low due to a control program initiated more than 50 years ago. However, in a prevalence study carried out in Sweden in 2012 over 237 sheep flocks, an overall

prevalence of SED of 17.6% around all the country was obtained. As scientific opinions and evaluation of on-farm control measures performed concluded that the impact of sheep associated *S. enterica* subsp. *diarizonae* on human health was very low, Swedish authorities decided to make an exemption for *S. enterica* subsp. *diarizonae* in sheep in the current Salmonella control measures (Sörén et al., 2015).

In a study carried out in Norway in 50 flocks, the prevalence was 14% (Sandberg et al., 2002) and in a subsequent work also performed in Norway on 133 farms, the herd prevalence was found to range from 10 to 45% (Alvseike and Skjerve, 2002). Unexpectedly, in our study, nine of the ten analyzed farms were positive (extrapolated collective prevalence of 90%) with an individual prevalence of 45.3%, although there was also high variability among farms, ranging from 0.0% to 80.0%. Therefore, it could be concluded that SED seems to be endemic in Spain, with a very high prevalence compared to what has been reported in other countries (Alvseike and Skjerve, 2002; Davies et al., 2001; Sandberg et al., 2002; Sörén et al., 2015). However, based on the results obtained in the present study, one would think that perhaps if in those studies carried out in northern Europe, samples had been taken from the nostrils in addition to faeces, the prevalence found would have been higher.

In other studies performed in abattoirs, the prevalence found was even lower, thus, in Britain, Milnes et al. (2008) found a prevalence of 1.0%, in Iceland, 2.0% (Hjartardottir et al., 2002), in Ethiopia, 3.0 to 17.6% (Woldemariam et al., 2005), in the United States, 26.9% (Dargatz et al., 2015) and in Switzerland from 11 to 43% (Bonke et al., 2012; Zweifel et al., 2004). Nevertheless, sheep analyzed in abattoir studies are generally elderly culling animals, which biases the results even more since in our study we observed that the oldest (older than six years) are the age range with the lowest percentage of isolation. This partially contrasts with the results obtained by Bonke et al. (2012) and

Sandberg et al. (2002), that related the age to the presence of SED in sheep, and both concluded that SED is most often isolated from animals older than two years.

In our study, the animals that showed positive results in nostrils had a high possibility of being positive also in faeces. This differs from the results reported by Bonke et al. (2012), that despite obtaining a high prevalence in tonsils, these same animals were negative in faeces. The authors justified the poor results of isolation in intestinal content with the small amount of sample analyzed and the poor conservation.

In addition, no substantial differences in SED isolates were found according to the season of the year, showing only significant differences in the lowest presence of positive isolates in faeces during autumn. Other published studies reported the highest percentage of isolations during spring, associating this with seasonality and lambing (Davies et al., 2001; Hjartardottir et al., 2002). Likewise, it has been described that there is greater isolation of the bacteria in animals in the last stage of pregnancy (Bonke et al., 2012). In other studies, the increase in the number of isolates is associated with stressful situations such as vaccinations (Lacasta et al., 2012), the transfer of animals to pastures in autumn (Hjartardottir et al., 2002) or related to management, production or immune system status (Long et al., 1978; Pritchard, 1990). By contrast, in the study of Sandberg et al. (2002), stress is dismissed as a risk factor.

Lastly, a significantly higher percentage of isolates of SED was found in our study in the flocks with previous cases of CPR than in those in which the disease had never been diagnosed. This could suggest that the infection pressure in the farm might favour the occurrence of clinical cases of the disease, since, as concluded in the experimental infection carried out by Lacasta et al. (2017), the simple presence of the bacteria in the nasal secretions is not enough to trigger clinical signs of the disease and other factors, yet to be discovered, are necessary for SED to pass through the epithelial cells of the nostrils

and elicit the inflammatory reaction. Further studies will be necessary to unravel why this saprophytic bacterium of the high respiratory tract is able to cross the epithelial barrier causing severe inflammation in some animals.

Funding

This study was supported by the Aragón Government and the European Social Fund (Construyendo Aragón 2016-20).

Acknowledgements

Authors would like to acknowledge veterinary colleagues and sheep farmers who have collaborated in the development of this work.

Conflict of interest statement

The authors have nothing to disclose.

References

1. Alvseike, O., Skjerve, E., 2002. Prevalence of a *Salmonella* subspecies *diarizonae* in Norwegian sheep herds. Prev. Vet. Med. 52, 277-285.
2. Bonke, R., Wacheck, S., Bumann, C., Thum, C., Stueber, E., Koenig, M., Stephan, R., Fredriksson- Ahomaa, M., 2012. High prevalence of *Salmonella enterica* subsp *diarizonae* in tonsils of sheep at slaughter. Food Res. Int. 45, 880-884.

3. Celeghini, E.C.C., Gregory, L., Pinheiro, E.S., Piva, F.M., Carneiro, P.A.B., Parapinski-Santos, B., Bianchi, M., Benesi, F.J., 2013. Orchiepididymitis in ram by *Salmonella enterica* sub *diarizonae*: first case in South America. Arq. Bras. Med. Vet. Zootec. 65, 139-144.
4. Chatzopoulos, D.C., Sarrou, S., Vasileiou, N.G.C., Ioannidi, K.S., Peteinaki, E., Valiakos, G., Tsokana, C.N., Papadopoulos, E., Spyrou, V., Mavrogianni, V.S., Giannakopoulos, A., Sbiraki, A., Lacasta, D., Bueso, J.P., Athanasiou, L.V., Billinis, C., Fthenakis, G.C., 2016. Dissemination of intestinal pathogens between lambs and puppies in sheep farms. Small Rumin. Res. 141, 5-10.
5. Dargatz, D.A., Marshall, K.L., Fedorka-Cray, P.J., Erdman, M.M., Kopral, C.A., 2015. Salmonella prevalence and antimicrobial susceptibility from the National Animal Health Monitoring System Sheep 2011 Study. Foodborne Pathog. Dis. 12, 953-957.
6. Davies, R.H., Evans, S.J., Preece, B.E., Chappell, S., Kidd, S., Jones, Y.E., 2001. Increase in *Salmonella enterica* subspecies *diarizonae* serovar 61:k:1,5,(7) in sheep. Vet. Rec. 149, 555-557.
7. Ferreras, M.d.C., Muñoz, M., Perez, V., Benavides, J., García-Pariente, C., Fuertes, M., Aduriz, G., García-Marin, J.F., 2007. Unilateral orchitis and epididymitis caused by *Salmonella enterica* subspecies diarizonae infection in a ram. J. Vet. Diagn. Invest. 19, 194-197.
8. Giner-Lamia, J., Vinuesa, P., Betancor, L., Silva, C., Bisio, J., Soleto, L., Chabalgoity, J.A., Puente, J.L., García-del Portillo, F. 2019. Genome analysis of *Salmonella enterica* subsp. *diarizonae* isolates from invasive human infections reveals enrichment of virulence-related functions in lineage ST1256. BMC Genomics 20:99.
9. Hall, M.L.M., Rowe, B., 1992. *Salmonella arizona* in the united-kingdom from 1966 to 1990. Epidemiol. Infect. 108, 59-65.
10. Hjartardottir, S., Gunnarsson, E., Sigvaldadottir, J., 2002. Salmonella in sheep in Iceland. Acta Vet. Scand. 43, 43-48.
11. Lacasta, D., Ferrer, L.M., Ramos, J.J., Bueso, J.P., Boborbia, M., de Arcaute, M.R., Figueras, L., Gonzalez-Sainz, J.M., De las Heras, M., 2012. Chronic proliferative rhinitis associated with *Salmonella enterica* subspecies *diarizonae* in sheep in Spain. J. Comp. Pathol. 146, 72-72.
12. Lacasta, D., Figueras, L., Bueso, J.P., De las Heras, M., Ramos, J.J., Ferrer, L.M., González, J.M., Ruiz de Arcaute, M., Ortín, A., Marteles, D., Navarro, T., Fernández, A., 2017. Experimental infection with *Salmonella enterica* subsp. *diarizonae* serotype 61:k:1,5,(7) in sheep: Study of cell mediated immune response. Small Rumin. Res. 149, 28-33.

13. Long, J.R., Finley, G.G., Clark, M.H., Rehmtulla, A.J., 1978. Ovine fetal infection due to *Salmonella arizonae*. Can. Vet. J. 19, 260-263.
14. Meehan, J.T., Brogden, K.A., Courtney, C., Cutlip, R.C., Lehmkuhl, H.D., 1992. Chronic proliferative rhinitis associated with *Salmonella arizonae* in sheep. Vet. Pathol. 29, 556-559.
15. Milnes, A.S., Stewart, I., Clifton-Hadley, F.A., Davies, R.H., Newell, D.G., Sayers, A.R., Cheasty, T., Cassar, C., Ridley, A., Cook, A.J.C., Evans, S.J., Teale, C.J., Smith, R.P., McNnally, A., Toszeghy, M., Futter, R., Kay, A., Paiba, G.A., 2008. Intestinal carriage of verocytotoxigenic Escherichia coli O157, *Salmonella, thermophilic Campylobacter* and *Yersinia enterocolitica*, in cattle, sheep and pigs at slaughter in Great Britain during 2003. Epidemiol. Infect. 136, 739-751.
16. Pritchard, J., 1990. Salmonella arizonae in sheep. Can. Vet. J. 31, 42-42.
17. Rubira, I., Figueras, L., De las Heras, M., Bueso, J.P., Castells, E., Climent, M., Lacasta, D., 2019. Chronic proliferative rhinitis in sheep: An update. Small Rumin. Res., 179, 21-25.
18. Sandberg, M., Alvseike, O., Skjerve, E., 2002. The prevalence and dynamics of *Salmonella enterica* IIIb 61:k:1,5,(7) in sheep flocks in Norway. Prev. Vet. Med. 52, 267-275.
19. Sojka, W.J., Wray, C., Shreeve, J.E., Bell, J.C., 1983. The incidence of Salmonella infection in sheep in England and Wales, 1975 To 1981. Br. Vet. J. 139, 386-392.
20. Sörén, K., Lindblad, M., Jernberg, C., Eriksson, E., Melin, L., Wahlstrom, H., Lundh, M., 2015. Changes in the risk management of *Salmonella enterica* subspecies *diarizonae* serovar 61:(k):1,5,(7) in Swedish sheep herds and sheep meat due to the results of a prevalence study 2012. Acta Vet. Scand. 3; 57:6.
21. Stokar-Regenscheit, N., Overesch, G., Giezendanner, R., Roos, S., Gurtner, C., 2017. *Salmonella enterica* subsp. *diarizonae* serotype 61:k:1,5,(7) associated with chronic proliferative rhinitis and high nasal colonization rates in a flock of Texel sheep in Switzerland. Prev. Vet. Med. 145, 78–82.
22. Woldemariam, E., Molla, B., Alemayehu, D., Muckle, A., 2005. Prevalence and distribution of Salmonella in apparently healthy slaughtered sheep and goats in Debre Zeit, Ethiopia. Small Rumin. Res. 58, 19-24.
23. Wolf, C., Schefers, J., 2017. Challenges posed by a flock problem of *Salmonella diarizonae* induced proliferative rhinitis. 9th International Sheep Veterinary Congress. Harrogate. United Kingdom.

24. Zweifel, C., Zychowska, M.A., Stephan, R., 2004. Prevalence and characteristics of Shiga toxin-producing *Escherichia coli*, *Salmonella* spp. and *Campylobacter* spp. isolated from slaughtered sheep in Switzerland. Int. J. Food Microbiol. 92, 45-53.

Table 1: Animal census per farm, number of analyzed sheep, positive animals per samples taken from nostrils and faeces and the total number of positive animals on each farm. Although the nostril samples were taken and analyzed individually from the right and left side, for the statistical study, both were analyzed together. Finally, estimated prevalence and confident interval 95% taking into account the population under study and the number of animals analyzed. In red, farms with CPR cases, in black, negative farms.

FARMS	Census	animals (n)	Pos. nostrils	Pos. feces	An. positive	Prevalence	CI 95%
Farm 1	2261	20	9/20	13/20	14	70.0%	50.01%, 89.99%
Farm 2	838	28	14/28	5/20	16	57.1%	39.08%, 75.12%
Farm 3	1492	20	4/20	0/20	4	20.0%	2.59%, 37.41%
Farm 4	732	20	9/20	4/20	9	45.0%	23.50%, 66.50%
Farm 5	1199	40	15/40	3/20	16	40.0%	25.07%, 54.93%
Farm 6	1906	20	14/20	6/20	16	80.0%	62.56%, 97.44%
Farm 7	1167	20	10/20	3/20	10	50.0%	28.25%, 71.72%
Farm 8	3200	20	0/20	0/20	0	0.0%	0.00%, 0.00%
Farm 9	358	20	9/20	7/20	11	55.0%	33.81%, 76.19%
Farm 10	1697	28	11/28	3/20	11	39.3%	21.36%, 57.24%
All farms	**14850**	**236**	**95/236**	**44/200**	**107**	**45.3%**	**39.00%, 51.60%**