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Improving the Diagnosis of Bacterial Rejections in Ovine Abattoirs by the Use of Simple Protocols

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Abstract
Veterinary inspection in abattoirs is extremely important either economic or public health point of view because a great amount of visceras are rejected in order to maintain a low risk for human. However, due to work dynamics in slaughterhouses, it is usually difficult to uncover this etiology. In this study, we applied simple protocols to determine the final diagnosis and the etiology of such rejections. Over the course of a year, organs rejected during meat inspection were sampled from an ovine slaughterhouse in central Spain that slaughtered both sheep and lambs. The application of these protocols were very useful in the identification of bacterial agents involved in those rejections that clinically were compatible with enzootic pneumonia and caseous lymphadenitis as well as abscesses, among others. The regular application of these protocols would provide valuable information to establish control measures of those processes that reach to the slaughterhouses and in consequence to avoid they reach the human food chain.
Keywords: Slaughter, bacterial rejections, diagnose, ovine, Public health

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INTRODUCTION

Bacterial processes affecting human and animals are usually multifactorial; however, there are one or two predominant agents involved in a great number of processes. For example, in the ovine enzootic pneumonia, excluding micoplasms, main bacterial agents are several strains of *M. haemolytica*, although from the lesions, occasionally, other organisms can be isolated: *Pasteurella multocida*, *Bordetella parapertussis* or *Branhamella catharralis* (Jones *et al.,* 1979; Arrigo *et al.,* 1984; Jubb *et al.,* 1993; Hervás *et al.,* 1996; Martin, 1996; Martin & Aitken, 2002). Similarly, *Fusobacterium necrophorum* is the most important agent involved in localised purulent processes in liver and lungs (Moreno, 2006) along with *Dichelobacter spp.* (Biberstein & Zee, 1994). The corinebacteria of intererst in meat inspection include *Arcanobacterium pyogenes*, agents of purulent infectious processes, and *Corynebacterium pseudotuberculosis*, which produces pseudotuberculosis or ovine caseous lymphadenitis (Moreno, 2006). The differential diagnostic with other pathologies that show up with abscesses of similar features is of high importance. Among them, infections caused by *Staphylococcus aureus*, *Streptococcus spp.* *Arcanobacterium pyogenes* (Burrel, 1980; Aleman & Spier, 2001; León *et al.,* 2002) should be considered. The microbiological studies of the disease of abscesses, also known as enfermedad de Morel, have determined that the etiological agent that causes the condition is *Staphylococcus aureus anaerobius* (De la Fuente *et al.,* 1985; De la Fuente, *et al.,* 2011).

In this context, there is a clear interest in establishing the underlying bacterial etiology of the rejections. The aim of this study was to applicate several protocols and basic laboratory techniques to identify common bacterial agents involved and equipment.

MATERIALS AND METHODS

From October 2010 to September 2011 in the central area of Spain. 40-50 lambs and 10-20 adult sheep older than two years of age were monthly sampled in a slaughterhouse in Madrid (Central Spain). A preliminary identification of each rejection were initially made by the official Veterinary inspector and immediately a portion of confiscated tissue was obtained with sterile procedures keeping samples at -20ºC until laboratory procedures.

According to the initial identification rejection samples: lesions compatible with enzootic pneumonia (105 cases in lambs and 17 in adults); with caseous lymphadenitis (24 cases in adults) and pulmonary abscesses; and with hepatic abscesses, caseous lymphadenitis, hepatitis and hepatic necrosis were processes following one of the protocols shown on Tables 1, 2 and 3, respectively. Any remaining bacterial species was rendered as unidentified and classified as "other". Samples with no bacterial growth were classified as "no growth".

In all cases bacterial identification entailed bacterial culture, bacterial staining from the lesion and the resulting culture, and biochemical testing (additional information may be seen in Vilallonga, 2013).

Table 1. Diagnostic protocol used in lesions compatible with ruminant enzootic pneumonia.

<table>
<tr><td colspan="4">Direct Gram stain from the lesion: Gram +, Gram -, cocci or bacillus</td></tr>
<tr><td colspan="2">Direct inoculation in 5% ovine blood agar from the lesion in aerobiosis (37ºC, 48/72 hours)</td><td colspan="2">Direct inoculation in agar McConkey from the lesion
(37ºC, 24/48 hours)</td></tr>
<tr><td>Growth</td><td>No growth</td><td>Growth</td><td>No growth</td></tr>
<tr><td>Possible positivity to</td><td>Negative to</td><td rowspan="2">B. parapertussis
M. haemolytica (+)</td><td rowspan="2">Negativo a
B. parapertussis
M. haemolytica (+)</td></tr>
<tr><td colspan="2">B. catarrhalis B. parapertussis M haemolytica P. multocida</td></tr>
<tr><td>Continue the identification</td><td>Other unidentified germs</td><td>Continue the identification</td><td>Continue the identification</td></tr>
<tr><td colspan="4">Growth in 5% ovine blood agar (reinoculation and isolation of compatible colonies if needed due to contamination)</td></tr>
<tr><td>- Blood agar: whitish or greyish colonies, small (1-2 mm) with hemolysis around the colony

- McConkey agar: faint growth (+) in dotted red colonies (lactose +)

- Gram -, bipolars (v), coccobacillus/small bacillus

- Catalase +

- Oxidase +</td><td>- Blood agar: small colonies (1-2 mm), smooth or mucousy, rounded, greyish or translucid, with no hemolysis, sweet smell

- McConkey agar: no growth

- Gram -, bipolars (v), coccobacillus/small bacillus

- Catalase +

- Oxidase +</td><td>- Blood agar: hemolysis (+) under the colony, medium size and greyish color

- McConkey agar: no growth

- Gram -, diplococcus

- Catalase +

- Oxidase +</td><td>- Blood agar: with hemolysis under the colony, medium size and greyish color

- McConkey agar: transparent colonies (lactose -)

- Gram -, small coccobacillus

- Catalase +

- Oxidase +</td></tr>
<tr><td>Mannheimia haemolytica</td><td>Pasteurella multocida</td><td>Branhamella catarrhalis</td><td>Bordetella parapertussis</td></tr>
</table>

(v) variable (+) more than 90%

Table 2. Diagnostic protocol used in lesions compatible with pulmonary caseous lymphadenitis and pulmonary abscesses.

Direct Gram stain from the lesion: Gram +, Gram -, cocci or bacillus					
Direct inoculation in 5% ovine blood agar from the lesion (37ºC, 48/72 hours)				Direct inoculation in agar McConkey from the lesion (37ºC, 24/48 hours)	
Growth in aerobiosis	No growth in aerobiosis	Growth in anaerobiosis	No growth in anaerobiosis	Growth	No growth
Possible	Negative to	Possible	Negative to	*P.aeruginosa* or contamination by Gram - enterobacteria	Negative to *P.aeruginosa* and no contamination by Gram - enterobacteria
A. pyogenes *C. pseudotuberculosis* *P.aeruginosa* *S aureus aureus* *Streptococcus spp.*					
Continue the identification	Other unidentified germs	Continue the identification	Other unidentified germs	Continue the identification	Continue the identification
Growth in 5% ovine blood agar (reinoculation and isolation of compatible colonies if needed due to contamination)					
Aerobiosis					Anaerobiosis
- Blood agar in aerobiosis/anaerobiosis: small colonies (1-2 mm), whitish-greyish and dry, with hemolysis after 48/72 hours - McConkey agar: No growth - Gram +, pleomorphic bacillus with club shape in palisades - Catalase + - Oxidase –	- Blood agar in aerobiosis/anaerobiosis: very small colonies (1 mm), white, opaque and shiny, with hemolysis after 48 hours - McConkey agar: No growth - Gram +, pleomorphic bacillus, single, in pairs (commonly in "V" shape) or short chains - Catalase – - Oxidase (v)	- Blood agar in aerobiosis: large (3-4 mm), fruitish odour, greyish-yellowish-greenish-brownish, uneven edges and metallic shine, with hemolysis (+) - McConkey agar: large colonies (3-4 mm), white-translucid or yellowish-greenish-brownish (lactose -) - Gram -, small bacillus - Catalase + - Oxidase +	- Blood agar in aerobiosis/ anaerobiosis: hemolysis (+), semi-transparent colonies - McConkey agar: No growth - Gram +, ovoid streptococcus, commonly in pairs or chains - Catalase – - Oxidase –	- Blood agar in aerobiosis/anaerobiosis: middle size colonies (2-3 mm), opaque, slightly convex, goldish color, orange or greyish, with hemolysis - McConkey agar: No growth - Gram +, stafilococcus - Catalase + - Oxidase –	- Blood agar in anaerobiosis: no pigments, with hemolysis - McConkey agar: No growth - Gram +, estafilococcus - Catalase – - Oxidase –
C. pseudotuberculosis	*A. pyogenes*	*P. aeruginosa*	*Streptococcus spp.*	*S. aureus aureus*	*S.aureus anaerobius*

(v) variable (+) more than 90%

Table 3. Diagnostic protocol used in lesions with hepatic abscesses, hepatic caseous lymphadenitis, hepatitis and hepatic necrosis.

<table>
<tr><td colspan="6">Direct Gram stain from the lesion: Gram +, Gram -, cocci or bacillus</td></tr>
<tr><td colspan="2">- Gram +
- Gram -, small bacillus</td><td colspan="2">- Gram - (faint), carbol fuchsin stain, cocoid-fusiform or filamentous bacteria, unevenly stained</td><td colspan="2">- Gram - (faint), carbol fuchsin stain, bacillus, straight or slightly curved, sometimes with thicker ends on both sides, single or in pairs</td></tr>
<tr><td colspan="2">Possible positivity to
C. pseudotuberculosis A. pyogenes
S. aureus aureus Streptococcus spp.
P. aeruginosa</td><td colspan="2">Fusobacterium spp.</td><td colspan="2">Dichelobacter spp.</td></tr>
<tr><td colspan="2">Continue the identification</td><td colspan="2">Diagnostic if these characterictics are found, otherwise considered unidentified germs</td><td colspan="2">Diagnostic if these characterictics are found, otherwise considered unidentified germs</td></tr>
<tr><td colspan="6">Direct inoculation in the culture from the lesion
(reinoculation and isolation of compatible colonies if needed due to contamination)</td></tr>
<tr><td colspan="3">5% ovine blood agar (37ºC, 48/72 hours, aerobiosis)</td><td colspan="3">MacConkey agar (37ºC, 24/48 hours)</td></tr>
<tr><td rowspan="2">- Growth in blood agar in aerobiosis/anaerobiosis with hemolysis

- Gram +</td><td colspan="2" rowspan="2">- Growth in blood agar in aerobiosis, large colonies (3-4 mm), fruity odour, greenish, uneven edges and metallic shine, with hemolysis (+)

- Growth in McConkey agar, large colonies (3-4 mm), whtish-translucid or yellowish-greenish-brownish (lactose -)

- Gram -, small bacillus

- Catalase +

- Oxidase +</td><td colspan="2">Growth</td><td>No growth</td></tr>
<tr><td colspan="2" rowspan="2">Pseudomonas aeruginosa
or
contamination by Gram - enterobacteria</td><td rowspan="2">Negative for Pseudomonas aeruginosa and no contamination by Gram - enterobacteria</td></tr>
<tr><td>C. pseudotuberculosis
A. pyogenes
S. aureus aureus
Streptococcus spp.</td><td colspan="2">Pseudomonas aeruginosa</td></tr>
<tr><td>Continue identification in Table nº 2</td><td colspan="2">Diagnostic if these characterictics are found, otherwise considered unidentified germs</td><td colspan="2">Continue the identification</td><td>Continue the identification</td></tr>
</table>

(v) variable (+) more than 90%

RESULTS

A total of 2,429 animals were inspected and 577 organs were rejected, the 60.14% of which had a bacterial condition as the cause of the condemnation whilst the remainder of the rejections had either a parasitic origin or some other cause (38.13% and 1.73%, Valcárcel and Villalonga, 2015).

The number of cultures and isolations were variable due the irregular presence in the abattoir. So, we processed (lambs/adults) 105/17 cases of enzootic pneumonia; 0/24 of caseous lymphadenitis; 3/16 of other lung processes; 39/23 of liver abscess; 14/8 of other liver processes; 0/1 of abscesse disease; 13/0 of cisticercosis; and fnally 0/22 of hydatidosis. The bacterial identified in each rejection are shown in Table 4.

Table 4. Bacterial identified in the different rejections sampled in an ovine abattoir in central Spain during October 2010 to September 2011.

	Enzootic pneumonia	Caseous lymphadenitis	Other lung processes	Abscesses in liver	Other liver processes	Hydatidosis	Cisticercosis
A. pyogenes	X	X	X	X		X	
B. catarrhalis	X						
B. parapertussis	X						
C. pseudotuberculosis		X	X	X	X	X	X
Dichelobacter spp.				X			
Fusobacterium spp.				X	X		
M. haemolytica	X		X				
P. aeruginosa			X				
P. multocida	X						
S. aureus		X	X	X	X		X
S. aureus anaerobius		X					
Streptococcus spp.			X	X		X	

The main pathogen isolated from lamb pneumonic tissue was *Manheimia haemolytica*, which was present in 78.10% of the cultures, followed by *Pasteurella multocida* and *Branhamella catarrhalis* and, at much lower levels, *Bordetella parapertussis* and *Arcanobacterium pyogenes*.

Staphylococcus aureus was isolated in over half of lung abcess cultures, followed distantly by *Fusobacterium spp.*, *Streptococcus spp.* and other unidentified pathogens. This pattern was similar in sheep and lambs, but in the case of adults, the unidentified microorganisms had a higher percentage of prevalence (17.95% and 34.78%, respectively).

Among the microorganisms isolated from the lesions caused by caseous lymphadenitis, *Corynebacterium pseudotuberculosis* was prominent and found in 100% of lesions consistent with caseous lymphadenitis, with occasional contamination by *Staphylococcus aureus* or other germs. We found one specific disease associated with caseous lymphadenitis, the abscesses disease, in one case from the spring. The culture of this condemnation rendered growth for *Staphylococcus aureus anaerobius* and *C. pseudotuberculosis*.

S. aureus was isolated in over half of hydatid cysts, followed by other pyogenic bacteria such as *Streptococcus spp.*, *A. pyogenes* and *Corynebacterium pseudotuberculosis*. Similarly, a quarter of lambs with abscesses also had cysticercosis, and 70% of the lesions caused by cysticercosis showed bacterial growth *Staphylococcus aureus*.

DISCUSSION

Despite the variety of reasons for condemnation, only a few bacterial diseases or processes - enzootic pneumonia, caseous lymphadenitis and abscesses in this study— are found in most rejections, as has been previously described (Jepson and Hinton; 1986; Vilallonga, 2013).

Enzootic pneumonia. The identification of bacteria by lamb pneumonic tissue culture appears to be variable. Our findings of *M. haemolytica* followed by *P. multocida* and *B. catarrhalis* and, at much lower levels, *B. parapertussis* and *A. pyogenes* are similar to those found by other authors (Pinto, 2011; Arrigo *et al.*, 1984). Interestingly, although there is generally less isolation in adults than in lambs, isolates of *M. haemolytica* remain the most frequent in lesions caused by enzootic pneumonia, followed by *P. multocida* and *B. catarrhalis*.

Abscesses. The high presence of *S. aureus*, *Streptococcus spp.*, *A. pyogenes* and *C. pseudotuberculosis* in cultured hydatid cysts and *S.aureus* in cysticercosis samples suggests a possible association between the presence of metacestodosis and liver and lung abscesses. The other isolates obtained from abscess cultures confirm the polymicrobial nature of suppurative infections, as demonstrated by the disparity found in the literature. For example, our data agree with those of Scanlan (1991) and Quinn *et al.* (2002), which highlight the isolation of two or more species of facultative anaerobes and/or obligate anaerobic, often including *F. necrophorum* and *Bacteroides spp.*

Fusobacterium spp. and *Dichelobacter spp.* constitute more than half of anaerobes isolated from mixed opportunistic infections (Moreno, 2006). However, the difficulties in their isolation and identification can lead to an underestimation of their involvement (Quinn *et al.*, 2002). This may have occured in the present study because the method employed for detection of these bacteria was not sufficiently specific for their complete identification.

Caseous lymphadenitis. *C. pseudotuberculosis* was isolated in all caseous lymphadenitis lesions, with *S. aureus* or other pathogens occasionally growing concomitantly. These results are in agreement with other studies that reported that both species were the most frequent isolates (Brown *et al.*, 1987; Ben Saïd *et al.*, 2002; Chikhaoui and Khoudja, 2013). Only abscess disease was concomitant to caseous lymphadenitis, that is; because it only manifested once, it does not appear to be relevant but rather a coincidential circumstance. Abscess disease has proven to be an uncommon finding and complicates the diagnosis during post-mortem inspection because it presents no characteristic lesion beyond a purulent abscess.

Other liver and lung processes. This group of lesions was used as a way to arrange a diversity of low incidence pathologies found during the research like necrosis, adhesions, hepatitis or pneumonitis. There was no clear pattern on their presentation probably due to their difference in origin. The bacterial isolations showed that most of these lesions were pyogenic in nature.

CONCLUSION

The bacteriological protocols proposed seemed to be be a practical, cost-effective and useful tool in the primary identification of common bacterial species involved in the principal pathologies found in slaughtherhouses. The results clearly show that the vast majority of the ovine bacterial pathologies are caused by a handful of bacterial species: *M. haemolytica, P. multocida, A. pyogenes, C. pseudotuberculosis* and *S.aureus*. Hence, the bacterial protocols mentioned above could be used extensively as a way to assess and better understand the situation of the national ovine livestock regarding the most common diseases found in this species. This not only would allow the implemmentation of specific animal health policies in order to decrease the prevalence of these pathologies and further investigate their epidemiology but also increase the quality of meat inspection procedures. Another consideration to bring is the fact that the protocols proposed imply the use of very simple and affordable laboratory equipment which would help the application of this system in undeveloped countries and satisfy the aforementioned goals.

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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