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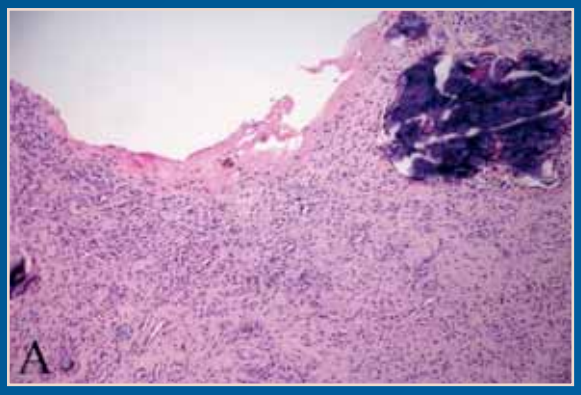

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HEALTH MANAGEMENT

Infezione da *Streptococcus agalactiae* in bovine da latte: epidemiologia e piano di controllo di regione Lombardia

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RIASSUNTO

L'infezione da *Streptococcus agalactiae* è la principale causa di mastite contagiosa subclinica con eventuali forme di cronicizzazione, che comporta l'innalzamento del conteggio di cellule somatiche, parametro di conformità igienico sanitaria che influisce negativamente nei processi di trasformazione casearia e che è normato dalla UE per la produzione di latte crudo.
DG Welfare di Regione Lombardia dal 2012 ha impostato un piano di controllo e di eradicazione dell'infezione con l'obiettivo di ridurre la prevalenza all'8% alla fine del 2014.
La prevalenza media iniziale del 17,24% nel 2012 si è ridotta al 11,69% nel 2014 e solo nel 2018 si è attestata al 7,27% con risultati più favorevoli nelle zone di pianura (2-7%), mentre nelle aree di montagna persistono livelli d'infezione elevati, dal 15 al 25%.
Questo lavoro descrive le caratteristiche epidemiologiche dell'agente eziologico e le modalità del suo controllo, illustrando l'andamento dell'applicazione del Piano e i suoi riflessi sul controllo dell'infezione dal 2012 al 2018, evidenziando in particolare le criticità, che non hanno ancora permesso la sua eradicazione; a tale fine è stato, infatti, rimodulato il piano Regionale 2019.

PAROLE CHIAVE

Streptococcus agalactiae; mastite bovina; Piano Regione Lombardia.

INTRODUZIONE

Tra le mastiti contagiose della bovina da latte, *Streptococcus agalactiae* rappresenta, insieme a *Staphylococcus aureus*, una delle cause più significative di perdita nella produzione di latte e di danno economico in quella di formaggio, poiché la sua presenza è generalmente associata a elevati conteggi di cellule somatiche[5].
A questo proposito, si ricorda che il Reg UE 853/2004 in tema di produzione di latte crudo include le cellule somatiche, quale parametro igienico sanitario da tenere sotto controllo e che esso è incluso da sempre nei sistemi di pagamento latte secondo qualità.
L'aumento della carica leucocitaria nel latte comporta, oltre al rischio di non commerciabilità del prodotto per il superamento dei limiti legali, una notevole perdita economica per mancata produzione. Infatti, l'effetto stimato ogni raddoppio di incremento delle cellule somatiche nel latte individuale, a partire da 50.000 cellule somatiche/mL, comporta la perdita di mezzo litro di latte, non considerando lo stadio di lattazione e il numero di lattazioni[15].
Dal punto di vista zoonosico, *Str. agalactiae* può essere fonte di contagio per l'uomo, anche se la via di trasmissione principale è il contatto con altri esseri umani, in cui può essere presente a livello di intestino, gola e vie urogenitali. Infatti, *Str. agalactiae* è presente nella microflora genitale, primariamente localizzato nel tratto gastrointestinale inferiore, da cui può colonizzare ad intermittenza le vie genitali o urinarie. I ceppi generalmente isolati nell'uomo presentano differenze genetiche con quelli bovini; gli studi filogenetici di questi ceppi fanno pensare che quelli umani e bovini abbiamo, comunque, progenitori comuni (bovini). Il problema del potenziale zoonosico è se realmente *Str. agalactiae* costituisca un pericolo per la salute umana con trasmissione diretta da animale a uomo o tramite l'evoluzione di ceppi patogeni per l'uomo derivanti dal bovino[4].
Uno studio svolto nel Nord Europa ha approfondito la possibile trasmissione interspecie, comparando ceppi umani e bovini con la sierotipizzazione molecolare e identificando 5 tipi di sequenze comuni[9]. La possibilità che l'uomo possa essere fonte d'infezione per il bovino, stante la presenza di *Str. agalactiae* nell'orofaringe tramite contatto diretto tra mani e mammella non è esclusa. Il quesito è determinare la direzionalità della trasmissione tra le specie ospiti (da bovino a uomo o da uomo a bovino?). Potenzialmente anche le acque di superficie e i liquami potrebbero essere possibili vie di trasmissione intra e inter-specie. Infatti, uno studio norvegese ha dimostrato la presenza di *Str. agalactiae* da tamponi rettali e ambientali in 13 di 19 allevamenti con almeno un campione di latte positivo o con episodi di mastite nell'anno precedente il campionamento. Pertanto, gli autori suggeriscono la possibile co-esistenza, oltre alla classica trasmissione contagiosa tramite il latte contaminato e la routine di mungitura, di un ciclo di trasmissione ambientale per via oro-fecale e tramite acqua contaminata[12].
La sintomatologia negli adulti di solito è lieve o asintomatica con infezioni cutanee o delle vie urogenitali. In categorie a ri-

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schio (anziani, diabetici, pazienti con deficit immunitari o gravi patologie debilitanti) può presentarsi anche con forme più gravi quali setticemia, artrite, polmonite ed endocardite. Inoltre, nelle prime ore di vita del neonato sono possibili sepsi o meningiti acute, dovute principalmente al contagio della madre in utero per via ascendente in prossimità del parto o subito dopo la nascita con inalazione di secreti infetti. L'origine dell'infezione tardiva (dai 7 ai 90 giorni di vita) non è stata ancora chiarita. In questi casi, i fattori di rischio sono madri portatrici, ambiente contaminato e nati prematuri[3].

Str. agalactiae è un batterio β-emolitico gram-positivo, parassita obbligato della ghiandola mammaria della bovina e altamente contagioso, che in genere ha una scarsa sopravvivenza nell'ambiente esterno. Lavori recenti[10,11] eseguiti con tecniche biomolecolari quantitative hanno, però, evidenziato la presenza dei medesimi ceppi di *Str. agalactiae* anche da campioni rettali, ambientali, sulla cute del capezzolo dimostrando la possibilità di sopravvivenza nell'ambiente. La via di entrata è il canale del capezzolo, che può infettarsi attraverso fomiti contaminati dal patogeno e perciò la mungitura è il punto critico della sua possibile diffusione all'interno della mandria.

Il controllo e successiva eradicazione di questa mastite contagiosa è raggiungibile tramite la corretta applicazione del Five-Point Mastitis Control Plan, che, oltre al rispetto di rigide misure di biosicurezza, include il trattamento e la registrazione dei casi clinici, la disinfezione del capezzolo post mungitura, l'idonea e mirata terapia in asciutta, la riforma dei casi cronici, recidivanti o refrattari alle cure e la manutenzione corretta della macchina mungitrice.

Infatti, il suo *reservoir* primario è la mammella infetta, da cui diffonde tra i quarti e tra bovine in lattazione durante la routine di mungitura, attraverso i gruppi di mungitura, le mani e i fazzoletti contaminati da latte di bovine infette. Le infezioni sono tipicamente sub-cliniche e facilmente cronicizzano, con episodi meno frequenti di sintomatologia conclamata e mastite acuta. Questa modalità di diffusione comporta solitamente la presenza di un singolo ceppo di *Str. agalactiae* nell'allevamento colpito. Per questa ragione sono stati condotti pochi studi di tipizzazione biomolecolare nei bovini per verificarne la persistenza, la via di trasmissione o le fonti di infezione[4].

L'isolamento da latte del microrganismo *Str. agalactiae* non rappresenta di per sé causa sufficiente per inquadrarlo nell'art. 5 del regolamento di Polizia Veterinaria D.P.R. 320/1954, in cui si prevede la denuncia di malattia infettiva come mastite catarrale contagiosa.

L'eradicazione dell'infezione da *Str. agalactiae* è stata storicamente uno degli obiettivi primari dell'intervento veterinario per la lotta alla mastite negli allevamenti di bovine da latte.

I motivi di questo particolare interessamento possono essere così sinteticamente elencati:

- la notevole **contagiosità** che determina il continuo diffondersi all'interno della mandria e l'interessamento di nuovi animali (*Str. agalactiae* è considerato un patogeno obbligato della mammella, poiché poco resistente nell'ambiente esterno);
- la sua **patogenicità** legata non tanto alla gravità delle forme cliniche acute, ma piuttosto alla frequente cronicizzazione delle infezioni mammarie che possono pregiudicare la futura carriera produttiva delle bovine;
- la rilevanza **sanitaria** sia in termini di Polizia Veterinaria (Mastite Catarrale Contagiosa) sia, più in generale, di Salute Pubblica (agente zoonosico) e di Sicurezza Alimentare (in particolare nel caso di produttori di latte crudo destinato alla vendita diretta);
- l'**impatto economico negativo** causato dai costi di terapia delle forme di mastite clinica, dalla penalizzazione nel pagamento del latte secondo qualità per l'innalzamento del tasso di cellule somatiche, dalla ridotta produttività delle bovine infette anche in forma sub-clinica. Particolarmente condizionanti sull'intera carriera produttiva sono poi le frequenti forme croniche;
- i costi degli **insuccessi terapeutici** che non dipendono da fenomeni di antibiotico-resistenza (fatto decisamente raro), ma ai già citati fenomeni cronici, alle recidive e alla continua comparsa di nuovi casi, che sono tipici degli allevamenti che "convivono" da anni con questa infezione;
- le crescenti limitazioni alla possibilità di **vendere animali vivi da produzione**, per gli allevamenti che non forniscono le necessarie garanzie d'indennità alla mastite contagiosa da *Str. agalactiae*, con l'alternativa di destinarli verso il macello con notevole deprezzamento.

Non è un caso, del resto, che proprio nei confronti di questa infezione si siano realizzati i primi interventi a metà degli anni '70 di assistenza sanitaria in provincia di Brescia, dagli albori della collaborazione tra IZSLER e Centro per il Miglioramento Qualità Latte Bovino (CMQLCB). Questi interventi, realizzati con professionalità, impegno, perseveranza negli anni hanno, in effetti, ridotto significativamente la prevalenza dell'infezione nella provincia di Brescia da circa il 70% negli anni '70 al di sotto del 10% negli anni '90.

Gli stessi metodi applicati anche nell'ultimo decennio non sono però riusciti a completare l'opera e ad arrivare al vero obiettivo finale: l'eradicazione di *Str. agalactiae* dall'intero territorio provinciale. Le ragioni di questa "crisi in prossimità del traguardo" sono diverse. Tra queste è fondamentale il carente coinvolgimento dei portatori di interesse della filiera produttiva, che non hanno incentivato l'eradicazione come un fattore di resa economica e di spendibilità della qualifica sanitaria nella filiera latte e nei confronti dei consumatori. Inoltre, la frequente cessazione di attività delle piccole-medie aziende ha comportato la vendita di animali infetti, che ha contribuito a reinfettare aziende negative e a mantenere stabile la prevalenza provinciale.

A ciò va aggiunto che, come in molte altre esperienze simili, è proprio quando si devono affrontare i casi meno reattivi, in cui i risultati appaiono meno determinanti in termini di costo-beneficio, che diviene più difficile ottenere risultati efficaci; non c'è dubbio, infatti che, a livello provinciale, soprattutto in pianura, il problema coinvolga direttamente ormai un numero limitato di allevamenti. Si sottolinea che la permanenza di allevamenti infetti non rappresenta un problema solo per i diretti interessati, ma costituisce un rischio potenziale per l'intero settore a causa dell'elevata probabilità di reintroduzione in allevamenti negativi (soprattutto in caso di monticazione degli animali o di compravendita di vacche e manze, queste ultime già infette ancor prima di entrare in lattazione).

EPIDEMIOLOGIA

A partire dagli anni '70, la mastite causata da *Str. agalactiae* è stata inquadrata come obiettivo primario della maggior parte dei piani di controllo e della ricerca scientifica. Il periodo si caratterizza come una vera e propria "era delle mastiti con-

tagiose", proseguita per almeno un ventennio. Il controllo dell'infezione (riduzione della prevalenza e dell'incidenza) si è basato su due metodi, scelti in base alla prevalenza dell'infezione in allevamento e alla strategia scelta dall'allevatore:
– esame batteriologico del latte e conseguente trattamento terapeutico della bovina in asciutta;
– eradicazione aggressiva con test batteriologico e trattamento immediato o riforma delle bovine infette.

La persistenza dell'infezione è solitamente legata ad una applicazione discontinua o inappropriata del pre dipping o della selezione delle bovine da trattare in asciutta, dell'ordine di mungitura e delle possibilità di segregare gli animali in gruppi differenziati.

Il primo sistema consente un risanamento più gestibile ed economico, sempre accompagnato da un appropriato programma di dipping del capezzolo, che richiede la costante applicazione di un programma di igiene della mungitura. Il pericolo da tenere sotto controllo sono le introduzioni di nuovi animali (biosicurezza), che vanno controllati prima dell'inserimento nella mandria.

La principale controindicazione è la maggior richiesta di tempo (fino ad uno-due anni) per eliminare tutte le infezioni e la necessità di controllare tutte le bovine fresche che rientrano in mungitura.

Questo sistema di eradicazione "soft" è stato quello maggiormente utilizzato nei piani di risanamento in Lombardia e richiede da parte dell'allevatore un'applicazione gestionale corretta e costante, preferibilmente con l'assistenza di un servizio tecnico del settore latte qualificato.

Il secondo sistema permette la rimozione più rapida dell'infezione, minimizzando le perdite produttive e riducendo il rischio di trasmissione. D'altro canto, esso richiede un costo inziale più elevato per il latte scartato delle bovine trattate anche in lattazione, il potenziale pericolo di presenza di sostanze inibenti nel latte aumenta ed è necessaria la disponibilità di un laboratorio in grado di sostenere l'attività diagnostica. A lungo termine, nonostante il costo iniziale, questo approccio può risultare più efficiente del primo[5].

La scelta tra i due metodi dipende dalla disponibilità dell'allevatore sia in termini economici sia di impegno gestionale nell'applicare le regole del risanamento e della biosicurezza. Sicuramente, la prevalenza dell'infezione intra-allevamento rappresenta un fondamentale fattore dirimente l'opzione di rimozione rapida, se è bassa, o a medio termine, se elevata.

Il conteggio delle cellule somatiche nel latte composito di singola bovina è un parametro associato alla ricerca di *Str. agalactiae* ed è utilizzato per impostare il piano di risanamento e individuare le bovine con mastite subclinica e, quindi, più probabilmente infette o croniche.

Dal punto di vista diagnostico, nonostante non esista un gold standard di referenza, l'esame colturale su latte di quarto o pool di quarti rappresenta il metodo più applicato, che presenta una sensibilità di circa il 95%, e una specificità di circa il 99%. L'esame batteriologico su latte di massa rispecchia la sensibilità nel latte di singola bovina, quando la prevalenza intra-allevamento è elevata, ma declina in maniera proporzionale con il livello di infezione. Questa diminuzione di capacità di rilevare l'infezione può essere ovviata pre-incubando il campione di latte, seminandone una quantità più elevata (0,05 mL) su terreni altamente selettivi oppure semplicemente ripetendo il prelievo e la coltura, soprattutto se si utilizza il metodo aggressivo del "test e terapia", in cui sono da evitare le false negatività[5].

Oppure si può ricorrere all'utilizzo di tecniche diagnostiche basate su PCR Real Time (RT) quantitativa, che presentano una maggior capacità di rilevare l'infezione, non inficiando la specificità diagnostica[6]. La scelta del cut-off (soglia) di lettura dipende dallo scopo del campionamento, vale a dire identificare tutte le bovine positive con rischio di rilevare anche falsi positivi o, in alternativa, quelle gravemente e realmente infette. In qualunque caso, il campionamento eseguito in sterilità diminuisce i falsi positivi[7].

Un altro studio conferma che sia l'esame colturale sia PCR RT sono metodi diagnostici similmente accurati in allevamenti con elevata prevalenza d'infezione (> 20%) e che PCR RT su latte di massa è un metodo di screening consigliabile in caso di infezioni a bassa prevalenza o per confermare l'indennità.

Sul latte di massa di 165 aziende monitorate, la PCR real-time ha individuato 90 campioni di latte di massa negativi e 75 positivi, mentre l'esame batteriologico ha evidenziato 78 positivi e 87 negativi. PCR RT *vs* metodo di riferimento "batteriologico su latte di massa" ha una Se del 96,5% (90-99,3) e una Sp del 94,4% (86,4-98,5) con un ottimo indice di concordanza (K = 0,91)[8].

Nelle fasi finali di un piano di eradicazione (prevalenza bassa) è consigliabile l'uso in parallelo dell'esame colturale su latte di massa aziendale con terreno selettivo Tallium Kristalviolette Tossin (TKT) e di PCR RT, poiché i due metodi aumentano la specificità diagnostica (capacità di identificare correttamente gli animali negativi), mentre nelle prime fasi è preferibile usare metodi diagnostici di screening altamente sensibili, perché l'obiettivo è di individuare gli allevamenti a rischio[1]. Infatti, con il decrescere della prevalenza d'infezione si riduce la capacità di rilevare *Str. agalactiae* con l'esame batteriologico nel latte di massa[5], al di sotto del limite di rilevabilità del metodo diagnostico, dovuta alla eliminazione intermittente del batterio e all'effetto diluizione.

L'utilizzo del latte di massa per la ricerca di *Str. agalactiae* è la scelta di elezione per il controllo periodico, almeno trimestrale, della conferma di negatività dell'allevamento, una volta raggiunta l'eradicazione. Ogni animale di nuova introduzione e ogni primipara che entra in produzione vanno sempre controllati con l'esame batteriologico o, preferibilmente, con PCR RT.

Il numero di allevamenti di bovine da latte in Lombardia negli ultimi vent'anni ha subito un decremento di quasi il 50% con un aumento di concentrazione di capi in aziende di grandi dimensioni, alimentato anche dallo spostamento e rimescolamento di bovine provenienti da aziende cessate non controllate prima dell'introduzione. I principi fondamentali del risanamento di un allevamento infetto da *Str. agalactiae* non sono cambiati nel tempo, ma la chiusura o la riduzione di servizi nel settore zootecnico a supporto del controllo da parte della componente veterinaria e tecnica nel settore del controllo delle mastiti, ha comportato la diminuzione di expertise sul territorio, del livello di attenzione e di aderenza a programmi consolidati di eradicazione, controllo e sorveglianza.

TRATTAMENTO E BIOSICUREZZA

La sensibilità *in vivo* di *Str. agalactiae* ad antimicrobici appartenenti alla famiglia dei beta lattamici è sempre stata elevata e costante nel tempo.

Una strategia di eradicazione a medio termine (uno-due anni) comporta un protocollo di rigorosa igiene nella routine di mungitura e la pratica costante della terapia in asciutta.
La strategia di eradicazione rapida prevede, invece, l'esecuzione dell'esame batteriologico seguito dal trattamento, anche in lattazione. La guarigione degli animali infetti che sono trattati va confermata con almeno due colture negative a distanza di tre settimane. Le bovine refrattarie al trattamento vanno riformate, poiché rappresentano potenziali *reservoir* di infezione in allevamento[5].
Per questa ragione, i protocolli di biosicurezza tra allevamenti devono essere stringenti e applicati costantemente, se si vuole evitare di introdurre nuovi ceppi di *Str. agalactiae*, che possono diffondere molto rapidamente in una popolazione suscettibile all'infezione. Il Piano di controllo di regione Lombardia prevede per il modello di accompagnamento di provenienza degli animali durante il trasporto la segnalazione di qualifica sanitaria dell'allevamento nei confronti di *Str. agalactiae*. Nuovi animali introdotti provenienti da allevamenti con stato sanitario sconosciuto sono da sottoporre preventivamente ad esame batteriologico o PCR RT prima di entrare nella mungitura ordinaria, altrimenti è consigliabile porli in isolamento, fino alla conferma di negatività.
Un altro fattore di rischio nelle zone di montagna è la pratica dell'alpeggio, da giugno a settembre, in cui bovini di aziende diverse vengono a contatto. L'inoltro alle malghe estive di animali provenienti da allevamenti con qualifica sanitaria negativa risulterebbe fondamentale. Il controllo pre e post alpeggio dei bovini è una pratica essenziale per verificare la possibile presenza di animali *reservoir* d'infezione o eventuali re-infezioni, per le quali vanno individuate l'origine delle cause attraverso una indagine epidemiologica.

MATERIALI E METODI

Il protocollo di campionamento del latte di massa da parte delle ATS lombarde ha previsto il suo prelievo con prelevatore automatico dal tank di raccolta aziendale previa agitazione di 15 minuti e raccolta di un campione di 120 mL in un contenitore sterile di plastica. I campioni sono stati immediatamente refrigerati e trasportati in tale stato al laboratorio, mantenuti a 4 ± 2°C fino ad inzio analisi. L'analisi batteriologica è stata eseguita entro le 24 ore dalla raccolta.
La semina di 0,1 mL di latte, previamente miscelato, è stata eseguita su piastra con terreno agar selettivo Tallium Kristalviolette Tossin, poi incubata per 24 ore a 37 ± 2°C in aerobiosi. La crescita di colonie di colore bianco-violetto e circondate da netto alone di emolisi completa e reazione negativa all'idrolisi dell'esculina è stata identificata come indicativa di presenza di *Str. agalactiae*. Un campione è stato classificato positivo per *Str. agalactiae* in base alla crescita di una o più colonie in piastra. Eventuali test di conferma su colonia isolata sono stati eseguiti applicando il metodo interno PCR RT per ricerca di *Str. agalactiae*, considerando un cut off di lettura di 40 cicli soglia[8].
L'analisi statistica è stata condotta con l'ausilio di fogli di calcolo (Excel®) e procedure statistiche sviluppate in R (R Core Team 2018). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/[14].
L'analisi ha permesso di calcolare la stima della significatività delle differenze fra medie (Tabella 2) mediante t-test fra medie e la stima della significatività della differenza fra proporzioni, con valutazione della significatività della tendenza e stima degli intervalli di confidenza delle proporzioni (Tabella 3) mediante z.test; i limiti superiore e inferiore sono stati calcolati con il metodo di Wilson. Per queste elaborazioni si è utilizzato il pacchetto R "binom" [Sundar Dorai-Raj (2014) binom: Binomial Confidence Intervals For Several Parameterizations. R package version 1.1-1. https://CRAN.project.org/package = binom][13].

Situazione epidemiologica dell'infezione da *Str. agalactiae* negli allevamenti da latte nella provincia di Brescia

I risultati del piano di monitoraggio quadriennale (2008-2011) eseguito in provincia di Brescia da IZSLER in collaborazione con CMQLB sono sintetizzati in Tabella 1.
L'andamento dell'infezione nel quadriennio considerato si è mantenuta su valori costanti.
La ragione di questo può essere individuata comparando i risultati del 2011 *vs* 2010, in cui si rileva che 1.077 aziende hanno mantenuto nel 2011 la negatività rilevata nel 2010 e 50 sono rimaste positive; 107 aziende (8,7%) hanno subito un cambiamento di stato sanitario, di cui 62 aziende negative sono diventate positive e 45 positive si sono negativizzate. Ciò dimostra che l'applicazione del risanamento è disomogenea e che le re-infezioni sono un evento tutt'altro che trascurabile.
Come si può notare in Tabella 2, la media del conteggio delle cellule somatiche nelle aziende positive è diminuita probabilmente per la progressiva consapevolezza del problema da parte degli allevatori e dell'avvio di azioni correttive nella gestione della mandria. In particolare, la media del conteggio di cellule somatiche nel 2010 in allevamenti positivi per *Str. agalactiae* era di 455.275 cellule/ml contro la media di 326.993 cellule/ml in allevamenti negativi, pur considerando l'ovvia variabilità interna ai due gruppi. La significatività delle differenze tra medie (t-test per le medie, assumendo uguale varianza) è stata confermata statisticamente significativa per $P > 0.01\%$.
Si evidenzia, perciò, che gli allevamenti infetti con *Str. agalactiae* presentano un valore maggiore del conteggio leucocitario del 39,2% rispetto a quelli negativi o indenni. Pur persistendo in un numero limitato di allevamenti (8,4%), il rialzo cellulare è consistente e, soprattutto, si attesta su un valore superiore al riferimento normativo per la commercializzazione del latte (Reg. CE n. 853/2004).

Tabella 1 - *Str. agalactiae*: allevamenti esaminati annualmente con esame colturale nel periodo 2008-2011 nella provincia di Brescia.

Anno	Allevamenti controllati	Allevamenti positivi	% positività
2008	1.411	107	7,6
*2009	672	81	12,0
2010	1.446	121	8,4
2011	1.234	112	9,1

*Controllo non eseguito nelle aree di montagna.

Tabella 2 - Comparazione della media delle cellule somatiche in aziende positive e non per *Str. agalactiae* nella provincia di Brescia; periodo 2008-2010.

Anno	Media conteggio cellule somatiche aziende positive	N° aziende	Media conteggio cellule somatiche aziende negative	N° aziende	$P(t_{obs})$ Pos > Neg
2008	585.196	107	304.112	1.304	> 99,9%
2009	574.582	81	298.271	591	> 99,9%
2010	455.275	121	326.993	1.325	> 99,9%

Quest'andamento, al di là degli aspetti particolari e di quelli tecnici organizzativi, descritti nel Piano proposto, supportava la prospettiva di perseguire l'obiettivo di "provincia ufficialmente indenne da *Str. agalactiae*" in tempi relativamente brevi (2-3 anni), aggiungendo valore sanitario e commerciale alla qualità del prodotto della provincia di Brescia.
Non da ultimo, ciò avrebbe rappresentato il degno riconoscimento di decenni d'impegno e di collaborazione tra IZSLER e CMQLCB nei confronti di questo problema sanitario ed economico al servizio della zootecnica bresciana, che si potrebbe fregiare di una ulteriore qualifica sanitaria.

Il Piano di Regione Lombardia di controllo ed eradicazione dell'infezione da *Str. agalactiae* negli allevamenti da riproduzione latte

Nel 2012 DG Welfare della Regione Lombardia, sulla base dei dati di prevalenza d'infezione in alcune province rappresentative (5% nel 2008 a Cremona; 8,4% nel 2010 a Brescia; 23% nel 2009 a Bergamo e il 50% nel 2011 in zone di montagna), attivava il piano triennale di controllo degli agenti di mastite, in particolare *Str. agalactiae*, all'interno del Piano Integrato Aziendale della Prevenzione Veterinaria 2012-2014, al fine di ridurre la prevalenza d'infezione al di sotto dell'8% sul territorio regionale, accreditare gli allevamenti di bovini da latte come ufficialmente indenni o negativi da *Str. agalactiae* in funzione dello *status* sanitario raggiunto, assicurare garanzie sanitarie supplementari nella compra-vendita di animali, valorizzare gli allevamenti della regione.
L'ulteriore preoccupazione per l'economia zootecnica lombarda e la commercializzazione del latte si focalizzava sull'aumento di cellule somatiche conseguente alla presenza di agenti mastidogeni, tra cui spiccava *Str. agalactiae*, e che si rifletteva sul rispetto dei limiti imposti dal reg. (CE) 853/2004 per la produzione latte crudo.
Il Piano era ad adesione volontaria e prevedeva il controllo del latte di massa, contestuale al controllo della brucellosi o rinotracheite infettiva, di tutti gli allevamenti di bovine da latte censiti e di codificare la qualifica sanitaria secondo i seguenti criteri:
– negativo: allevamento da riproduzione latte con tre prelievi consecutivi negativi a distanza di almeno tre mesi sul latte di massa per *Str. agalactiae*;
– indenne: allevamento da riproduzione latte con sei campioni negativi, effettuati sul latte di massa almeno a distanza di tre mesi e controllo finale con esito negativo sul latte dei singoli capi in lattazione presenti in azienda;
– positivo: allevamento riproduzione latte con un prelievo su latte di massa con isolamento di *Str. agalactiae*;
– stato sanitario non disponibile: allevamento senza analisi effettuate su latte di massa con ricerca di *Str. agalactiae*.

Il Piano prevedeva di segnalare la qualifica sanitaria sul modello di provenienza in caso di movimentazione dei capi, per la monticazione e/o il pascolo, di movimentazione di baliotti destinati ad un allevamento da riproduzione latte.
La segnalazione sul modello aveva lo scopo di informare l'allevatore sullo stato sanitario dell'allevamento di provenienza per operare scelte consapevoli nella tutela della biosicurezza del proprio allevamento.
Per quanto concerneva la movimentazione di bovini era previsto il controllo prima e dopo 8 giorni la loro introduzione, mantenendoli separati dalla mandria. Nel caso di alpeggio era consentita la monticazione di bovini positivi per *Str. agalactiae*, previo trattamento terapeutico e controllo batteriologico due volte a distanza di otto giorni, con esito negativo. I costi dei controlli degli accertamenti sanitari per la movimentazione degli animali erano a carico della regione Lombardia, mentre quelli di controllo sui singoli animali necessari al risanamento dell'infezione erano a carico dell'allevatore.
Inoltre, nel Piano era indicato un protocollo di intervento in azienda a seguito positività latte di massa. Compito dei Dipartimenti Veterinari era anche quello di effettuare attività di formazione/informazione sulle norme di biosicurezza da adottare per prevenire o eradicare la patologia mammaria.
Alla fine del Piano mastiti (sorveglianza dei batteri contagiosi alla stalla), nel 2014 la prevalenza media aziendale regionale si attestava al 11,7% (5-22%), con valori intorno al 20% nelle aree di montagna e del 6-7% in pianura. L'obiettivo di ridurre la prevalenza al di sotto dell'8% non era ancora raggiunto.
Nel 2015 Regione Lombardia decide di aggiungere, sempre in regime di adesione volontaria, la obbligatorietà del controllo sul latte di massa, al fine di confermare lo stato sanitario favorevole acquisito dalle aziende negative o indenni e di registrare le qualifiche sanitarie nella Anagrafe Regionale.
In Tabella 3 è illustrato l'andamento del Piano dal 2012 al 2018. Si evidenzia che dal 2015 la copertura dei controlli è passata dal 65 al 88% e la prevalenza media dal 2017 si è attestata al di sotto del 8%, iniziale obiettivo del piano triennale partito nel 2012.
Il test di significatività della differenza fra proporzioni (z.test) ha evidenziato una differenza significativa fra le stesse (P > 0.01%), confermando anche la tendenza in decrescita.
Ciò nonostante, rimangono diverse criticità che non consentono ancora di poter considerare "raggiunto" l'obiettivo complessivo del Piano e che possono riassumersi nei seguenti punti:
– il piano di eradicazione è ancora volontario, nonostante più del 90% delle aziende sia negativa o indenne, anche se è diventato obbligatorio il controllo sul latte di massa aziendale;
– è necessario l'aggiornamento del numero di allevamenti da latte controllabili presente in anagrafe;

Tabella 3 - Andamento del Piano di controllo e di eradicazione di *Str. agalactiae* in regione Lombardia; periodo 2012-2018 (fonte dati SEL - IZSLER).

Anno	Aziende presenti	Aziende controllate	% copertura	Aziende negative	Aziende positive	% positive	Limiti inferiore e superiore (%)	
2012	6.834	2.940	43,02	2.433	507	17,2	15,9	18,7
2013	6.605	4.310	65,25	3.781	529	12,3	11,3	13,3
2014	6.427	4.234	65,88	3.739	495	11,7	10,8	12,7
2015	6.329	5.572	88,04	4.920	652	11,7	10,9	12,6
2016	6.226	5.531	88,84	4.935	596	10,8	10,0	11,6
2017	6.065	5.196	85,67	4.808	388	7,5	6,8	8,2
2018	5.907	5.049	85,47	4.682	367	7,3	6,6	8,0

– la compra-vendita di animali provenienti da allevamenti positivi è segnalata sul modulo di accompagnamento, ma non è vietata/condizionata/limitata;
– l'alpeggio e la possibile promiscuità tra animali provenienti da allevamenti con diverse qualifiche sanitarie non possono ancora essere considerati sotto pieno controllo;
– il coinvolgimento nel Piano delle Associazioni Allevatori deve essere ancor più incentivato;
– la prospettiva di essere una provincia o Regione indenne da *Str. agalactiae* andrebbe maggiormente valorizzata in termini di spendibilità del settore lattiero-caseario nei confronti sia dei primi Acquirenti Latte sia del consumatore.

Sulla base di questi risultati e di queste considerazioni, il Piano è stato rimodulato nel Piano Integrato Aziendale della Prevenzione Veterinaria del 2019, prevedendo i seguenti interventi:
– aggiornamento dell'anagrafe bovina in termini di tipologia produttiva e allevamenti con assenza di capi, al fine di aggiornare il denominatore e di calcolare correttamente la percentuale di copertura degli allevamenti controllabili, la prevalenza e incidenza dell'infezione;
– mantenimento del controllo annuale su tutti gli allevamenti di produzione latte e della segnalazione sulla modulistica di accompagnamento dei campioni dell'adesione al Piano *Str. agalactiae* e della qualifica sanitaria aziendale;
– registrazione della qualifica sanitaria nel sistema informativo veterinario e comunicazione dello *status sanitario* all'azienda. Le ATS forniscono indicazioni sulla corretta applicazione delle misure di biosicurezza, al fine di evitare la reintroduzione dell'agente patogeno e non vanificare gli sforzi effettuati per raggiungere la qualifica. Particolare attenzione va prestata all'introduzione di nuovi animali in azienda, quando non sia correttamente riportata la qualifica sul modello di compravendita. In tale situazione, è consigliato il controllo batteriologico sul latte dei singoli animali introdotti, che vanno segregati fino ad esito negativo;
– esecuzione dell'indagine epidemiologica volta ad individuare la possibile origine dell'infezione e dei fattori di rischio in caso di reinfezione in aziende negative/ indenni o di aziende sotto controllo (piano di risanamento) o che risultano persistentemente positive;
– le aziende positive devono predisporre un piano di eradicazione concordato con il Veterinario Aziendale e che deve essere notificato alle autorità competenti;
– gli allevamenti che non aderiscono al piano sono inseriti nell'elenco delle aziende a rischio e come tali sono campionati al fine di verificare il rispetto di quanto previsto dal Reg (CE) 853/2004 sulla produzione latte e dal D.lgs. 193/06 sull'utilizzo di farmaci;
– valutazione annuale dei risultati del Piano con definizione di opportune misure correttive, che possono prevedere anche vincoli nella movimentazione degli animali e interventi di risanamento d'ufficio.

CONCLUSIONE

In generale, le campagne di controllo delle malattie infettive necessitano di motivazione, comunicazione e formazione che coinvolga tutti i portatori di interesse, chiamati a lavorare in modo coordinato e sostenibile e con comportamenti di aderenza costante ai principi e alle regole condivise per gli obiettivi a medio-lungo termine. L'infezione da *Str. agalactiae* non si sottrae a questi concetti basilari, tanto più che rappresenta una mastite a lungo studiata ed eradicabile a breve-medio termine.

Proprio in quest'ottica, il Piano di controllo dell'infezione da *Str. agalactiae* della Regione Lombardia è stato rimodulato nel 2019, poiché il target originario di fine 2014 è stato raggiunto solo nel 2017.

La qualifica sanitaria di azienda negativa o indenne raggiunta da oltre il 90% degli allevamenti nelle zone di pianura va tutelata e le residue sacche d'infezione vanno rimosse. In questo caso, l'obiettivo di eradicazione è ampiamente alla portata, mentre la criticità maggiore insiste nelle zone di montagna, per le quali risulta problematica la gestione dell'alpeggio e il rischio di re-infezione si mantiene elevato.

A questo proposito, è fondamentale valutare l'opportunità *in itinere* di intervenire con misure più stringenti, che incidano sui fattori di rischio della movimentazione di animali infetti o loro promiscuità.

Streptococcus agalactiae epidemiology in dairy herds and control plan of Lombardy Region

SUMMARY

Streptococcus agalactiae is the most cause of contagious mastitis, mainly subclinic or chronic, increasing somatic cells count, that is a hygienic parameter regulated by UE for the raw milk production and that adversely affects cheese production and milk composition.

In 2012 DG Welfare of Lombardy Region programmed a control plan of the *Streptococcus agalactiae* infection with the goal to reduce dairy herd prevalence in three years from 17.24% below 8%.
In 2014 at the end of this plan the average prevalence was 11.69%; only during the following three years, mantaining the surveillance plan the prevalence decreased to 7.47%, with values 2-7% in lowland areas and 15-25% in mountainous territories.
This paper describes epidemiologial characteristics of *Streptococcus agalactiae* and its control methods, showing the infection trend from 2012 to 2018 and the results of the Control Plan carried out in Lombardy Region. Furthermore, some critical issues are discussed about eradication, taking in account the main news in the Regional Plan published in 2019.

KEY WORDS
Streptococcus agalactiae; epidemiology; bovine mastitis; Lombardy Region Mastitis Plan.

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EVENTI SIVAR 2020

Reproductive problems in small ruminants (Sheep and goats): a substantial economic loss in the world

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SUMMARY

The primary fortitude of this study is to overview the environmental, nutritional and also some main infectious causes related to reproduction in small ruminants mainly sheep and goat, that caused milk, meat and wool production loss globally. Mostly 90% small ruminants are reared by rural household globally. The major issues of which are poverty, lack of new techniques and improper management skills. Families usually own few small ruminants, which are used for religious celebrations, serve as savings or emergency cash or provide meat or milk. Reproductive problems mainly arise due to nutritional deficiencies and some pathogens, including bacteria, viruses, parasites and also by environmental stress conditions. The primary reproductive diseases which are commonly associated with the reproductive system of small ruminants are brucellosis, leptospirosis, toxoplasmosis fever, listeriosis, campylobacteriosis along with nutritional deficiencies, socio-sexual and photoperiods also affect the reproductive system. Vaccination and nutrition are mainly advised to control these issues of reproduction. The environment control system can also compensate for this economic loss. This paper represents a limited contribution for the review of reproductive problems in small ruminants' primarily focusing on sheep and goat which was neglected in past at worldwide level.

KEY WORDS

Reproductive issues, nutritional deficiencies, photoperiods, socio-sexual effect, pathogen.

INTRODUCTION

Small ruminants (sheep and goats) play an important role in the survival, economic, and social livelihoods of many humans, more significantly in developing countries[1]. According to FAO (2010), roughly 95.7% of all goats and 63.3% of all ewes are located in developing countries and embody more than 70% of total animal production in the world[2]. Agreeing to the Food and Agriculture Organization (FAO), in 2011, there were 875.5 million goats and one billion sheep in the world. However, around 80% of all small ruminants were in developing countries, many of them placed in tropical areas. So, signifying more than 70% of their production of these species in developing countries (FAOSTAT 2011)[3]. Several diseases which affect the small ruminants mostly causing abortion, delayed estrous and reduced fertility. The diseases which cause abortion are campylobacteriosis and enzootic abortion. Toxoplasmosis, contamination caused by a *Toxoplasma (T.) gondii* apicomplexan protozoan, is common in humans an animal species, having already been testified in many countries and different weathers. Goats had significantly higher ($p<0.01$) prevalence (25.4%) as compared to that of sheep (11.2%); and higher ($p<0.01$) in female (24%) than in the males (19%) for both species[4]. Despite the status of small ruminants breeding in developing countries, milk/meat productivity remains insufficient. Communicable diseases such as leptospirosis, brucellosis, and small ruminant lentiviruses (SRLVs), add to this scenario[2]. Developing countries, due to the social, soil, and weather features, present a suitable scenario for development and increase of goats and sheep breeding. In the last decades, although the high number of small ruminant population in developing countries, its yield remains lower than that in developed ones[3].

Infectious diseases, particularly those of the reproductive domain, play a dramatic role in this scenario, causal important economic threats in livestock[5]. But it may vary according to management and related areal climate changes. Animals have developed reproductive strategies for enough food coincides with pregnancy and lactation. The economic and public health effect of brucellosis remained of a concern in developing countries. The diseases that generally cause a significant loss of productivity through abortion, stillbirth, low herd infertility and comparatively low milk production[6]. Under the name "Malta fever," the disease now called brucellosis first came to the attention of British medical officers in the 1850s in Malta during the Crimean War. Brucellosis is a zoonotic disease that leads to considerable illness. Also, it was characterized by abortion in females and epididymitis and orchitis in males[7].

The economic and public health effect of brucellosis remained serious concern in developing countries[8]. In general brucellosis can cause significant loss of productivity through abortion, stillbirth, low herd fertility and comparatively low milk production. It is thought that this can be achieved by linking sexual activity to changes in the photoperiod, a reliable predictor of the seasons and future food supplies, however, this is not applicable due to 3 reasons[1] photoperiods can change in, the region that, are close to the equator[2]. In many

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regions, the fodder availability can be determined by many factors other than photoperiods[3]. In semi-arid regions, the pattern of food supply mainly changes year to year due to unpredictable rain falls. However, low mineral intake or availability to small ruminant's results to reduce productivity. Natural grazing is also a source of obtaining minerals from forage plants. Thus, feeding places and seasons also affect the mineral status. Families commonly own a few small ruminants, which are used for religious festivities, serve as savings or spare cash or provide meat or milk. Consequently, due to the reduction of reproductive performance of the herd, whole milk, as well as meat production, tends to decrease, demonstrating an important hazard to farmers. The acceptance of adequate programs to reduce the incidence of infectious diseases[2]. Inadequate management practices and poor propagative performance have been reported as vital factors in decreasing productivity level in livestock. Diverse pathogens have been reported in small ruminants' herds/groups with reproductive failures. Subclinical infection is mainly categorized by reproductive problems, such as infertility, abortion, occurrence of stillbirths, and weak lambs/goat kids[3]. Reproductive problems caused by nutritional deficiencies and infectious diseases and other environmental stresses are mentioned here.

ENVIRONMENTAL CAUSES (PHOTO-PERIOD, SOCIO-SEXUAL)

Mostly reproductive response to environmental factors are coordinated at brain level, where all external and internal inputs ultimately converge into a final common pathway that controls secretion of gonadotropin-releasing hormone. This neurohormone controls the secretion of gonadotropins pituitary hormones that determined activity of reproductive axis[9]. The mechanism of feed flush has been done in sheep management for many years to increase breeding duration[10]. Thermal stress decreases the intensity of sexual behavior and also cause in failure of the animal to maintain a pregnancy and mating[11]. Sometimes heat effect causes a reduction in fetal growth[12]. If stress is imposed in the follicular phase of sheep, it causes to decrease in estrogen level concentration and also effect on late preovulatory GnRH/LH surge[13]. When ewes are under or above the critical temperature, their oestrus duration is delayed that is due to thermal stress[14] or when these are transported then due to transport stress[10].

For proper reproductive efficiency follicle must grow at proper rate in ovaries, ovulation should occur and related hormones that need to be produced not to control releasing but also to make uterus ready for conception. All these events are controlled by endocrine coordination which can be disturbed in stressed conditions[15]. The activities of the neurons are coordinated and synchronized so GnRH is released as a stream of pulses, the frequency of which is critical: a high frequency promotes the gonadal activity and a low frequency permits gonadal activity to minimize. Every pulse of GnRH releases a pulse as of gonadotropins luteinizing hormones from the pituitary glands in the males each LH pulse stimulates the ledying cells in the testes to release a pulse of testosterone which completes the loop by exerting "negative feedback k" on the hypothalamus system to minimize the frequency of GnRH and LH pulses[16]. The timing events in the reproductive process using male effect in sheep and goats, the sudden introduction of new males can induce ovulation in females that are reproductively quiet[16]. The male effect also work for advancing the 1st cycle in young females. Among the environmental factor affecting the reproductive system in sheep and goat the level and type of nutrition is also the most important factor which has also impact on reproduction[17].

EFFECT OF NUTRITION ON THE REPRODUCTION IN SMALL RUMINANTS

Limited feeding resources may cause a decrease in reproductive performance according to the required limit and reproductive status[18]. High feed intake can increases the reproductive output. While for short and long term under-nutrition may exert a negative effect on ovarian activity in goats[19]. The feed flushing system has been incorporated into the sheep and goat management system to increase the seasonal breeding period[15]. The interaction between nutrition and reproduction having a major role in sheep reproductive performance[17]. The relationship between ovary functioning and nutrition is a fundamental rule to maximize reproductive efficiency[20]. Deficient nutrition having long term effect on reproductive capabilities as pregnant ewes fed 50% of its requirement after mating from 28 to 78 days resulted offspring having lower stress response at one year of age, it also cause to lessen progesterone level in the luteal phase and also reduction in fertility when compared to control ewes[21]. Feed restriction is causing a decrease in GnRH amplitude, pulse and the capability of low amplitude GnRH pulses to generate a consequence LH pulse in ovariectomized ewes[10]. The magnitude of LH and FSH surge in pre-ovulatory stage may be diminished in fasting in ewes[18]. FSH and LH level tend to be decreased in fasted ewes[22]. Fasting decrease plasma concentration and FSH pulse amplitude in comparison to that of control significantly[23]. Short-term fed on high protein and energy diet causes an increase in blood glucose and insulin concentration in cyclic ewes and also affect the follicular environment particularly follicular fluid glucose level. The follicular fluid concentration of progesterone was negatively correlated with the follicular fluid concentration of glucose[17]. Fasting effects pituitary-hypothalamus ovarian axis that can be stimulated by metabolic meditators like glucose, insulin, GH, IGF-1 and IGFBP.

Nutritional effect on reproductive performance may be inter-linked via changes in the IGF-1 mechanism (IGF-1 and IGFBP) which is secreted by the liver and may be present in other reproductive tissues[24]. An acute nutrition restriction till the IGF-1 level decrease then it may cause a reduction in FSH-receptor expression by developing follicle to respond FSH[18]. Insulin and glucose are necessary as a source of nutritional folliculogenesis stimulation[17]. Progesterone synthesis and its release by ovary are also regulated pituitary gonadotrophin as well as by IGF-1 which is an important factor for ovarian secretory and granulosa cell proliferation activity. Glycaemia and insulinemia having a role in the regulation of ovarian follicles responsive to gonadotropin[25]. The insulin-dependent glucose transporter is also present in theca and granulosa cells of follicles in sheep. Insulin is also affecting granulosa and theca cell function. An increase in in-

sulin-mediated glucose is critical when up taken by follicle cells for growth and protection from atresia thus increasing ovulatory follicles in numbers. On the other hand, ovulation rate can also be affected by nutrition[22]. Fasting reduced the ovulation rate significantly in ewes[23]. Increasing dietary intake may affect the stimulation of follicular development and increases the ovulation rate in sheep. Acute feed restriction in the luteal phase of the estrous cycle in ewes cause the changes in endocrine functions that may affect the timing of LH surge and ovulation rate also[18]. Short-term fed on high energy and protein may cause changes in feedback mechanism between FSH and Estradiol[17]. Energy-deficient feed may cause lipid to break down which result into an increased level of non-esterified fatty acids and beta-hydroxybutyric acid including low glucose concentration in serum[27]. The resulted metabolites affect the endocrine signaling pathway and also affect the quality and number of oocytes[28]. Increased plasma non-esterified fatty acid level as a sign of negative energy balance that is linked to decreasing in fertility in goats[19]. Circulating urea and non-esterified fatty acids affect the quality of granulosa cells and also oocytes[27].

While short-term fasting may decrease the follicular growth, high plasma progesterone level and lowers the magnitude of LH and FSH surge in sheep[18]. Fasting causing a decrease in average plasma FSH level compared to the control group significantly[23]. The short term dietary treatments lead to changes in blood concentrations of glucose, fatty acids, insulins and leptins as well as the cerebrospinal fluid concentration of glucose, insulin, leptins and some amino acids. Some crucial factors seen to be fatty acids, insulin and leptin, all of which can maximize GnRH pulse frequency. Current studies showed that there are neuroanatomical connections between the centers that control the reproduction[9]. The acute fasting is having a negative effect on ovulation rate in ewes. It also causes a decrease in the level of LH, FSH, Leptin and changes in the signaling pathway of GH[23]. The increase leptin concentration is having an effect on LH secretion from pituitary cells within physiological range in-vitro. The synthesis of steroid hormones depends on the availability of cholesterol metabolites to ovarian theca and granulosa cells[29]. Intracellular cholesterol is transported by steroidogenic acute regulatory protein (**STAR**) from the outer mitochondrial membrane to the inner mitochondrial membrane[30]. Circulating urea has been negatively associated with fertility[31]. Dangerous effects on reproduction are stimulated by the direct effect of urea on the nuclear and cytoplasmic development of the follicle having oocyte[32].

INFECTIOUS CAUSES

Toxoplasmosis

Toxoplasmosis is a parasitic disease causing reproductive problems and enormous economic loss to the sheep industry all over the world[33]. Toxoplasmosis is an infection caused by *T. gondii* blood protozoan that is widespread in animal species reported many countries and many different climate conditions. Goats are having high prevalence than sheep and highly significant incidence in females than males[34]. The considerable incidence has also been noted in males than females[35]. Toxoplasmosis is protozoal a parasitic blood disease that causes severe reproductive issues and economic losses to the small ruminants all over the world[33]. *Toxoplasma gondii* has been recognized as an essential cause of lambing loss and food hazards (Innes et al., 2010). Prevalence of toxoplasmosis is varied from country to country and from climate to climate changes within a state[37].

The worldwide seroprevalence of toxoplasmosis in sheep is 30% while in goats 15%[38]. Age of animal is also a determining factor for the prevalence of toxoplasmosis in animals. Incidence is highest (38.88%) in the age group of 16-28 months and lowest (8.51%) in the age, group of 68-80 months and *T. gondii* is higher in younger animals than adults and body weight of the animal is also a key factor for prevalence[38]. *Toxoplasma gondii* is common in sheep's and goat's one of the significant causes of abortion in many countries. It can also affect fetus of pregnant females (AH/sheep/19). Within livestock sheep and goats are more easily infected with *Toxoplasma gondii* although infection has also been reported in cattle. Moreover, goats infected by *T. gondii* also represent a vital source of human disease due to ingestion meat and milk from infected animals[39]. It may cause resorption or mummified fetus if infected late in gestation may cause abortion or death soon after birth. Female are immune after abortion (AH/sheep/19). Cats are a primary reservoir and definite host, usually spread it only for few weeks as a kitten. Cats and rodent shed agent into hay and feed by their feces which are later ingested by sheep and goats. The disease is transmitted by ingestion of contaminated feed having oocytes and cyst of *Toxoplasma gondii*[38]. Uncooked meat and milk having cysts and oocytes may spread the disease to human by eating such food. Some studies show that sheep and goats are significant sources of infection in a human by eating uncooked meat and milk that cause a massive loss for a small ruminant breeder in many countries.

T. gondii has also been found in mutton and beef in many developed countries[40]. Keep cats away from feed storage and feeding areas. Some producer it best to maintain one adult cat which keeps younger cats away from the field. Burn aborted products[41]. Wear rubber gloves when handling with aborted fluid and tissues. It is one of the leading cause of abortion in many countries. Infection during early gestation may cause of reabsorbing of the fetus while in later stages abortions are most common. The abortion manner and disease are different from the bacterial cause of abortion.

Preventive Measures - Keep dog and cat away from feed storage and feeding areas. Some producers have found it best to maintain one adult, neutered cat which then tends to keep younger, stray cats away from the area. Because cats are reservoir of infecting agent.

Leptospirosis

Leptospirosis is a global zoonotic disease caused by pathogenic spirochetes *Leptospira interrogans* that belongs to genus Leptospira. Genus Leptospira consists of pathogenic and non-pathogenic according to DNA related species[42]. Leptospira colonies in the kidney in various animals that are excreted in urine[3]. Following period of leptospiremia 7-10 days it localizes and persists in renal tubules[43]. Transmission of leptospirosis occurred by exposure to water or soil contaminated by the urine of infected animals or by direct contact with infected animals[44], i.e., rodents, ruminants, swine and canines. The environment also considered being a source of maintenance for leptospirosis in tropical

conditions[3]. Transmission also occurs when the animal is contacted with standing water like a lake or pond. Leptospirosis in small ruminants may present in acute form with increased body temperature, anorexia depression, jaundice and anemic or hemorrhagic syndrome[44] and chronic form with impaired fertility neonatal death abortions and decreased milk outputs occurred more frequently a major economic loss[45].

A subclinical infection characterized by infertility, repeat breeding, abortion, weak lambs and stillbirths. Leptospirosis is a neglected disease that's why, its effects on animals are undetermined. The lack of study on leptospirosis in small ruminants and poor study on outbreaks and surveys contributed to limited contribution for leptospirosis in animals[3]. It determined that leptospirosis in goats a main cause of abortion showing strong symptom of the syndrome[45]. It is cared and spread in the urine of infected animals. Avoid using contaminated water isolation of aborted animals and vaccination of the animals. Serological studies elaborated that Leptospira infection in goats and sheep is common worldwide, for many years small ruminants had been considered as accidental host of leptospirosis being affected only for incidental serovars, carried by other domestic and wild species. Leptospirosis in small ruminants commonly associated with strains belonging to serogroup serovars hardjo[46]. Leptospirosis shows an important infectious disease that affects the reproduction in small ruminants[2]. Many risk factors may influence the occurrence of Leptospira infection in animals. Leptospirosis is most common in cattle, an elaborative study with sufficient analysis of those factors regarding infection in the sheep has never conducted[47]. In several countries Leptospira infection in sheep and goats is determined by serological methods. Leptospirosis seroative is 25.9% in goats while 47.4% in sheep[2]. Agglutination test is the most important use for leptospirosis in the world[3]. Diagnosis can also be made by urine test of the aborted dam, aborted placenta and fetus. Vaccination play in a key role for control of leptospirosis in the herd[5].

Preventive Measures - Avoid using leptospira contaminated water as a drinking water for healthy. Isolation of animals ill or aborting from vaccinated animals. Vaccination plays an important role in the control of leptospirosis and may reduce significantly occurrence of clinical symptoms (abortions) in herd. Control of leptospirosis in small ruminants involves measures such as identification and treatment of carriers and other sources of infection. Quarantine is necessary for new herd/flock[5]. Burn or bury aborted products. Wear rubber gloves while handling tissues and fluids[3].

Q fever

Q fever is a ubiquitous zoonotic disease caused by pathogen *Coxiella burnetii* responsible for acute and chronic clinical signs[48] especially in sheep and goats causing abortion, anorexia and lesion. Cattle, sheep and goat are primary reservoirs[49]. *Coxiella burnetii* is a small obligate intracellular gram-negative that is prevalent throughout the world. Genus Coxiella a classified based on the gene sequence analysis in order of Legionellales family Coxiellaeceae with Rickettsia and Aquicella. This agent has highly osmotic resistant[50]. Ranch animals and pets are the key reservoirs of infection and transmission to human beings is mainly able through inhalation of contaminated aerosols, this bacteria also shed in milk, urine, feces, placental debris and amniotic fluid and causing illness associated with a wide clinical spectrum from asymptomatic to fatal disease[51]. Mostly introduction of bacteria is through aerosol route in farm animals[52]. To prevent from oral infection with *C. burnetii* in human is required to pasteurize the milk before use[53]. In small ruminants mainly causing abortion and stillbirth in late pregnancy without clinical signs[49]. *Coxiella burnetii* on inoculation to pregnant goats the trophoblast cells of the allanto-chorion are primary target cells for *C. burnetii*[54]. Almost in all cases trophoblasts of inner-cotyledons allantochorion and also the base of cotyledonary villi are affected[55]. Abortion in dairy goat increased by incidence of metritis. Abortion mostly occurs with no proceeding clinical signs[53]. Many studies indicate that human Q fever risk factors are more living near to farm has a history of abortions through *C. burnetii*[56]. In small ruminants premature birth, weak offspring deliver, stillbirth and abortion are reported[53]. Chronic fatigue is infrequently also associated with *C. burnetii* infection[57]. It is yet not clear that *C. burnetii* is present other than placenta or not[54]. Induction of long-lasting immune response against many diseases is facilitated by *C. burnetii*. Immune response to *C. burnetii* infection is better studied in goats than in sheep[58] but result from work in goats could also be applicable in sheep.

After inoculation of *C. burnetii* specific antibodies both IgM, IgG can be detected after two weeks and remained for up to 13 weeks. Prevalence of *C. burnetii* in sheep or goats have been described on the basis of analysis different body fluids and tissues, seroprevalence have been described for many countries but these data are difficult to compare because of the differences of the methodology of studies[15]. Sheep, goats and cattle are common hosts but many other species may be infected. Excreted in milk, urine, feces and with especially high numbers in the birth fluids and fetal membranes. Ticks can assist as a reservoir and also spread the disease (Seshadri et al., 2003). *C. burnetii* is also transmitted between sheep, goats, cat, cattle and dogs by biting of ticks or by contact with contaminated excreta. Prevention, burn or bury the reproductive organs and fluids. Infection in closely confined animals is almost universal. The level in research flocks can be reduced by periodically testing their serum and culling all infected animals (Gikas et al., 2001). This disease is present at worldwide level affecting a wide range of domestic and wild animals[59]. The presence of *C. burnetii* is worldwide except in New Zealand[60]. In many countries Q fever is epidemic in rural outbreaks and occupationally it is endemic[61]. No ovine abortion is observed in human Q fever outbreak related to sheep[62]. Analysis of human Q fever outbreak indicates that it is related to small ruminants instead of cattle[63]. In humans *C. burnetii* infection unnoticed and with signs flu-like illness, pneumonia, or as hepatitis. In 1 to 5% cases disease proceed to chronic stage mainly to endocarditis or vascular infections. A few studies have been done on ruminants and zoo animals for clinical demonstration, despite having zoonotic importance occurrence of infection information is scarce[64].

European Commission concern about the high risk of Human Q fever associated with small ruminant herds in urban areas[65]. Q fever incidence and prevalence is still not well known and undetermined in many years till now[51]. Increased numbers of lambing in lambing seasons cause an increase introduction and transmission of the pathogen in the popula-

tion or small ruminants[60]. Another factor including visiting and working professionals and animal supply also cause an increase transmission of the pathogen on the farm[56]. *C. burnetii* DNA detection by PCR is done by milk tank from dairy goats having the reproduction problem. An association between *C. burnetii* and reproductive problems prevalence is reported in some studies[58]. *C. burnetii* is having a sensitivity to oxytetracycline in vitro and demonstrated to reduce the abortion rate. Globally prevalence of *C. burnetii* in mixed herd is 38.4%, in sheep herd is 37.5%, and in goats, the herd is 28.8%. While seroprevalence 74% in sheep herd in Spain[66] and Turkey 83%[59]. Overall mean prevalence was 15% in sheep and 27% in goats.

Preventive Measures - During an outbreak screening of animal is advisable with identified risk factor[48]. Eradication of Q fever from a herd is not currently straightforward for a range of reasons, including chronic infection in a small number of animals, presence of shedding, but test-negative animals are also potential for shedding of agent[67]. Reduction of excretion has been reported using a phase 1 *C. burnetii* vaccine for animals, however, this could be affected by herd infection status and the timing of vaccination[68]. To minimize human health risks, vaccination of animals must need to be conducted in combination with repeated testing. For this, first require training of health workers and laboratory staff to strengthening laboratory analysis[69].

Prevention of shedding and abortion can be achieved by vaccination. Some vaccines claim to prevent abortion and to contribute for reduction of shedding in vaginal charges, feces and milk. Vaccination seems to be most operative when administered in non-infected small ruminants before their first pregnancy[70,71]. Pasteurization of milk from infected farms is recommended to prevent oral infection of humans[51]. Placentas and foetuses should be collected, properly stored and destroyed. Wear protective clothing, although it has been demonstrated that this does not completely prevent infection for humans[72]. General hygiene measures can also reduce exposure to animals from infecting agent.

Campylobacteriosis

The main infectious agent that causes campylobacteriosis is vibriosis (Campylobacter). Campylobacter is not much investigated in small ruminants as in other farm animals. Campylobacter is isolated from ovine meat, liver, carcass, gut, and feces[73]. Carcass contamination rate is different according to the age of animal that was 94% lambs, 63% goats, 78% kids and 72% for sheep. On an average Campylobacter presence were 30% of intestinal contents and 70% of carcass and liver surface[74]. The primary agent for abortion is *Campylobacter fetus* subspecies fetus, abortion and stillbirth occurred in late pregnancy. Remove all the aborted fetus, placenta, to avoid contamination of feed and water of other uninfected animals. It causes abortion in sheep but occasionally affects goats; *Campylobacter fetus* is a primary agent. It causes abortions and stillbirths in late-term pregnancy. It causes inflamed placenta, necrotic cotyledons, and the leathery area between cotyledons. It is spread by the aborted fetus, tissues and all discharges. Moreover, the digestive tract is probably a long term reservoir. Campylobacter is a gastrointestinal bacterial pathogen that is commonly reported in human by European Union[51]. For human infection it is confirmed by the 72-78% of sheep liver and carcass in comprised of campylobacter[75]. Human campylobacteriosis is highly acquired by consuming undercooked contaminated foods[76]. Carcass and liver of animal become colonized by Campylobacter once exposed to a contaminated external environment but remain mostly asymptomatic intestinal carriers[77].

According to EFSA, existing data on Campylobacter in goats and sheep are primarily from clinical investigations since no surveillance so far has been carried out. Moreover, the largest goat population and the fourth largest sheep population in the EU[51]. The majority of sheep and goats at slaughter carried Campylobacter present on meat and liver. This indicates that meat and offal not only of sheep but also of goat origin are commonly contaminated with a diverse population of thermophilic campylobacters, and serve as a vehicle for human infection (Lazou et al., 2014). Revealed resistance to tetracycline (47.9%) monitored by streptomycin (22.9%) and ciprofloxacin alongside nalidixic acid (18.3%) Isolates exhibited low opposition to erythromycin (2.5%) and were liable to gentamicin[74]. Now vaccines are available to prevent Campylobacter infections.

Preventive Measures - Ovine vaccines are available but should be used prior to exposure. They may not protect for all kinds of strains. Antibiotics can be fed prophylactically during pregnancy (tetracycline at 100 mg/head/day). Feed in mangers and water in troughs to avoid contamination of feed and water by aborted material of infected animals. Remove aborting ewes, fetus, and placenta from lambing area. Parenteral injection of oxytetracycline (antibiotic) followed with repeated injections, will decrease the abortion loss.

Brucellosis

"Malta fever" now called brucellosis firstly came from British during Crimean War[6]. Brucellosis is having zoonotic importance worldwide high impact on rural livelihood and underestimated toward febrile illness[78]. The economic and public health impact by brucellosis is considered the main concern in developing countries. This disease causes a significant loss of production by abortion, low herd fertility, stillbirth and low milk[8]. Due to zoonotic impact it leads to morbidity. It is considered most common zoonotic disease is occurring globally[79]. Brucellosis is the world's most common considered bacterial disease[80]. Brucella species are intracellular gram-negative coccobacillus that can infect many animal species including human also[81]. *Brucella melitensis* infects goats and sheep common in many countries. Primary clinical effect of brucellosis is related to production problems in livestock[6]. It is a zoonotic disease.

Brucella abortus primarily infects cattle but can infect sheep and goat also. *B. ovis* cause brucellosis with no zoonotic impact with genital lesions in rams, placentitis and abortion in ewes. The agent can be secreted by vaginal discharge and milk to other related animals in a herd. In some countries, *B. abortus* is reported in cattle[82] as well as in small ruminants[83]. But mixed infection by both has also been reported[84]. *B. melitensis* (sheep, goats, biovars1-3). *B. ovis* (sheep) among these *B. melitensis* having a high risk for human infection which is followed by *B. suis* and *B. abortus* and others are virulent for human health[85]. The serological test has been done only in sheep *B. ovis* having low affinity for the host. This disease causes abortion in late gestation, retained placenta, perhaps some weak newborn animals. It can be spread by

oral ingestion of the organism from the fetus, placenta, uterine discharges or contaminated feed and water; it can also enters through the conjunctiva, mucous membranes, wound and intact skins. In dairy farms, mostly dog use contaminated milk and carcass, aborted fetus, placenta and vaginal debris then serve as a basis of infection for canine brucellosis and can also transmit to livestock[86]. It is prevented by involving testing and testing of slaughtering of animals, isolate aborted animals, clean up and burn the aborted fetus and placenta, contaminated bedding manure. Keep all aborted tissues and fluids away from dogs and birds. Clean and disinfect floors, feed bunks, building, and equipment's. The most widely used diagnostic techniques are serological tests such as complement fixation, agar gel immunodiffusion (AGID), and indirect ELISA. Although seroreactivity to an agent does not necessarily mean that the animal was clinically affected by that pathogen. Furthermore, we inferred that these pathogens contributed to the decreased productivity of these animals[2].

Quarantine the new herd until further serological testing reveals no new cases. In general, brucellosis causes huge loss of production by abortion and stillbirth and low milk. If brucellosis is highly prevalent in a country then improved diagnosis can be helpful for proper treatment[78]. By regression analysis, it is reported that association of antibody against brucellosis is having high potential risk for age, history of abortion and party number for *B. melitensis* infection in small ruminants[6]. In Nigeria, reported seroprevalence is 0-76% in small ruminants and humans; it is 0-74%[69]. Brucellosis primary affects on livestock, but it can also affect humans by ingestion of contaminated milk or meat and by close contact with infected animals[87]. Animal-to-human transmission may occur through a direct connection with the vaginal and placental fluid and material and aborted fetuses of infected animals, or via consumption of raw milk or unpasteurized dairy products from these animals[69]. Brucellosis is endemic among Mediterranean countries of Europe. Northern and eastern Africa, India, central Asia, Mexico and South America almost occurred in the whole world. Signs and symptoms of brucellosis in human are not specified and be confused with febrile illness especially malaria[88]. Poor diagnosis can result in the treatment of malaria for brucellosis and unnecessary use of antimalarial medicine in human[88]. Some studies are conducted in high-risk groups like a farmer, veterinary professionals, meat inspectors and artificial insemination technicians in Amhara Regional State[86]. The importance of brucellosis in equine, swine and wild animals has also not been addressed to date[6].

Preventive Measures - Brucellosis can transmit to human by consuming unpasteurized products or direct contact with infected animals[89]. Vaccination was applied to overcome extensive bovine abortions in government-owned farms and local production by a liquid S19 vaccine started. A test and killing policy was also implemented[69]. Many countries have eradicated brucellosis by this method. Isolate the aborted animal from normal ones. Clean up and burn the aborted foetus and placenta, contaminated bedding, manure. Keep all aborted tissues and fluids away from dogs and birds. Clean and disinfect floors, feed bunks, buildings and equipment. Use serologic tests to identify infected animals and if positive for brucellosis send to slaughter.

Quarantine the new incoming animals for almost 30 days. Buy all replacement animals from herds or flocks that have been certified free of brucellosis or animals tested as serologically negative prior to bring them into new herds or flocks which are free of brucellosis.

Listeriosis

It is triggered by *Listeria monocytogenes*, a bacterial disease. Listeria species are small gram-positive rod-shaped having 1-2 micron in length and 0.5 micron width. Optimal growth temperature for these bacteria ranges from 30-37celcius. But organism can also grow and reproduce at a temperature of -0.4 and 45 celciuse with a pH of 4.5-9.6 in aerobic or microaerophilic conditions. Common in goats but sheep affected occasionally. *L. monocytogenes* is a universal organism for which soil is the most important cave for its transmission and persistence to animals[90]. The risk factor for listeriosis is associated with winter season by using silage listeria can grow at this low temperature while another pathogen cannot grow, and their growth is inhibited at that temperature. The incidence rate for this may reach 9%, but rarely it is up to 2%. It is isolated from livestock[91]. *Listeria monocytogenes* mostly distributed in soil, feces and environment of animals. Upon certain conditions, it becomes highly pathogenic and causes serious disease. It is clinically characterized by abortion, decomposed fetus, necrosis of cotyledons and necrotic foci in the lungs and liver. May have a severe uterine infection after an abortion; abortion commonly occurs during late pregnancy spread of agent by spoil silages with soil and rodent contamination. Other clinical signs for listeriosis are septicemia, abortion, mastitis and gastroenteritis. The diseased animal can shed the organism in milk and feces. It is also zoonotic one that's why infection from milk and meat is of real concern[92]. Abortions usually occurred in the last of gestation. Fever, depression, metritis may also occur in it. The main clinical sign is of nervous system unilateral or bilateral. The outbreak of listeriosis can also occur by without any silage feeding but can occur by feeding low-quality pasture or vegetation to animals. In ruminants, sheep are one of the most commonly affected one clinical characterized by encephalitis, septicemia, abortion, mastitis and gastroenteritis[92]. It can be prevented by improving the storage and management of the silage or other forages. Antibiotic can be administered for a long period be a cause of recovery period for almost one month. Prevalence of organism is higher in cattle than sheep[93]. Removal of *L. monocytogenes* is an unrealistic challenge because it can survive in very stressful conditions of the environment. But efficient management and control measure can help to reduce food contamination[94].

Preventive Measures - Make improvement in storage and handling of silages as well as forages because these are sources of infecting agent.

Trypanosoma (Dutton Ella) vivax

Trypanosoma vivax is a blood parasite that is normally associated with anemia and permanent febrile status causing a loss in production, weakness of related animals and sometime may causing the death of the infected animal[95]. Trypanosomosis is a disease of economic importance caused by blood protozoan *Trypanosoma* spp. That affects most of the areas of South America, Africa, and Central America causing a huge loss in the cattle industry. The Trypanosoma

species members are parasites having two hosts involved in their life cycle. Bloodsucking insects are the main vectors for transmission of trypanosome[96]. This parasite initially named by *T. guyanense* later on according to genetic similarity with *T. vivax* was high so considered it as a separate group of the parasite[95]. For transmission, by the natural way the vector is tsetse fly and by mechanical it is directly transmitted from one animal to other by blood-sucking parasites, i.e. Tabanidae and Stomoxydae[97]. It can also be transmitted by invertebrates which sucks blood, i.e., Vampire bats and by the mechanical way by contaminated needles by infected blood[98]. Trypanosoma caused by *T. vivax* may spread in a new herd that is never before exposed to blood-sucking parasite can also be infected within 6months[99]. *T. vivax* infection triggered by high temperature, anorexia, anemia and also degenerative changes at testicular and epididymis level[100]. It can also affect semen quality and may cause infertility or sterility (Bittar et al., 2015). It may result in multi-focal epididymitis and hyperplasia of epididymis tissue. Beside this in female sheep, *T. Vivax* also results into the degeneration of hypothalamus, hypophysis and gonads that results into hormonal disturbance and lower its concentration in blood plasma which is necessary the basic fundamental reproductive processes like gestation. These conditions might cause an increase in corticosteroid, estrogen; prostaglandins results in abortion and luteolysis[35].

Reproductive failure includes irregular oestrous, infertility, abortion, neonatal death, intrauterine infection, and also abortion in goats. If it is occurred in the last third of the gestation period may give rise to placental retention, perinatal mortality, and next to that disruption of the estrous cycle[95]. Damage by protozoa to placenta may cause a disturbance in placental progesterone secretion[35]. Diagnostic tools are important for the detection of the parasite to increase sensibility and specificity. It is of paramount status to prepare field professionally for differential diagnosis in reproductive failures[95]. Different parasitological, immunological and molecular techniques for diagnosis of Trypanosomosis. The simplest method for Trypanosomosis is by blood examination under a light microscope. Most important techniques for detection of trypanosome are ELISA, immunofluorescence PCR, and restriction fragments length polymorphic[96]. Trypanosoma gets different genetic variability during the stages of infection making it difficult for vaccination development[39].

Preventive Measures - Trypanosomosis is mostly confused with babesiosis, anaplasmosis, theileriosis, and even with erlichiosis (OIE, 2013). Diagnosis must consider the herd's history, the region's epidemiology and an adequate coordination between the veterinarian in charge and the diagnostic laboratory so this can allow to define the best sample and test available which can help identifying causative agent[102]. It is very important to eliminate possibility of iatrogenic transmission, using specific needles for each animal; plus, attention must be paid to fly control, especially in times of year when their population increase[95].

The Economic losses in small ruminants are mainly because of infectious causes are in the form of production loss, animal loss and production quality and quantity loss. Here we have given different reports of these losses for each infectious disease.

Disease	Losses	References
Brucellosis	Infertility, abortion, milk loss, zoonotic, 20% financial loss of total production system, weak new-borns	(89)(69) (79)(95) (103)
Q fever	In chronic high death rate, productivity loss, costly vaccination, abortion, stillbirth, public health issue	(104)(59) (63)
Trypanosomiosis	Abortion, repeat estrous, repeat breeding, retained placenta, delay uterine involution, infertility, mortality, sterility	(100)(95) (101)
Compylobacteriosis	Abortion, infertility, late pregnancy, stillbirth	(73)(95)
Listeriosis	Abortion, septicemia	(95)
Leptospirosis	Infertility, stillbirth, abortion	(105)
Toxoplasmosis	Meat loss due to infection, infertility, abortion	(95)(106)

CONCLUSION

These reproductive problems cause an economic loss in the world. Inadequate management and nutritional deficiencies cause poor production. All these reproductive problems should be resolved by positive improvements in nutritional, photoperiod & infectious causes to compensate these losses. These losses can be nullified by improvement in management and vaccination should be done to avoid zoonosis of some these reproductive diseases. The efficiency of small ruminants can be increased by the identification of the cause of disease, quarantine and active immunization by the commercially available vaccine. The more research is to be done in diagnosis and molecular basis of these reproductive diseases that were neglected in past. Which have made high impact on countries economy.

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DATABASE EPIDEMIOLOGICO SANITARIO
Raccoglie dati sanitari ed epidemiologici di cui dispone solo il veterinario d'azienda

DDD

DEFINED DAILY DOSE
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ISCRIVITI ALLA SIVAR PER IL 2020 E RICHIEDI L'ABILITAZIONE AI SERVIZI DEL PORTALE VETERINARIO DI FIDUCIA: AVRAI ACCESSO A DES, DDD E FORUM
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Identificazione e caratterizzazione molecolare di *Atypical Porcine Pestivirus (APPV)* nel centro Italia

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RIASSUNTO

Scoperto per la prima volta in USA nel 2015, APPV (Atypical Porcine Pestivirus) o Pestivirus K è stato successivamente riscontrato anche in Europa e in Asia, sia nei suini domestici che nei cinghiali, talvolta in associazione a forme di tremore congenito (CT) dei suinetti. Questo studio riporta i risultati di una analisi virologica retrospettiva condotta allo scopo di accertare la presenza del virus nel centro Italia. Su un totale di 1665 sieri prelevati nel triennio 2016-2018 e provenienti da Umbria, Marche, Abruzzo e Lazio, un solo campione è risultato positivo. Esso, denominato APPV-LA/4911/2016, deriva da un siero di un suino adulto e asintomatico proveniente da una azienda da riproduzione del Lazio, che non riporta alcuna notizia anamnestica di CT. L'analisi filogenetica delle regioni NS5B e NS3 dimostra che APPV-LA/4911/2016 clusterizza con il *clade* degli stipiti tedeschi, e mostra la più elevata similarità con gli stipiti ungheresi. Inoltre, limitatamente alla regione NS3, appare piuttosto simile anche ad isolati italiani precedentemente identificati, mentre è geneticamente distante dal cluster degli stipiti cinesi, a conferma della elevata variabilità genetica di APPV. Questi dati dimostrano che APPV circola in Italia almeno dal 2016, probabilmente introdotto in seguito a scambi commerciali intracomunitari di suini e/o prodotti derivati. Inoltre, la presenza del virus in suini asintomatici potrebbe essere dovuta alla sua capacità di indurre una infezione persistente, che permette il mantenimento e la diffusione della infezione. Infine, non è da sottovalutare il probabile ruolo di APPV come *door-opener*, in caso di co-infezione con altri agenti patogeni del suino.

PAROLE CHIAVE

Atypical Porcine Pestivirus; virus emergente; Italia; caratterizzazione molecolare.

INTRODUZIONE

Al genere *Pestivirus,* famiglia *Flaviviridae*, appartengono virus dal notevole impatto socio-economico sul comparto zootecnico. Le quattro specie principali sono rappresentate dal virus della Diarrea virale bovina (BVDV-1 e BVDV-2), dal virus della Peste suina classica (PSC) e dal virus della Border disease (BD)[1]. Nel 2017 queste specie sono state rinominate, rispettivamente, come Pestivirus A, Pestivirus B, Pestivirus C e Pestivirus D[2]. A questi si sono aggiunti, nel corso del tempo, nuovi e distinti *Pestivirus*, in grado di infettare specie animali diverse con segni clinici variabili: Pestivirus F (Bungowannah virus), Pestivirus G (giraffe Pestivirus), Pestivirus I (Aydin-like), Pestivirus J (rat Pestivirus), Pestivirus E (pronghorn antelope Pestivirus), Pestivirus H (Hobi-like), e Pestivirus K (Atypical porcine pestivirus, APPV)[2-5]. Quest'ultimo è stato individuato per la prima volta nel 2015 negli USA attraverso metodiche metagenomiche in campioni di siero suino[5]. Nel 2016 la sua presenza è stata associata a forme di tremore congenito (CT) in suinetti neonati mediante riproduzione sperimentale della malattia[6]. In caso di CT, a causa della ipomielinizzazione del sistema nervoso, i suinetti manifestano tremori muscolari, incapacità nel muoversi/alzarsi e nell'alimentarsi col latte materno[7,8]; i neonati più gravemente ammalati vengono a morte in breve tempo. Alcuni autori hanno dimostrato la presenza di APPV anche in suini apparentemente sani[9,10], oltre che in suinetti affetti da CT[11,12]. Tra gli anni 2015 e 2018, APPV è stato identificato nei suini domestici in Germania[13], Austria[14], Svizzera[15], Spagna[9], Paesi Bassi[7], Brasile[16], Cina[17], Ungheria[18], Canada[19], Regno Unito[20], Svezia[21], nonché nei cinghiali in Spagna[22], Germania e Serbia[23], dimostrando che il virus è ampiamente distribuito in tutto il mondo. In Italia APPV è stato segnalato per la prima volta nel 2017, in sieri di suini clinicamente sani[10] ed è stato identificato anche in organi di feti suini abortiti (Dr.ssa E. Sozzi, comunicazione personale). APPV possiede un genoma a RNA singolo filamento a polarità positiva (+ SS), dalla lunghezza variabile tra 11-12 kb. Un lungo Open Reading Frame (ORF) codifica per una poliproteina costituita da circa 3635 amminoacidi, che viene processata in 4 proteine strutturali (C, Erns, E1, E2) e 8 non strutturali (Npro, P7, NS2, NS3, NS4A, NS4B, NS5A, NS5B)[5]. Le regioni maggiormente indagate dal punto di vista filogenetico sono NS2, NS3, NS5, anche se non mancano lavori che riportano indagini molecolari su altre regioni, come ORF, Npro ed Erns[24]. I diversi stipiti di APPV finora indagati sono significativamente distinguibili dagli altri *Pestivirus* nella loro sequenza genomica[25]. Al contempo, diversi studi di sequenziamento hanno dimostrato la notevole variabilità genetica di APPV[10]. In particolare, è stata evidenziata una variabilità

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elevata, tra 81% e 87%, all'interno di una stessa area geografica, per esempio tra stipiti provenienti da differenti aziende di un territorio, mentre, per quanto riguarda gli stipiti circolanti nella stessa azienda, in alcuni casi è stata evidenziata una netta omologia virale, in altri è stata dimostrata la presenza contemporanea di differenti stipiti virali[13,26]. L'obiettivo di questo lavoro è stato quello di effettuare una indagine virologica volta ad accertare la presenza del virus APPV in allevamenti del Centro Italia, con particolare riferimento ad aziende da riproduzione.

MATERIALI E METODI

Campioni di sangue

Sono stati analizzati retrospettivamente 1665 sieri di suini adulti, provenienti da aziende da riproduzione casualmente selezionate nelle regioni Umbria e Marche (1280 campioni), Lazio (49 campioni) e Abruzzo (336 campioni) e prelevati nel triennio 2016-2018.

Scheda di indagine epidemiologica

È stata creata una apposita scheda di indagine epidemiologica, da impiegare a posteriori in caso di positività, suddivisa in 8 sezioni. Le prime tre contengono informazioni generali (dati anagrafi, gestione dei nati, censimento e distribuzione dei suini in azienda); la sezione 4 raccoglie i dati relativi all'applicazione di misure di biosicurezza nella conduzione aziendale; le sezioni 5 (stato sanitario) e 6 (dinamica di infezione per APPV) raccolgono i dati anamnestici relativi sia alle patologie riscontrate in azienda sia alla eventuale presenza di CT; le ultime due sezioni comprendono la planimetria dell'azienda e le conclusioni del veterinario compilatore.

Estrazione, amplificazione, sequenziamento ed analisi filogenetica

I campioni prelevati sono stati sottoposti dapprima ad estrazione dell'RNA virale in pool da 8 sieri cadauno, mediante QIAamp UltraSens Virus Kit (Qiagen) secondo le indicazioni del produttore; in caso di positività del pool, i singoli campioni sono stati estratti mediante QIAamp Viral RNA Mini Kit (Qiagen), seguendo le istruzioni dello stesso. Gli estratti sono stati inizialmente analizzati mediante una real-time RT-PCR APPV specifica di screening[26]; successivamente i campioni positivi sono stati confermati attraverso una ulteriore real-time RT-PCR APPV[6] e caratterizzati mediante amplificazione delle regioni NS5B[13] e NS3[26]. Primers e probes impiegati in questo lavoro sono riportati nella Tabella 1. I prodotti di PCR attesi sono stati ritagliati, purificati, quantificati e sequenziati direttamente, utilizzando il kit ABI PRISM® Big Dye® Terminator v3.1 in un ABI PRISM 3130 Genetic Analizer (Applied Biosystems). Le sequenze sono state assemblate usando il software Lasergene SeqMan (DNAstar USA), ed ogni dataset è stato allineato con le sequenze di riferimento presenti in GenBank usando il metodo Clustal X V.1.83. I datasets di sequenze sono stati analizzati mediante il software BioEdit V.2.1. Per ogni regione indagata, l'albero filogenetico è stato costruito con il software Mega V.7, utilizzando il metodo di Maximum Likelihood (ML). La validità dell'analisi filogenetica e la robustezza degli alberi sono state determinate effettuando una analisi di bootstrapping su 10.000 replicati.

RISULTATI

Lo screening effettuato nelle Regioni Umbria e Marche non ha evidenziato alcuna positività virologica per APPV, così come i campioni dell'Abruzzo, che si sono rivelati tutti negativi. Al contrario, l'analisi dei campioni provenienti dal Lazio ha dimostrato la presenza di APPV in un unico suino, rappresentato da un lattone di circa 70 giorni di età. L'azienda di origine esegue cicli di riproduzione aperti, con vendita di riproduttori. La successiva indagine epidemiologica ha dimostrato l'assenza di forme di CT, attuali e pregresse nei suinetti neonati, nonché l'assenza di segni correlati, riferibili agli animali adulti. Il campione positivo, denominato APPV-LA/4911/2016, è stato sottoposto ad analisi filogenetica per le due regioni genomiche di APPV maggiormente informative, NS5B (560 bp) e NS3 (805 bp). Lo stipite APPV-LA/4911/2016 clusterizza con isolati precedentemente identificati in Germania[14], con una percentuale di identità del 95,40-98,10% (Fig. 1). L'analisi della regione NS3 (Fig. 2) conferma l'attribuzione al cluster rappresentato da isolati provenienti dalla Germania e dall'Ungheria[18]; in particolare lo stipite APPV-LA/4911/2016 mostra la più elevata identità con l'isolato HUN Jasz 1 (98,80%). Inoltre l'isolato APPV-LA/4911/2016 mostra una certa divergenza con gli stipiti virali precedentemente identificati in Italia[10], con una percentuale di similarità del 90,20-94,70%. Per entrambe le regioni, APPV-

Tabella 1 - Primers e probes usate per PCR diagnostiche e di sequenziamento.

Primer & probe	Sequenza (5'-3')	Target	Impiego	Referenza
APPV_5587_fw	CAGAGRAAAGGKCGAGTGGG	NS3	PCR screening	26
APPV_5703_rev	ACCATAYTCTTGGGCCTGSAG			
APPV_CT-59 probe	[6FAM] ACTACTATCCTTCGGGGGTAGTACCGA [BHQ1]			
Pesti_6332_F	TGCCTGGTATTCGTGGC	NS3	PCR conferma	6
Pesti_6455_R	TCATCCCATGTTCCAGAGT			
Pesti_6351_Probe	/5Cy5/CCTCCGTCTCCGCGGCTTCTTTGG /3BHQ_2/			
Pesti_11453_F	ACAGCMATRCCAAARAATGAGAA	NS5	Sequenziamento	13
PestiV NS5-R	AAGCCRTCRTCICCRCASACGTG			
APPV_4186-fw	GTGCGGCCTCCCAACTGTAG	NS3	Sequenziamento	26
APPV_5169-rev	ACGTCACCCTCTTTCCGCTC			

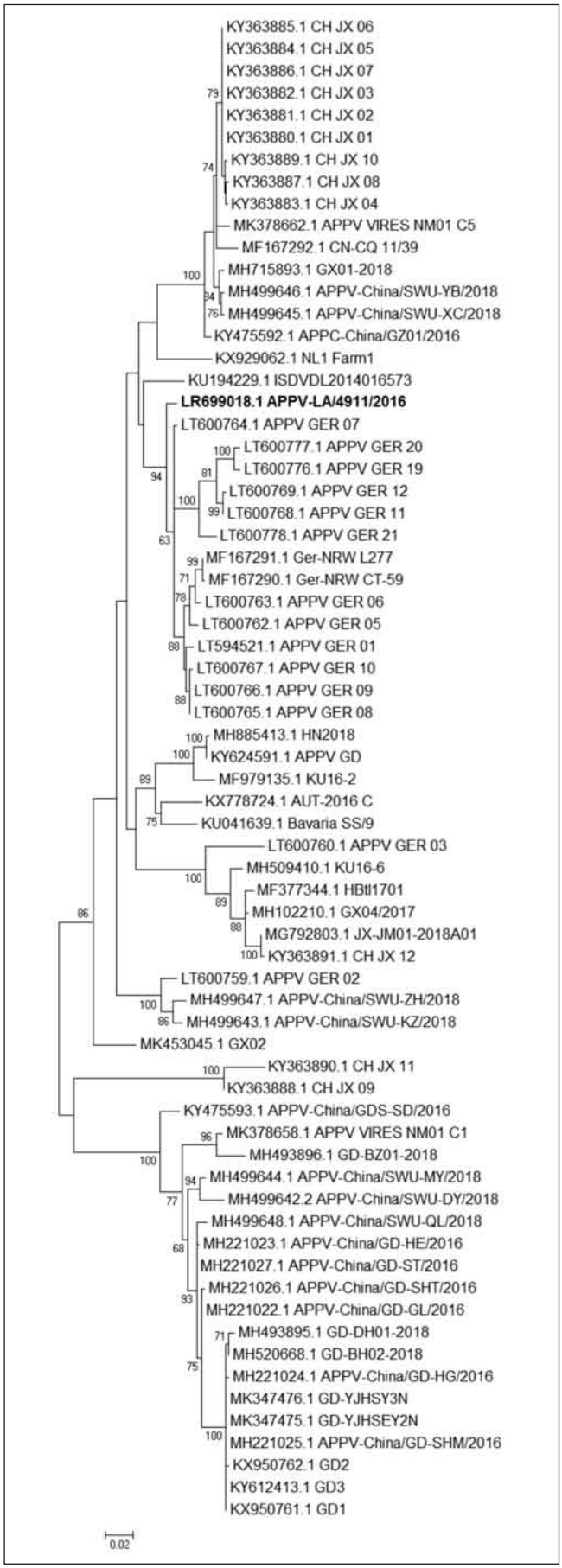

Figura 1 - Albero filogenetico costruito su una porzione (801 nt) del gene codificante la poliproteina NS5B di APPV mediante il metodo Maximum Likelihood (ML) utilizzando il software MEGA v.7.0. Sono mostrati i valori di bootstrap > al 60% (in % su 10.000 replicati). Bar: numero di sostituzioni per sito. In grassetto il campione caratterizzato nello studio.

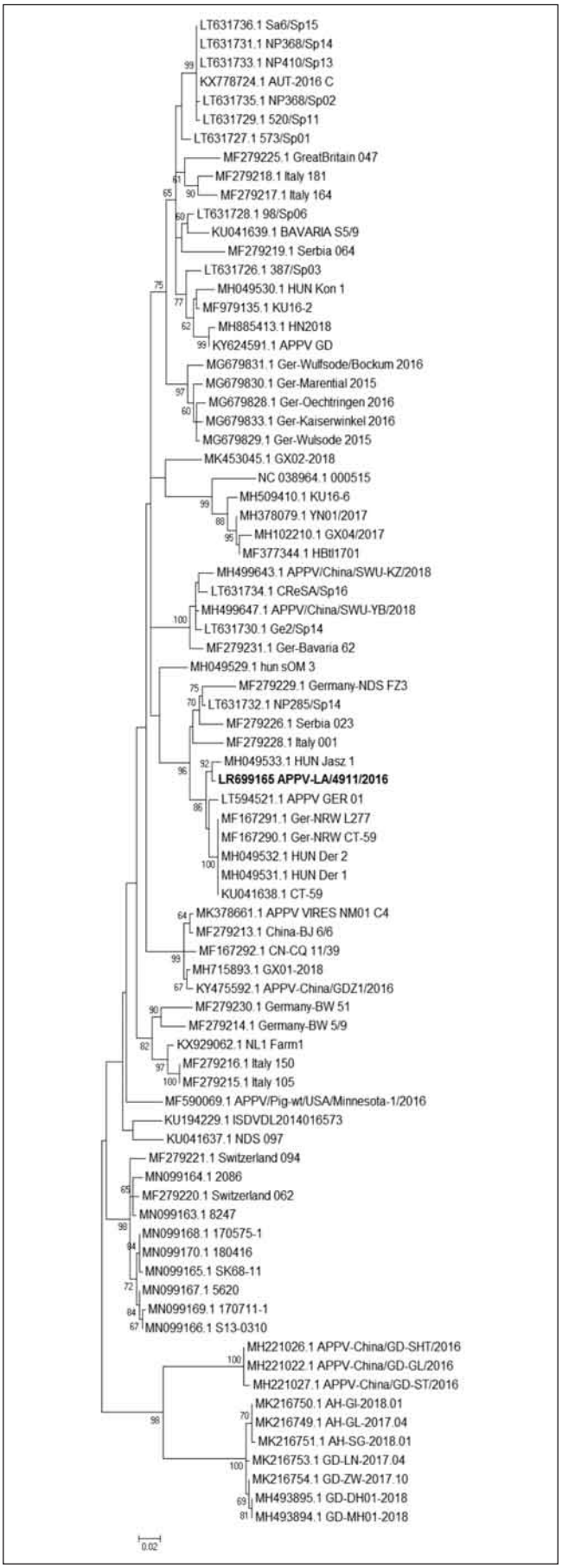

Figura 2 - Albero filogenetico costruito su una porzione (400 nt) del gene codificante la poliproteina NS3 di APPV mediante il metodo Maximum Likelihood (ML) utilizzando il software MEGA v.7.0. Sono mostrati i valori di bootstrap > al 60% (in % su 10.000 replicati). Bar: numero di sostituzioni per sito. In grassetto il campione caratterizzato nello studio.

LA/4911/2016 mostra invece la più elevata divergenza con isolati cinesi (rispettivamente, 83,10% in NS5B e 84,40-85,20% in NS3). Le sequenze sono state depositate in GenBank (acc. nrs. LR 699018 e LR699165).

DISCUSSIONE

APPV è un Pestivirus emergente, individuato per la prima volta nel suino nel 2015 mediante tecniche di metagenomica. In questo studio, in seguito a una indagine randomizzata, è stato identificato e caratterizzato nel 2016 uno stipite di APPV da un suino apparentemente sano proveniente da un allevamento del centro Italia, denominato APPV-LA/4911/2016. Nel nostro Paese, APPV era già stato segnalato in precedenza e questo lavoro ne conferma la presenza nella popolazione suina. D'altra parte, ad oggi, l'esistenza di APPV è stata documentata in più di 10 paesi in Europa, Asia e Americhe. Sebbene la scoperta di APPV sia avvenuta recentemente, ciò non implica che il virus non abbia potuto circolare nelle decadi precedenti, come è stato anche ipotizzato da alcuni autori[18]. Studi retrospettivi realizzati in Svizzera[15] e in Spagna[9] hanno dimostrato la presenza di APPV in campioni collezionati negli anni '80 e '90, rispettivamente. In questo lavoro, su un totale di 1665 sieri testati, uno solo è stato riscontrato come APPV positivo. La bassa prevalenza riscontrata potrebbe spiegarsi col fatto che nelle aziende da riproduzione, più che in quelle da ingrasso, le misure di biosicurezza sono in genere applicate con maggiore severità, per evitare l'introduzione di patogeni dall'esterno. Inoltre, la probabilità di trovare una positività in soggetti adulti è ragionevolmente attesa come inferiore. APPV viene associato a forme di CT nei suinetti neonati, sebbene anche suini e cinghiali adulti, apparentemente sani, siano stati trovati positivi. Il campione APPV-LA/4911/2016 deriva da un capo asintomatico proveniente da una azienda che non riferisce una storia anamnestica di CT, e resta da spiegare il ruolo svolto da APPV in questi casi. Come per altri *Pestivirus*, è stato ipotizzato che anche APPV possa essere in grado di stabilire la condizione di infezione persistente che giocherebbe un ruolo fondamentale nella trasmissione e nel mantenimento della infezione in azienda[25]. I sintomi clinici di CT nei suinetti neonati compaiono nei casi di infezione del feto prima che questo sviluppi competenza immunitaria. Nei suinetti che sopravvivono, i sintomi scompaiono con la crescita, ma l'animale eliminerà il virus nell'ambiente per un periodo di tempo variabile e ancora non definito[15]. In particolare, alcuni studi hanno dimostrato che suini di circa 15 giorni non presentavano segni clinici riferibili a CT pur essendo viremici[9]. In questa condizione, essi potrebbero crescere senza evidenza di sintomi clinici né produzione di anticorpi e, nel contempo, diffondere il virus nell'ambiente, mantenendo l'infezione. APPV presenta un elevato grado di variabilità genetica tra gli stipiti, anche all'interno di uno stesso territorio. Sebbene due degli stipiti virali identificati in Italia, Italy 001/2015 e APPV-LA/4911/2016, risultino correlati tra loro, facendo supporre una probabile origine comune, altri stipiti italiani sono invece geneticamente divergenti, facendo ipotizzare più di una introduzione anche nel nostro Paese. Sulla base dei risultati ottenuti in questo studio, lo stipite APPV-LA/4911/2016 è risultato strettamente legato dal punto di vista genetico ad isolati identificati precedentemente in Germania ed in Ungheria. Considerati i frequenti rapporti commerciali che l'Italia intrattiene, in special modo con la Germania, si può speculare sul fatto che tale stipite possa essere stato introdotto da uno di questi Paesi, sebbene non esista alcuna riprova di ciò. Lo stipite APPV-LA/4911/2016, d'altro canto, è geneticamente molto distante dal gruppo degli isolati cinesi, avvalorando la tesi di una origine univoca, almeno per i ceppi europei che risultano simili tra loro. Se ciò fosse vero, il *clade* degli isolati cinesi potrebbe pertanto derivare da un comune e diverso *ancestor* genetico. In generale, la elevata diversità genetica dimostrata da APPV potrebbe essere la conseguenza non solo del tasso di evoluzione virale ma anche del processo di globalizzazione in zootecnia, che ne ha favorito la diffusione mediante la movimentazione di animali vivi e di carni suine.

CONCLUSIONI

APPV appare ben stabilito nella popolazione suina domestica di differenti paesi in Europa, Asia e Americhe, dove probabilmente risiede da qualche decennio, sebbene sia stato possibile rilevarlo solo negli ultimi anni. Una così ampia diffusione a livello globale sarebbe pertanto da attribuire alla commercializzazione su vasta scala di animali e prodotti animali, che è andata aumentando esponenzialmente nel tempo. La presenza del virus nella popolazione suinicola di tre continenti rende necessario quantificare l'impatto economico della infezione da APPV. Il virus è stato per il momento associato a forme di CT, una condizione che interferisce con l'alimentazione dei suinetti neonati e ne incrementa il tasso di mortalità sotto scrofa. La presenza di animali viremici e apparentemente sani pone inoltre importanti interrogativi sul reale ruolo patogenetico del virus che non è ancora sufficientemente chiaro. In particolare, l'associazione talvolta riscontrata con altri patogeni del suino[21,27,28,29], pone la questione circa la possibilità che APPV possa svolgere un effetto *door opener* o sinergico nel determinismo di alcune patologie, come già è stato ampiamente dimostrato per altri *Pestivirus*. A questo riguardo, ulteriori studi si renderanno pertanto necessari per approfondire, tra gli altri, temi quali origine, diffusione e ruolo patogenetico di questo virus emergente.

Identification and molecular characterization of *Atypical Porcine Pestivirus (APPV)* in central Italy

SUMMARY

A novel atypical porcine pestivirus (APPV), firstly discovered in USA in 2015 and now called Pestivirus K, was subsequently reported in Europe and Asia, both in pigs and in wild boars, sometimes associated with congenital tremor (CT) clinical signs in piglets. In this study, a virological survey was performed in central Italy to investigate the presence of APPV genome. One APPV strain was detected by Real Time RT-PCR and named APPV-LA/4911/2016. It comes from an adult and asintomatic pig, living in a breeding herd from Lazio region, without any anamnestic CT history. Sequences from NS5B and NS3 genomic regions were submitted to phylogenetic analysis. APPV-LA/4911/2016 clustered to-

gether with the corresponding NS5B and NS3 fragments of some German APPV strains and showed the highest similarity with APPV strains from Hungary. In addition, it is very genetically divergent from Chinese APPV strains. These data confirm the high APPV genetic diversity, not being able to cluster this virus according to the geographic area. Of course, APPV-LA/4911/2016 is similar to some Italian strains, already identified, for NS3 region. These results showed that APPV has been circulating in Italy at least since 2016 and it has been involved in a probable multiple introduction in our country. The animal and swine products trade movements and the global pig transport could have allowed the spread of APPV in different countries of three continents, as well as the introduction of APPV in Italy. Therefore, APPV presence in asintomatic pigs could be assignated mainly to its capability to induce a persistent infection, required for disease manteinance and spread. In the same time, APPV could be a door opener virus, like the most of Pestivirus, in case of co-infections with other swine pathogen agents. Further investigation should be performed to evaluate origin, epidemiological distribution, molecular evolution and pathogenetical role of this emerging swine virus.

KEY WORDS

Atypical porcine pestivirus; emerging virus; Italy; molecular characterization.

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BOEHRINGER

Comparison between synovial fluid cytology and joint capsule histopathology in horses with chronic osteochondritis dissecans

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SUMMARY

Introduction and objective - The osteochondrosis dissecans (OCD) is a disease that affects humans and animals and its aetiology and pathogenesis have been investigated for long time in human and veterinary medicine. OCD can cause slight changes in viscosity, mild increase in total protein and mild to severe increase in cell count, depending on the stage of the disease, in the synovial fluid of affected joint. Histological examination of the articular cartilage of horses with OCD showed areas of disorganization with reduction of glycosaminoglycans, including chondroitin sulphate, compared to normal horses. The purpose of the present work was to study the relationship between synovial fluid cytology and histopathologic examination of the articular capsule of horses with OCD.

Materials and methods - Fifteen horses of different breed, age and sex with OCD were included in the study and all of them underwent arthroscopy. Synovial fluid samples and osteochondral, or synovial capsule, samples were collected from 22 joints affected by OCD during arthroscopic surgery. For each synovial fluid sample was assessed the following parameters: synovial fluid turbidity, viscosity, mucin clot, total protein (TP), total nucleated cell count (TNCC) and differential cell count. Furthermore, osteochondral or synovial capsule samples were evaluated on histologic examination. The samples were retrospectively divided into two groups according to the results of the differential cell count of synovial fluid: a) group 1: hypocellular synovial fluid (few lymphocytes); b) group 2: cellular synovial fluid. Data were expressed as average and standard deviation. T-test was applied to verify differences between group 1vs 2 for TNNC and TP, while chi-square test was used for turbidity, viscosity and mucin clot test.

Results - Ten/22 joint samples were classified into the group 1 and 12/22 into the group 2. No significant differences were found between the 2 groups for turbidity, viscosity and mucin clot, while differences were obtained for TNCC and TP. Histological examination was normal in 16/22 joints and abnormal in 6/22.

Conclusions - The lower viscosity, abnormal mucin clot test and increasing in turbidity might be related to a dysregulation of pathways involving inflammation, and matrix damage, similar to those found in osteoarthritis. Comparing the two groups, TP were higher in the group 2 vs group 1, supporting the hypothesis of different grade of inflammation in the two group (low vs mild). Synovial TNCC results and the cytological assessment are suggestive of degenerative inflammatory changes in both groups. The prevalences of histological alterations support the hypothesis that the histological evaluation of the cartilages is not useful in the diagnosis of degenerative joint diseases, in line with previous studies. In conclusion, the assessment of synovial fluid cytology seems to be more sensitive than the histological exam in the diagnosis of degenerative joint diseases, as reported by others[4].

KEY WORDS

Synovial fluid; OCD; Horse; Cytology; Histology.

INTRODUCTION

Osteochondrosis (OC) is a multifocal pathology, which takes place in both articular-epiphyseal cartilage complex and growth plate in a variety of mammalian species[1;2;3]. The disorder is characterized by failure of endochondral ossification and is considered as one of the most common primary causes of degenerative joint disease in domestic animals[2-6]. OC has been believed to be caused by a combination of factors[6-7] such as traumatic lesions, ischemia and failure of cartilaginous canals and blood vessels, dyschondroplasia and alteration of the cartilaginous matrix, rapid growth, heredity and dietary imbalances[8].

One of the most recurrent manifestations of OC is the osteochondrosis dissecans (OCD)[2]. It is a non-septic degenerative disease caused by a failure of cellular differentiation in growing cartilage, leading to its dramatic thickening or retention, emergence of fissures and eventual focal loss of cartilage flaps into the joint cavity[9-10]. The detached fragments may be responsible of severe joint inflammation, which can lead to subsequent development of secondary osteoarthritis (OA)[2;11-12].

OCD clinical signs may develop when the joint surface is involved by the dissecting lesions[2]. Sometimes the OCD frag-

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ments and debris released into the joint lead to synovitis, joint inflammation and clinical signs of pain and lameness[2;13]. In some cases, no defect in the subchondral bone are present, no evident clinical signs of the disease can be pointed out[2;13].
OCD medical treatment depends on the severity of the disease. Some younger foals with larger cartilage flaps and little subchondral bone attachment may be amenable to reattachment to underlying subchondral bone via arthroscopically placed polydioxanone (PDS) pins[13]. In some cases, treatment with mesenchymal stem cells, platelet-rich plasma, or other biologics may be appropriate, depending on the type of OC lesion[13]. Arthroscopic removal of osteochondral chips or fragments is generally recommended after foals have reached a year of age, reducing predominant intra-articular fractures and accurately diagnosing ligamentous and meniscal lesions[2;14].
When alterations occur in the articular cartilage, meniscus, ligament or synovial membrane, some molecules may be released into the synovial fluid, whereas the biomarkers of bone tissue will generally be released into the blood, if the underlying bone of a joint is involved[13;15-16].
Analysis of cartilaginous explants bearing OCD lesions as well as serum and synovial biomarker measurements of animals suffering from OCD, revealed a significant increase in collagen turnover[14;16-17]. Synovial fluid biomarkers associated with OC are mainly products of collagen (CPII:C2C) and proteoglycan degradation products, mainly represented by Chondroitin Sulphate epitope 846 (CS846) and glycosaminoglycan (GAG)[2;13;16].
These biomarkers linearly correlate with severity grade of osteochondral lesions, indicate that this epitope is closely associated with OCD and suggest involvement of increased synthesis of cartilaginous aggrecan and procollagen type II during the pathophysiological development of this condition[6;18].
Histopathological results from articular cartilage samples reveal pathological cellular changes represented by increased cell volume, cell death, proteolysis of collagen and thus the reduction of its content in early OC[18]. Lecocq and colleagues (2008)[7] demonstrated that type II collagen synthesis is altered in OC cartilage and may be an initiating factor in weakening of cartilage matrix near cartilage canals and the osteochondral junction. Increased expression of matrix metalloproteinase (MMP-13 and MMP-3) may also results in biomechanical weakening of matrix in these locations[16;19-20].
The aim of the present study was to assess synovial fluid cytology and histopathological articular cartilage samples for the detection of OCD in horses.

MATERIALS AND METHODS

A cohort of 15 horses affected by OCD were included in this two-years study (2009-2010). Seven out of fifteen were female, five out of fifteen were male and three out of fifteen were geldings. The median age was 3 years (range 1-7 years). Eight/15 horses were Trotters, 4/15 Thoroughbreds and 3/15 were Warmbloods.
All the horses underwent orthopaedic visit and local anaesthesia in order to recognise the specific lesion location; after that the x-ray exam and the arthroscopy surgery were performed for each pathological joint, in order to remove OCD fragments. During surgical procedures, the synovial fluid and histopathological articular cartilage samples were collected from 22 pathological joint. The synovial fluid was collected in plain tubes without anticoagulant and in EDTA tubes and analysed within 1h after collection. Mucin clot test and total protein (TP) concentration were evaluated using samples collected in plain tubes[15;21-22].
TP were assessed using a rephractometer[21-22]. Turbidity, viscosity and Total Nucleated Cell Count (TNCC) were evaluated using synovial fluid samples collected in EDTA tubes[15]. Turbidity and viscosity were qualitative assessed by others[21-22]. TNCC was quantified after hyaluronidase treatment[23] to reduce viscosity using an automated cell counter (Hecovet C 01030360/ITA, and CAL-SEAC 71010810 multiparametric haematology calibrator, SEAC-RADIM Co, Italy). Cytological smears were prepared starting from EDTA synovial fluid samples. Smears were cytocentrifuged at 1500 gpm for 5 minutes (Cytofuge 2, StatSpin, USA) to improve smear quality[23], air-dried, coloured using a modified Romanowsky staining technique (Diff Quik®, DADA, Milan) and observed at 40X and 100X for differential cell count evaluation (expressed as percentage)[15;21-23].
Two groups were retrospectively and arbitrarily detected, based on differential cell count. Group 1: hypocellular synovial fluid (few lymphocytes); group 2: cellular synovial fluid (presence of inflammatory cells).
Histopathological articular cartilage samples were stored in 10% formalin solution and routinely processed. Histologic smears were stained with haematoxylin and eosin and observed at 10X and 20X.
Data relating TP, TNCC, and differential cells count were expressed as mean and standard deviation. Differences between group 1 vs 2 were assessed using a T-test for unpaired data for TP and TNCC. The Chi-Square test with Yates correction, was applied to compare group 1 vs 2 for turbidity, viscosity and mucin clot test. Significance level was set at $P < 0.05$. Statistical analysis was performed with a commercial software.

RESULTS

No lameness and/or no joint effusion were detected in 5/15 horses (33.3%), while 10/15 horses (66.7%) were lame. Twenty-two pathological joint were detected: 15/22 were tarso-crural joint (68.2%); 4/22 were hind limb fetlock joints (18.2%); 2/22 were front limb fetlock joints (9.1%); 1/22 was distal interphalangeal joint in front limb (4.5%). Only one pathological joint was detected in 9/15 horses (60%), 5/15 (33.3%) subjects showed two pathological joints and 1/15 (6.7%) had three pathological joints (Table 1).
No statistically differences were found between group 1 vs 2 for turbidity, viscosity and mucin clot test. Synovial fluid turbidity was found increased in 16/22 joint (72.7%) and normal in 6/22 (27.3%). Synovial fluid viscosity was found lower in 8/22 joints (36.4%) and normal in 14/22 (63.6%). Mucin clot test was resulted lower in 2/22 joints (9.1%) and normal in 20/22 (90.9%).
Ten out of 22 (45.5%) articular samples were included in group 1 and 12/22 (54.5%) in group 2. Data on synovial TP, TNCC (groups 1 and 2) and leukocyte cells (neutrophils, macrophages, lymphocytes) (group 2) were reported in Table 2. TNCC for both groups 1 and 2 and the percentage of inflammatory cells for group 2 were within reference in-

Table 1 - Lesions' distribution within the cohort of 15 horses included in the study.

Horses	Tarso-crural Joint	Fore Fetlock Joint	Hind Fetlock Joint	Fore Distal Interphalangeal Joint
1	Present	–		–
2	Present	–	–	–
3	Present		Present	–
4	Present	–	–	–
5	Present	–	Present	–
6	Present		–	Present
7	Present	–	–	–
8	Present	–	–	–
9	Present	Present	Present	–
10	Present	–	–	–
11	Present	–	–	–
12	Present	–	–	–
13	Present	–	Present	–
14	Present	–	–	–
15	Present	Present	–	–

Table 2 - Data on synovial total protein concentration (TP), total nucleated cell count (TNCC) and leukocyte cells (neutrophils, macrophages, lymphocytes) were reported.

	Group 1	Group 2
TP (gr/dL)	2.2±1.2*	2.2±0.9*
TNCC (cells/μL)	216±185*	433±115*
Neutrophils	0±0	9±11.9
Macrophages	0±0	25.4±11.1
Lymphocytes	1±0.1	65.7±14.2

Legend: * within the same row means ≠.

tervals[23] for all the joint analysed. Statistical differences were found between group 1 vs 2 for TP and TNCC. Figure 1C and D showed synovial fluid cytology of one horse owned to group 1 and 2, respectively.

Histopathological articular cartilage samples were found normal in 16/22 joints (72.7%) and abnormal in 6/22 (27.3%). Bone inflammation was present in 3/6 joints (50%); in 2/6 joints (33.3%) fibrous cartilage was detected; 1/6 joints (16.7%) showed fibrous cartilage degeneration and synovial membrane inflammation. All the horses with abnormal histopathological findings belonged to group 2 (Figure 1A, B).

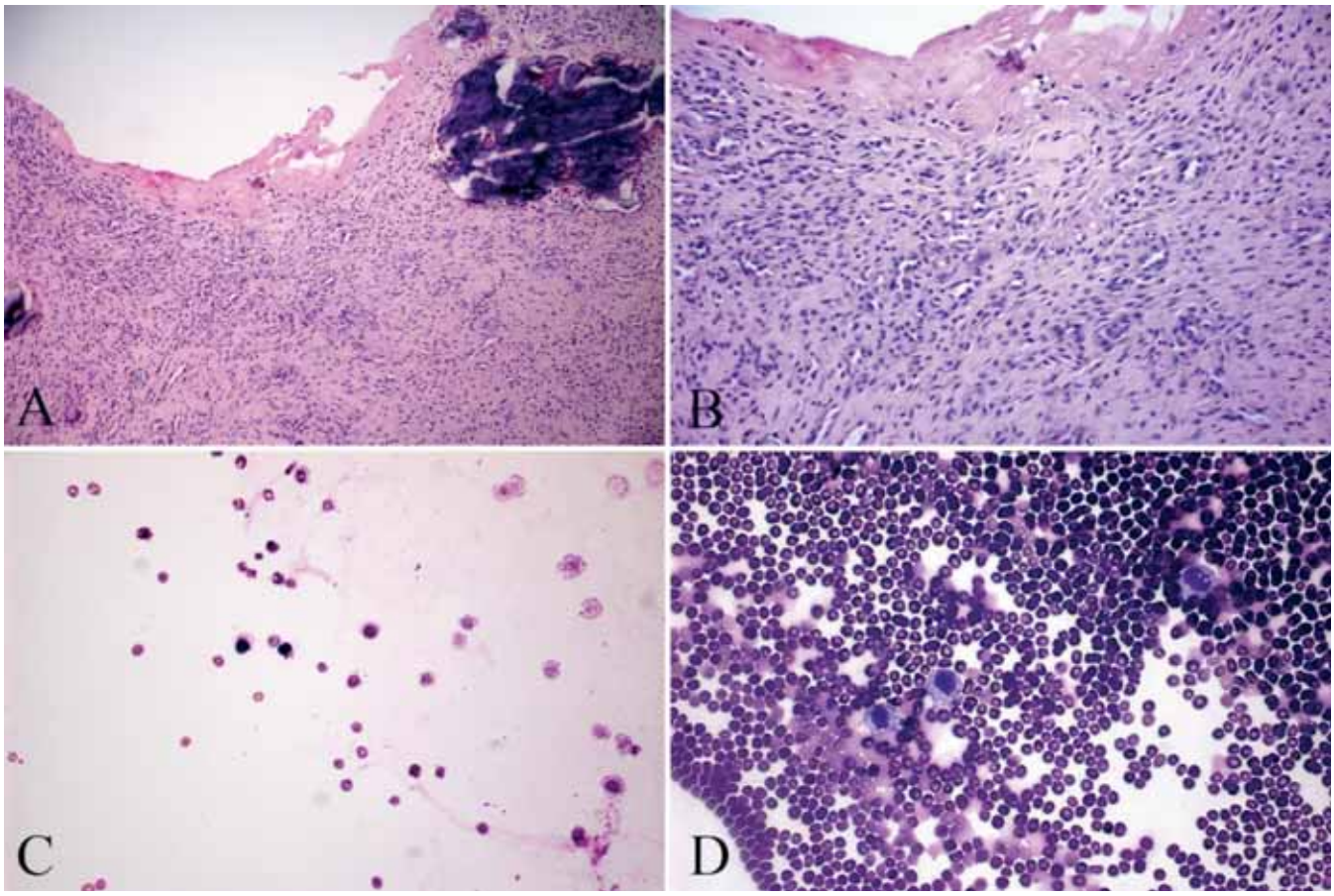

Figure 1 - **A)** The articular biopsy of one horse, included in group 2. Synovial surface necrosis and thickening of the articular capsule, due to stromal cells proliferation, with bony metaplasia areas (Hematoxylin-Eosin., Ob. 10X). **B)** The articular biopsy of one horse, included in group 2. It represents Figure 1A higher magnification. Histological exam shows stromal cells proliferation of the articular capsule, evident neoangiogenesis and chronic inflammation, represented by macrophages small lymphocytes and mature neutrophil (Hematoxylin-Eosin., Ob. 20X). **C)** The synovial fluid cytological exam of one horse, included in group 1. Slight background colouring due to high protein component. The smear shows red blood cells and few small lymphocytes (Diff Quik, Ob 40X). **D)** The synovial fluid cytological exam of one horse, included in group 2. Numerous red blood cells and macrophages, with many vacuoles within the cytoplasm, mature neutrophil and small size lymphocytes (Diff-Quik, Ob 40X).

DISCUSSION AND CONCLUSION

During pathological processes of joint diseases, the articular environment dramatically changes due to synovial membrane impairment[12-13;21]. In particular, synovial fluid GAG (i.e. hyaluronic acid and chondroitin sulphate) concentration increased, and molecular weight of hyaluronic acid and fluid viscosity decreased[14]. Diagnosis usually is performed based on clinical signs and diagnostic imaging.

The assessment of synovial fluid turbidity, viscosity and mucin clot test, the fluid cellularity (TNCC, differential cell count) and the TP concentration are important to differentiate septic or degenerative joint diseases.

One of the most frequent degenerative joint diseases in the horse is OCD. This pathology affects 20,000 to 25,000 foals every year in the northwestern Europe; therefore, it is one of the most important of the so-called 'developmental orthopaedic diseases' in horses[2].

The aim of the present study was to assess synovial fluid cytology and histopathological articular cartilage samples for the detection of OCD in horses.

All the horses affected by OCD and included in this study showed TNCC and differential cell count values, and TP concentration compatible with the diagnosis of a degenerative joint disease[22], such the OCD.

No differences in viscosity and turbidity and mucin clot test have been found between horses with hypocellular or cellular synovial fluid. In particular, viscosity and mucin clot test were decreased, and turbidity was increased in both groups with similar prevalences' distribution. These results are in agreement with literature[21-22]. The alteration of the quality of synovial fluid usually is due to the presence of fibrin and/or cartilage fragments within articular space, but the inflammation is low-mild in degenerative joint diseases than to septic ones[21]. The lower viscosity, abnormal mucin clot test and increasing in turbidity might be related to a dysregulation of pathways involving inflammation, and matrix damage, similar to those found in osteoarthritis[13].

Total protein concentrations did not exceed 3 gr/dL concentration[15;21-22]. This finding is consistent with other studies[22] and it agrees with normal value found during degenerative joint disease. Comparing the two groups, TP were higher in the group 2 vs group 1, supporting the hypothesis of different grade of inflammation in the two group (low vs mild).

Synovial TNCC was found to be less than 600 cells/μL in both groups and the cytological exam detects no significant neutrophilic percentage. According to the literature, these results are suggestive of degenerative inflammatory changes[15;22].

Finally, the histological exam did not show pathological alterations in 72.7% examined joint, while it revealed pathological lesions in 27.3%. These prevalences are in line with previous studies[2;6] assessing the histological alterations of cartilage in degenerative joint diseases. Our results seem to support the hypothesis that the histological evaluation of the cartilages is not useful in the diagnosis of degenerative joint diseases. Probably this is due to difficulties in sampling a truly representative slice of the whole articular cartilage surface[8].

In conclusion, the assessment of synovial fluid cytology seems to be more sensitive than the histological exam in the diagnosis of degenerative joint diseases, as reported by others[21].

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LA SALUTE ANIMALE PER LA SALUTE DELL'UOMO

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Survey on dead on arrival of broiler chickens under commercial transport conditions

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SUMMARY

Transportation is a major component of the global commercial poultry production system and it can lead to various levels of stress in chickens, even under optimal conditions. It can cause harm ranging from slight disorders to death. Birds that die between loading at the farm and slaughtering are described as 'dead on arrival' (DOA). DOA is an important indicator of animal welfare and financial losses. It can be influenced by various factors such as ambient temperature, stocking density in crates, transport distance, lairage time in the holding barn and slaughter age. The aim of this survey was to determine the effects of some factors on the incidence of the phenomenon 'dead on arrival' in broiler chickens under commercial transport conditions. This survey was carried out in a commercial slaughterhouse on the basis of data for 4,062 transfers and 12,723,444 broilers under commercial conditions during 2018. The data related to slaughter age, transport distance, lairage duration, ambient temperature at the slaughterhouse and the incidence of DOA of broiler chickens was recorded by staff throughout the study. Slaughter age was divided into four groups (up to 39 days, 40 to 42 days, 43 to 44 days, 45 days or more); lairage duration was divided into five groups (up to 60 min, 61 to 120 min, 121 to 180 min, 181 to 240 min, 241 min or more); transport distance was divided into four groups (up to 15 km, 16 to 50 km, 51 to 101 km, 100 to 200 km) and ambient temperature at the slaughterhouse was divided into six groups (–5°C to 0°C, 0.1°C to 5°C, 5.1°C to 10°C, 10.1°C to 15°C, 15.1°C to 20°C and 20.1°C to 28°C).

Across the present study, the overall DOA rate was 0.389%. The effects of slaughter age, transport distance, lairage duration and temperature on the DOA rate were all significant ($P<0.001$). The DOA rate at ≤ 39 days slaughter age was higher than that of the other groups ($P<0.001$). Furthermore, the DOA rate for transport distance up to 15 km (0.448%) was higher than that for the other distance intervals ($P<0.001$). As the transport distance increased, the DOA rate usually increased ($P<0.001$). In addition, there was a positive relationship between DOA rate and lairage duration ($P<0.001$). As the lairage duration increased, the DOA rate increased. In addition, the DOA rate was highest (0.622%) at cold ambient temperatures (–5°C - 0°C) and lowest at 5.1°C to 10°C (0.334%).

In conclusion, the results of the current study regarding DOA rates clearly showed that short or long distance transport and long lairage duration were extremely detrimental to the health of broiler chickens. It is therefore important to avoid long or short distance transportation and long lairage duration, especially in adverse environmental conditions such as sub-zero and high temperatures. In addition, broiler chickens of up to 40 days of age were more susceptible to pre-slaughter stress than other ages. Therefore, more attention should be paid to the management of broilers in this age range in the pre slaughter period.

KEY WORDS

Animal welfare, dead on arrival, broiler chicken, transport, ambient temperature.

INTRODUCTION

Transportation is a major element of the global commercial poultry production system, and can induce stress in chickens, even under optimal conditions, with harm ranging from slight stress to death[1]. The death of broiler chickens may happen after loading at the farm, during transportation to the slaughterhouse or during lairage in a holding barn[2]. The death of birds between the catching stage at the farm and unloading from their crates at the slaughterhouse is described as 'dead on arrival' (DOA), which is an important indicator of both animal health and welfare[3]. Chickens in the DOA category also represent a financial loss to the producer because they are unacceptable for human consumption and are therefore condemned[4]. The DOA rate may be affected by various factors such as ambient temperature[3,5], stocking density in crates[4], transport distance[6], lairage duration in holding barn[7] and slaughter age[2]. Extreme ambient temperatures and humidity are major reasons for mortality; 40% of DOA was associated with heat or cold stress[1,8]. The effects of ambient temperature and relative humidity may vary according to season, stocking density in crates, design of vehicle and conditions in the holding barn[9]. The DOA rate has been reported as 0.15% Mitchell[10], by Petracci et al. as 0.35%[11], by Nijdam et al. as 0.46%[3] and by Ritz et al. as 0.68%[8].

Earlier studies have investigated the effects of one or two factors on DOA in broiler chickens. In addition, these studies were carried out with small numbers of individuals in the groups. In contrast, the current study, which was carried out on an extremely large number of animals, investigated

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the effects of four factors on DOA rate simultaneously. In specific terms, the aim was to determine the effects of some factors on DOA in broiler chickens under commercial transport conditions.

MATERIALS AND METHODS

The survey was carried out in a commercial slaughterhouse and covered 4,062 transfers and 12,723,444 broilers under commercial conditions during 2018. The data related to the crating, transport and lairage at the slaughterhouse and the DOA rate of broiler chickens were recorded by staff during the course of the study. Feeding at the rearing farms was withdrawn 8 hours before the loading of all flocks. The Ross 308 broiler chickens were manually caught and loaded into plastic crates on the trailer by three staff members. The dimensions of the crates were length (80 cm) × width (45 cm) × height (30 cm). Stocking densities in the crates for all transfers were within the range recommended by the FAWC[12]. The type of the trailers was similar. The top, front and rear of the trailers were closed but the sides were open throughout the year. The trailers were transported by road to the slaughterhouse after loading. The broiler chickens were transported from different locations to the slaughterhouse (latitude 41°03 N, longitude 36°05 E and 514 m above sea level) in the city of Samsun, Turkey. The slaughtering period started at 11:30 p.m. and finished at 8:00 a.m., according to the workload. The vehicles were weighed on arrival at the slaughterhouse. The total weight of the broiler chickens was divided by their total number to obtain the mean live weight of a broiler chicken. When the vehicles loaded with broilers arrived at the slaughterhouse, they were parked in a holding barn for lairage. The lairage duration was different for each truck. The holding barn capacity was 6 trucks and the fans worked at maximum capacity during the summer period. A data logger (Testo 174H) was placed in the holding barn and the data were collected at 1 h intervals for one year. The vehicle was driven to the unloading area of slaughterhouse for ante-mortem inspection after the lairage period. After that, the crates were manually unloaded from the trailer and the broiler chickens were placed on a shackle line. At this stage, the number of dead broiler chickens was recorded as the mortality rate per transfer.

In order to evaluate the independent effects of the four factors on the mortality of the broiler chickens, the slaughter age was divided into four groups (up to 39 days, 40 to 42 days, 43 to 44 days, 45 days or more); lairage duration was divided into five groups (up to 60 min, 61 to 120 min, 121 to 180 min, 181 to 240 min, 241 min or more); transport distance was divided into four groups (up to 15 km, 16 to 50 km, 51 to 101 km, 100 to 200 km) and the ambient night temperature was divided into six groups (–5°C to 0°C, 0.1°C to 5°C, 5.1°C to 10°C, 10.1°C to 15°C, 15.1°C to 20°C and 20.1°C to 28°C). The numbers of transfers, the numbers of transported broiler chickens and the numbers of dead broiler chickens were recorded and the mortality percentages were calculated for these intervals.

Statistical analyses

The data were analysed with the Proc GENMOD procedure of SAS[13]. The model included the fixed effects of slaughter age, lairage duration, transport distance and ambient temperature. The Tukey's multiple comparison test procedure was used to assess differences between means.

Table 1 - Means (± SEM) for some pre slaughter conditions.

Characteristics	Mean	± SEM
Slaughter age (d)	41.79	0.058
Stocking density (m^2/bird)	0.046	0.001
FAWC value (m^2/bird)	0.037	0.001
Transport distance (km)	55.76	0.771
Lairage duration (min)	143.21	1.146
Ambient temperature (°C)	10.74	0.093
Ambient humidity (%)	87.09	0.203
Pre-slaughter live weight (kg/bird)	2.346	0.005
DOA rate (%)	0.389	0.010

RESULTS

The data for some pre slaughter parameters or the broiler chickens in the current study in Table 1. The overall DOA rate in the present study was 0.389%. The effects of slaughter age, transport distance, lairage duration and temperature on the DOA rate were significant ($P<0.001$). The mean DOA rate by slaughter age is given in Fig. 1. The DOA rate at ≤ 39 days slaughter age was significantly higher than that of the other slaughter age groups ($P<0.001$). The mean DOA rates for the transport distance intervals are presented in Fig. 2. The DOA rate for transport distance up to 15 km (0.448%) was significantly higher than that for other transport distance intervals ($P<0.001$). As the transport distance increased, the DOA rate generally increased. The mean DOA rates for lairage duration are given in Fig. 3. There was a positive relationship between DOA rate and lairage duration. As the lairage duration increased, the mean DOA rate increased significantly ($P<0.001$). The mean DOA rate by temperature is presented in Fig. 4. The DOA rate was highest (0.622%) at cold ambient temperatures (–5°C - 0°C) and lowest (0.334%) in the temperature range from 5.1°C to 10°C in present study.

DISCUSSION

DOA rate

The DOA rate is an important indicator of both animal welfare and financial losses. Therefore, the DOA level is a major factor in the economics of the broiler industry. The DOA rate was reported as 0.35% in Italy[11], 0.37% in the Czech Republic[14], 0.41% in Turkey[15] and 0.46% in the Netherlands[3]. A similar result (0.389%) was also found in the current study. These rates were below the maximum level recommended by the EU which is less than 0.5%[16]. On the other hand, the DOA rate was reported as 0.68% in the United States[8]. The differences in the DOA rate between studies may be due to differences in ambient temperature and humidity, feed withdrawal duration, stocking density, airflow characteristics during transport and / or lairage duration.

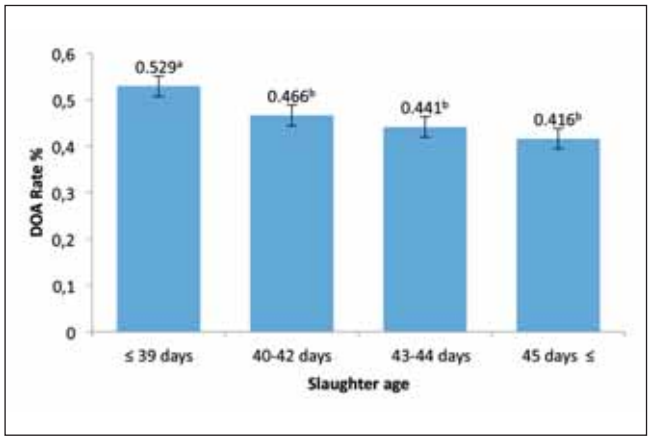

Figure 1 - Mean DOA rate by slaughter age.
Means in the same column with different superscripts are significantly different (P<0.001).

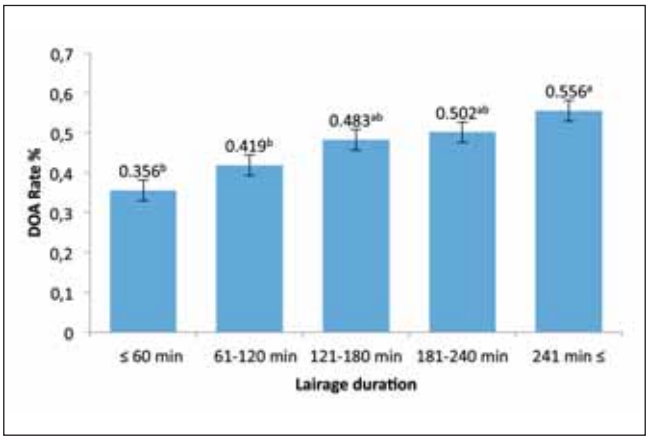

Figure 3 - Mean DOA rate by lairage duration.
Means in the same column with different superscripts are significantly different (P<0.001).

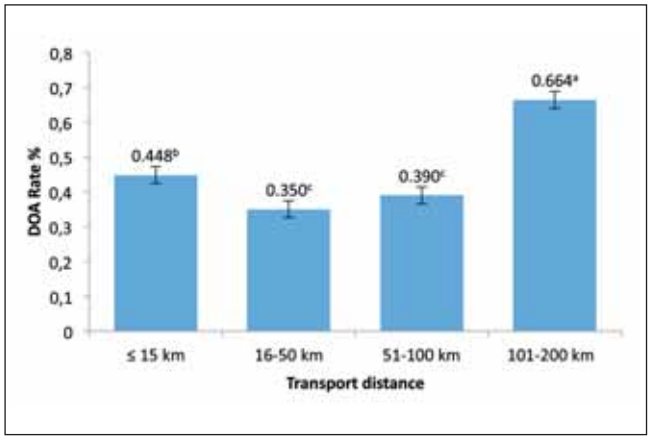

Figure 2 - Mean DOA rate by transport distance.
Means in the same column with different superscripts are significantly different (P<0.001).

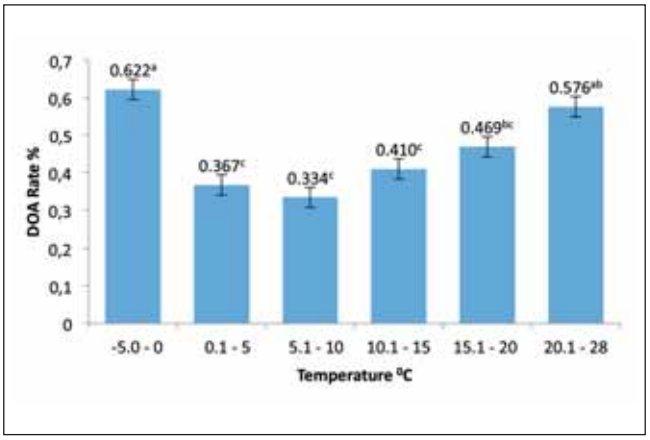

Figure 4 - Mean DOA rate by temperature category.
Means in the same column with different superscripts are significantly different (P<0.001).

Slaughter age

Caffrey et al.[2] reported that the mortality rate in a study in Canada was lower at older ages (more than 40-45 days) than for younger ages (33-37 days of age) and the difference was significant. They also stated that the mortality rate was higher for the 33-37 days slaughter age period than for the 38-39 days period. Chauvin et al.[17] stated that the mortality rate in a study in France was higher at ≤ 40 days of age than at older ages (41-47 days of age) and the difference was significant. In the present study, the DOA percentage at ≤ 39 days slaughter age was higher than for the other slaughter age groups and the difference was significant (P<0.001). The results obtained in the current study were similar to those reported for the two earlier studies. Older birds have higher body weight and greater feather coverage of the body. This issue appears to be related (at least in part) to surface to volume ratio; the smaller an animal is, the higher is its surface to volume ratio and hence its propensity to lose body heat. The body form of older birds better insulates them against cold weather conditions. That means they can better maintain their body temperature in cold weather conditions and therefore have higher a survival rate than younger birds[18]. In addition, the significant effect of age of broiler chickens on DOA was also accordance with previous studies[3,17,19,20,21,22].

Transport distance

Vecerek et al.[6] stated that the DOA rate in a study in Czech Republic increased from 0.247% to 0.862% as the transport distance increased from 150 km to 300 km. Warriss et al.[25] analysed the relationship between mortality in broiler chickens and distance of transport to slaughterhouse in a study in the UK. They reported that the DOA rate was 1.81 times higher when broiler chickens were transported for more than 4 h than less than 4 h (0.283% vs 0.156%). Aral et al.[15] found in a study in Turkey that the mortality rate rose in broiler chickens from 0.29% to 0.46% when the transport duration increased from less than 2 h to longer than 10 h. Separately, Vecerek et al.[14] found that the mean DOA percentage was 2 times higher when broilers were transported less than 100 km when compared with more than 300 km. Cockram[26] stated that in a study in Canada the quality of transport was more important than transport duration for the welfare of broiler chickens. In the present study, the DOA rate increased as the transport distance increased, except for a distance up to 15 km. Overall, there was a positive and significant relationship between DOA rate and transport distance (P<0.001). This result is in accordance with the results of previous studies[6,14,15,30]. If transport takes a long time, broiler chickens deplete the glycogen store in their muscles and this

situation results in fatigue. Thus, broiler chickens may die due to sudden death syndrome, congestive heart failure or generalized circulatory collapse[25]. Therefore, Nijdam et al.[3] suggested that slaughterhouses should be built within 2 h travel distance from farms in order to decrease the DOA rate. However, it seems more logical to build farms near the slaughterhouse because the slaughterhouse would normally be more capital intense.

Vecerek et al.[14] reported that high mortality rate in broiler chickens is not only related to long distance transport but also to short distance transport. They found that the DOA rate for transport distance up to 50 km was higher than that for broiler chickens transported for distances from 51 to 100 km. In the present study, the DOA rate for transport distance up to 15 km (0.448%) was higher than that for 16-50 km (0.350%) or 51-100 km (0.390%). The result produced by this study was accordance with the result reported by Vecerek et al.[14]. Longer distance transport provides relatively adequate time for the recovery of broiler chickens from loading. Therefore, broiler chickens need at least one hour transport duration[28,29].

Lairage duration

Lairage duration can be defined as the period between the arrival of the broiler chickens at the holding area in the slaughterhouse and their slaughter. An appropriate lairage period after transport decreases thermal stress and contributes to broiler welfare. Lairage also assists animals to adjust to their new environment. Lairage reduces the effects of physiological stress before slaughter and thus improves meat quality[27]. Most researchers have recommended a lairage duration of less than 2 h. Warriss et al.[7] proposed a maximum lairage duration of 1 h in a holding barn, but if the conditions in the holding area were good, this time can be extended to 2 h. Hunter et al.[28] found that the optimal lairage duration period was up to 2 h, if sufficient ventilation and appropriate thermal conditions were supplied. Bayliss and Hinton[29] investigated the effect of lairage duration on the DOA rate. They concluded that the DOA percentage significantly increased if the lairage duration was 4 h. Vieira et al.[30] studied three different lairage durations (between 1 h and 2 h, 2 and 3 h, and more than 3 h) and reported that the mean DOA rate was 0.33% and it decreased by about 0.1% as the lairage duration increased from below 1 hour to 3 hours. They concluded that optimal lairage duration was between 1 and 2 hours when the ambient temperature was below 21°C. A different study reported that liver glycogen was depleted to maintain body temperature when the ambient temperature was high or low, such as under summer or winter conditions. If this situation persists, the animal may die. Therefore, the recommended lairage duration was between 1 h and 2 h[7]. Chauvin et al.[17] studied 403 broiler chicken flocks and reported that the DOA rate increased significantly in broiler chickens lairaged for 260 min or more before slaughter. In the present study, the DOA rate increased as lairage duration increased, especially after 2 h, which is in accordance with the results of previous studies[7,28,29,30,31].

Temperature

Thermal stress is a main reason for mortality in broiler chickens and it causes economic losses in most countries. Ritz et al.[8] evaluated broiler DOA in the UK by necropsy and concluded that 40% of DOA was associated with heat or cold stress. Warriss et al.[5] investigated the relationship between temperature and DOA rate due to transport from farms to a commercial slaughterhouse. They found average mortality rates of 0.10%, 0.13%, 0.26% and 0.66% for temperature ranges of 14-17°C, 17-19.9°C, 20-22.9°C and 23-27°C, respectively. The DOA rate was very high for the 23°C to 27°C interval in their study. Petracci et al.[11] investigated the relationship between DOA rate and season. They found DOA rates of 0.47%, 0.28%, 0.35% and 0.32% for summer, autumn, winter and spring, respectively. The DOA rate was highest in summer. In another study, DOA records were investigated by Vecerek et al.[12] who studied the relationship between DOA percentage and ambient temperature. They found that the DOA rate was highest (about 0.80%) at ambient temperatures between –6°C and –3.1°C. This temperature interval was the lowest in their study. Vosmerova et al.[16] noted that broiler chickens transported in cold weather were very stressed and further stated that their plasma corticosterone levels when transported at lower ambient temperatures (–5°C to 5°C) were higher than in the higher temperature intervals. Vecerek et al.[6] and Caffrey et al.[2] analyzed the relationship between mortality rate and season in the Czech Republic and Canada, respectively. They found that the DOA percentage was higher in both summer and winter months than that in spring and autumn. Nijdam et al.[3] reported a significant association between ambient temperature and DOA rate. They stated that if the ambient temperature was relatively high (>15°C) or low (<5°C), the DOA percentage increased. In the present study, the DOA rate was highest (0.622%) at low ambient temperatures (–5°C - 0°C) and also very high (0.576%) at high ambient temperatures (20.1°C - 28°C). On the other hand, the DOA rate was lowest when the temperature ranged from 5.1°C to 10°C (0.334%). These results are in agreement with those reported by previous studies[2,3,5,6,12]. Increased DOA rates in summer and winter are most likely related to the more extreme ambient temperatures in those seasons, when high or low temperatures adversely affect the welfare of transported broiler chickens. If the weather conditions are very hot, cold or humid, broiler chickens may not be able to maintain thermoregulation and may die from hyperthermia or hypothermia[5,27].

CONCLUSIONS

In conclusion, the results for the DOA rates in the present study clearly showed that very short and long distance transport and long lairage duration were harmful to broiler chickens. Birds did not have enough time to recover after very short distance transport. Long distance transport and long lairage time, or both, could have led to decreased blood glucose levels, overfatigue and even death in broiler chickens. It is especially important to avoid long distance transport and long lairage duration under adverse environmental conditions such as low and high temperatures as broiler chickens struggle to maintain their body temperature in the optimum range and are therefore more vulnerable to premature death. In addition, broiler chickens of up to 40 days of age were more susceptible to pre-slaughter stress than other ages. This may again reflect a lesser ability of younger broilers to maintain their body temperature in the optimum range when un-

der stress. More attention should be paid to this age range in the pre slaughter period because younger broiler chickens are more negatively affected by cold ambient conditions. Therefore, in addition to these measures, stocking density in crates of broiler chickens of up to 40 days of age should be increased in cold weather conditions to reduce the possibility of hypothermia.

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Characteristics of by-product and animal waste: a review

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SUMMARY

Animal waste is one of the waste products from the livestock industry's production process. The waste can be either solid, liquid or gas. Solid waste is all solid waste such as livestock, animal carcasses or the remainder of the slaughter process at slaughterhouses. Liquid waste is waste in the form of liquid that comes from the urine of animal as well as remnants from the washing process of cages and animal itself. Gas waste is all the results of a gas phase discharge. The animal waste has a great influence on humans and the environment, but also has very important benefits. Animal waste can used as a source of nutrition for plants, energy sources in the form of methane gas, alternative animal feed sources and growing media for earthworms. The Animal waste that not processed optimally can be cause environmental pollution.

KEY WORDS

By-product, animal waste, livestock industry.

INTRODUCTION

Livestock is one of the creatures intended to meet human food needs. Therefore, we must make maximum use of it to meet human needs. Human needs for fulfillment of food, especially animal food, are constantly increasing along with the increasing human population. As an illustration, from only one meat-producing livestock, only around 30-35% of the meat can be obtained as the main product, while the remaining 65-70% is the by-product which until now has not been utilized optimally. Livestock by-product and waste have the connotation meaning as "harmful waste material". However, if you get a touch of technology, it will have a very beneficial impact on human benefit and the environment[1,2,3]. The terms by product and waste are different in terms of how to obtain them. Livestock waste is the residual product in the form of biomass that has been obtained since the on-farm process, such as: faeces and urine, while the follow-up results are obtained from the rest of the slaughtering process such as skin, bones, blood, innards, fur, horn and the organs that make up the digestive tract.

MANAGEMENT AND UTILIZATION OF LIVESTOCK WASTE

Animal manure and urine are waste groups. Waste in its form always has a negative connotation. Waste has a bad influence on humans. Wastes are containing toxic substances or pollutants exceeding the level of tolerance limits adversely affect humans. This can also occur in the survival of various other biodiversity. The waste treatment process is one source of livelihood for the poor in developing countries. Contamination of waste has a very significant impact on the health of workers[4]. Lately, livestock businesses in developing countries such as in Indonesia have been quite advanced and growing rapidly compared to the last three decades. This is as a result of the entry of superior seeds from abroad that have been intensively developed. One result of the development of the livestock business is the accumulation of animal waste in the form of feces and urine.

Stool produced by ruminants (cows, buffaloes, goats and sheep) brings problems in global warming, especially the production of methane gas. At present, feces and urine as well as other organic material remnants have been utilized by the world community as the main raw material for producing methane gas. It is formed as a result of the aerobic metabolism of microorganisms in the hulls of animal. This model was later developed by people in the world to design biogas reactor systems[5].

The use of biogas is now growing rapidly as one of the solutions to obtain alternative energy sources. The energy crisis in the 1970s caused economic problems in several countries in the world. Many countries that are classified as poor are still dependent on imported oil and natural gas products. Biogas can be used on a household scale for cooking, heating and lighting. Biogas can be produced from raw materials for lignocellulose biomass such as feces and agricultural waste[6]. In addition, it can also be used by larger industries as heaters or as the main energy source. The animal feces used as raw materials for biogas installations. However, it can also using feces from human, municipal wastes, plant and vegetables wastes. These ingredients are rich in nutrients and suitable for the growth of several an-aerobic bacteria. The composition of biogas are depends on the composition of the raw material, the load of organic matter that included in the digester, time and temperature of an-aerobic and then decom-

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position process[7]. The production of energy sourced from biogas in several countries in the world in full was presented in Table 1.

Feces from the animal has been developed as a base for producing biogas by adding several types of organic waste, lime fertilizer and ethanol industrial waste known as vinasse. The use of additional materials in the biogas production process is an effort to improve and utilize the potential of waste. At present, approximately 22.4 gigiter of vinasse material is produced worldwide. This amount has the potential to produce 407.68 gigabytes of biogas[8].

Biogas is the result of anaerobic digestion from organic waste which can be used as an alternative energy source. One type of organic waste used is faeces from livestock. Increasing production of organic waste will have an impact on increasing the potential for energy production. The use of biogas as a renewable energy source has been developed on a very large scale in South Africa and Germany[9].

The use of organic materials to produce biogas using renewable hydrogen aid has been developed by several previous researchers. This process involves the activity of CO_2 as a carbon source[10,11].

Besides as biogas, animal feces has also used as a alternative natural energy source in the form of bio-charcoal briquettes. In China, the biomass combustion process is a concern because of its enormous effect on the environment, especially

Table 1 - Comparison of energy production from biogas in several countries in the world in 2015[7].

	Electricity capacity	Average capacity	Electricity production	Heat production	Derived heat
EU	MW	kW	GWh	TJ	TJ
Belgium	183	897	955	4272	388
Bulgaria	20	1818	120	182	24
Czech Republic	368	664	2611	6491	623
Denmark	102	671	485	3265	2099
Germany	4803	443	33,073	69,047	9285
Estonia	11	611	50	286	112
Ireland	53	1828	202	370	0
Greece	49	1750	230	661	0
Spain	224	1612	982	2474	0
France	320	446	1783	6859	1432
Croatia	28	1217	177	219	219
Italy	1336	859	8212	10,469	8604
Cyprus	10	769	51	214	51
Latvia	60	1017	391	1256	892
Lithuania	21	583	86	403	91
Luxembourg	12	400	62	390	80
Hungary	69	972	293	667	131
Malta	3	1500	7	30	6
Netherlands	239	892	1036	0	48
Austria	194	437	624	2036	145
Poland	216	780	906	3703	436
Portugal	66	1031	294	336	0
Romania	14	1273	61	303	156
Slovenia	32	1231	132	383	304
Slovakia	91	650	541	2122	473
Finland	0	0	358	1600	763
Sweden	95	337	62	2150	274
UK	1488	2845	7189	6641	0
Switzerland	74	116	303	1342	1199
Iceland	0	0	0	37	0
Norway	17	138	7	834	118
FYROM	4	1333	20	37	0
Serbia	5	714	23	45	0
Moldova	3	750	15	159	11
Ukraine	18	1125	10	282	360
EU	10,107	609	60,973	126,829	26,636
Europe	10,228	588	61,351	129,565	28,324

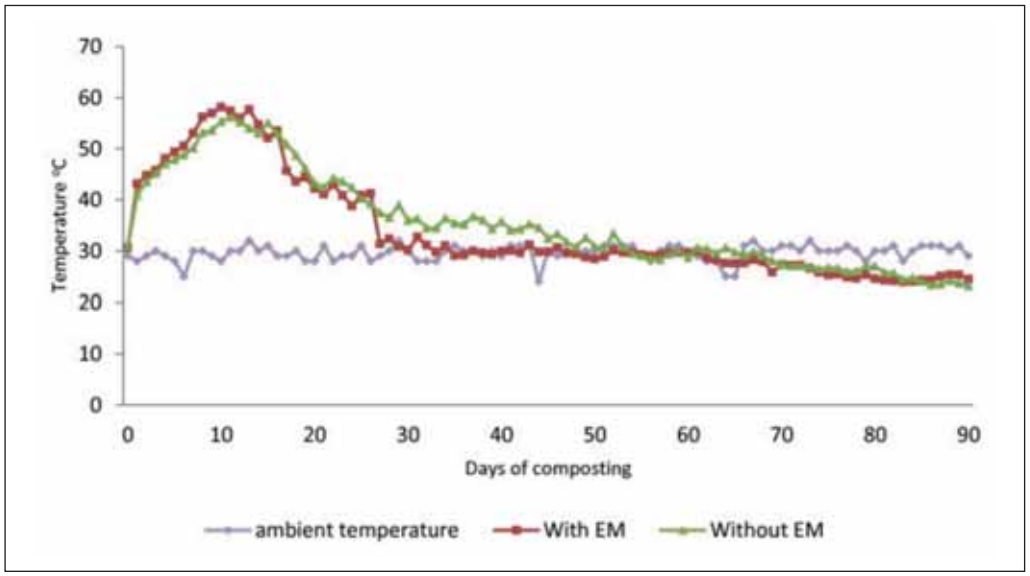

Figure 1
Changes in compost temperature during the composting process[17].

related to air emissions[12,13,14,15,16]. The process of forming bio-charcoal briquettes was done through the process of burning dry biomass without using air (pyrolysis). Some of the advantages of using bio-charcoal briquettes are: 1) the heating system produced is natural or traditional heating (without using oil), 2) bio-charcoal briquettes are safe for human health because they do not contain smoke, soot and odor, 3) production costs relatively cheaper than kerosene or wood charcoal, 4) fuel power is much longer with high enough heat energy, 5) its use is relatively safer because it does not contain emissions of sulfur and nitrogen free into the air, 6) briquette has produce a distinctive aroma, 7) reducing environmental pollution.

The other uses of animal feces are raw material in making organic fertilizers. The application of organic fertilizer to the soil has carried out by humans. This is done to improve soil fertility and productivity on agricultural and plantation land. The use of effective microorganism (EM) in the compost production process has been widely applied. The use of goat feces as raw material for fermented compost using EM has been developed by researchers previously. The use of EM affects the temperature of fermentation. This can be seen in Figure 1.

Based on Figure 1, it can be seen that the temperature of the compost gradually increases until the tenth day and then decreases. In fermentation for 30-35 days the temperature of the compost with EM decreases and stabilizes near the ambient temperature. Compost without EM down at 48-52 days of fermentation[17].

In Indonesia, several types of fertilizer production have been developed. For example manure, green manure and compost. At present, feces was produced by cattle, buffalo, horses, goats, sheep, ducks, chickens, etc. The feces have been widely used as raw material in the process of making organic fertilizers. The business of producing organic fertilizer is currently growing rapidly along with the development of organic farming systems. The use of feces as fertilizer for plants is done by giving directly to plants after passing the drying period. In addition, it can also be processed into a compost product. Composting is a process and effort to decompose and stabilize biologically organic substrates under conditions that allow for the development of thermophilic bacteria. The end result is heat production and stability, free of pathogenic bacteria and plant residues, and can be used as natural fertilizer.

The process of composting using a mixture of water hyacinth (Eichhornia crassipes), livestock feces and sawdust has been developed by researchers. This is done to reduce the use of chemical fertilizers. In addition to reducing the population of water hyacinth that is developing rapidly. Livestock feces are used as carbon sources. Nutrient content (nitrogen, phosphorus, Na, K, and Ca) increases significantly in the final process of composting. The content of pathogenic bacteria, especially coliform, is significantly reduced. This is an indicator of the presence of pathogenic bacteria in compost[18]. Data related to changes in physicochemical properties during the composting process in several locations were presented in Table 2.

In the past few years, the type of animal waste in the form of urine has been developed and used as a raw material in the manufacture of liquid organic fertilizer (LOF). This is because the material contains elements that are needed by plants in full[19].

An adult cow of a particular breed can produce as much as 8 liters of urine perday. For the livestock industry, urine is a very potential commodity to produce high economic value. The urine from the cow has contains chemical elements that needed by plants, such as N, P, K, Ca, and Mg, which are bound in the form of organic compounds, including: urea, ammonia, creatinine and keratin, uric acid, amino acids, alantoin, chloride, sulfate, phosphate, vitamins, hormones, and enzymes. Early research related to the potential urine of rabbits as a LOF base material has been developed by the author using fecal extract and banana buds as natural decomposers.

MANAGEMENT AND USE OF BY-PRODUCT OF ANIMAL

Animal by-product has a positive correlation with the rate of slaughter of animal. The types of by-products produced from slaughterhouses business activities include: skin, blood, bones, fat and offals. The potential by-product will have economic value and impact on humans if it is processed by involving technological processes.

Table 2 - Changes in physico-chemical parameters during the composting process (Mean ± SD, n = 3)[18].

Days	Nutrients concentration							
	BRS Total Nitrogen (%)	AS	BLS	IS	BRS NH4-N (mg/kg)	AS	BLS	IS
0	0.85 ± 0.13	0.83 ± 0.07	1.05 ± 0.07	0.64 ± 0.02	253.49 ± 0.26	42.26 ± 0.53	223.31 ± 4.38	70.67 ± 0.17
3	1.05 ± 0.21	0.74 ± 0.02	1.05 ± 0.07	0.64 ± 0.03	377.9 ± 1.23	43.33 ± 0.32	206.04 ± 1.88	74.21 ± 0.03
6	1.05 ± 0.07	0.84 ± 0.14	1.05 ± 0.21	0.68 ± 0.02	156.36 ± 0.09	42.46 ± 0.0	193.03 ± 4.52	78.78 ± 0.06
9	0.98 ± 0.14	0.81 ± 0.03	1.19 ± 0.07	0.68 ± 0.02	167.69 ± 8.88	35.61 ± 0.0	182.79 ± 2.59	60.5 ± 0.13
12	1.12 ± 0.14	0.84 ± 0.0	1.26 ± 0.14	0.77 ± 0.07	105.13 ± 6.51	32.95 ± 0.50	94.08 ± 2.59	54.24 ± 0.08
15	1.19 ± 0.07	0.98 ± 0.14	1.47 ± 0.07	0.91 ± 0.07	93.32 ± 0.06	32.48 ± 0.09	92.20 ± 1.43	51.61 ± 0.34
18	1.33 ± 0.07	0.98 ± 0.0	1.33 ± 0.21	0.98 ± 0.0	91.86 ± 0.04	26.76 ± 0.57	99.18 ± 0.54	50.85 ± 0.03
21	1.26 ± 0.14	0.91 ± 0.07	1.19 ± 0.07	0.98 ± 0.0	85.3 ± 0.04	26.79 ± 4.26	94.045 ± 1.2	50.76 ± 0.10
24	1.33 ± 0.07	1.05 ± 0.07	1.267 ± 0.01	0.98 ± 0.0	69.42 ± 0.03	29.19 ± 0.07	96.49 ± 3.76	42.71 ± 0.14
27	1.47 ± 0.07	1.12 ± 0.14	1.4 ± 0.14	0.98 ± 0.0	67.76 ± 6.41	27.12 ± 0.08	103.83 ± 4.38	36.28 ± 0.35
30	1.56 ± 0.02	1.19 ± 0.1	1.47 ± 0.07	1.05 ± 0.07	61.91 ± 0.10	21.53 ± 0.01	94.30 ± 2.82	34.82 ± 0.07
Days	Total phosphorus (g/kg)				Available phosphorus (g/kg)			
0	3.05 ± 0.24	1.57 ± 0.14	3.14 ± 0.12	2.19 ± 0.0	2.8 ± 0.05	1.32 ± 0.08	1.59 ± 0.05	1.43 ± 0.14
3	3.16 ± 0.19	1.7 ± 0.01	3.41 ± 0.01	2.22 ± 0.02	3.07 ± 0.07	1.55 ± 0.16	1.51 ± 0.03	1.52 ± 0.07
6	3.16 ± 0.23	1.96 ± 0.0	3.92 ± 0.0	2.51 ± 0.0	3.1 ± 0.05	1.52 ± 0.01	1.61 ± 0.03	1.54 ± 0.08
9	3.22 ± 0.29	2.02 ± 0.01	4.04 ± 0.01	2.6 ± 0.0	3.1 ± 0.24	1.44 ± 0.04	1.64 ± 0.02	1.6 ± 0.09
12	3.38 ± 0.51	2.26 ± 0.01	4.53 ± 0.01	2.73 ± 0.01	3.01 ± 0.03	1.28 ± 0.04	1.75 ± 0.01	1.63 ± 0.03
15	3.50 ± 0.23	2.27 ± 0.03	4.53 ± 0.05	2.87 ± 0.01	3.13 ± 0.03	1.49 ± 0.17	1.81 ± 0.01	1.67 ± 0.01
18	3.46 ± 0.2	2.28 ± 0.01	4.56 ± 0.02	2.95 ± 0.0	3.45 ± 0.07	1.57 ± 0.13	1.88 ± 0.09	1.69 ± 0.0
21	3.03 ± 0.21	2.38 ± 0.0	4.76 ± 0.01	3.04 ± 0.01	3.52 ± 0.04	1.63 ± 0.14	2.37 ± 0.02	1.8 ± 0.04
24	3.53 ± 0.26	2.54 ± 0.02	5.07 ± 0.04	3.16 ± 0.0	3.64 ± 0.05	1.57 ± 0.03	2.33 ± 0.04	1.9 ± 0.11
27	4.0 ± 0.23	2.54 ± 0.0	5.07 ± 0.0	3.2 ± 0.0	3.53 ± 0.07	1.89 ± 0.11	1.95 ± 0.0	2.28 ± 0.10
30	5.37 ± 0.79	2.76 ± 0.01	5.52 ± 0.02	3.37 ± 0.0	3.57 ± 0.07	2.2 ± 0.23	1.91 ± 0.09	2.38 ± 0.10

Note: BRS = Bharalu river site; AS = low-lying area near agriculture site; BLS = Boragaon landfill site; IS = Amingoan industrial site.

Leather

Skin in livestock is the largest biological protector that serves to protect from dehydration, injury, environment and microbes. Besides that, it also functions as an immune response organ. The skin epidermis consists of layered and multilevel epithelial cells that regenerate through cell proliferation and differentiation[20].

The leather processing industry is one of the strategic industries that has the potential to develop in countries rich in potential ruminants. Every leather processing industry certainly has needed sufficient raw materials so that the industry can continue to run. In general, the potential of leather raw materials can be predicted by looking at the potential availability of raw materials in an area. The skin consists mostly of collagen protein. Skin needs strength and flexibility. The skin contains most of the water. Higher moisture content with greater flexibility results in greater lateral distances between collagen molecules. When collagen molecules are aligned, the skin tightens. Collagen molecules close together will reduce the ability of molecules to move relative to each other (Figure 2)[21].

Utilization of livestock skin by-products is not only for non-food purposes. However, the use for food purposes lately has been widely used and developed both in the form of research and mass-produced. One example is gelatin. Efforts to find alternative materials through research using the region's potential have developed rapidly. The hide of cattle and goat as one of the most abundant plasma livestock has the potential to be a source of gelatin. Gelatin is a hydrocolloid compound obtained from the hydrolysis of animal protein compounds partially which has hydrophilic properties. Various physicochemical properties possessed caused gelatin to be applied to various industries such as: food industry (foam forming, stabilizer, binder and emulsifier), pharmaceutical and laboratory fields (capsule shells and agar media), cosmetics industry, printing industry and photography. Gelatin extracted from goat skin can be applied as a basic ingredient in making hard capsules.

Environmental factors are the main problem experienced by the leather processing industry. Waste produced from the leather processing industry is liquid or solid. Based on existing data that for one ton of wet skin produces about 650 kg of solid waste. The resulting waste is in the form of pieces of leather or the result of liming process[22].

The use of vegetable tanners is considered to reduce the production of chemical waste. The type of vegetable tanner that can be used is tannin. This material can be obtained from quebracho and mimosa extracts. Tanin with higher reactivity produces more stable skin. This happens because it uses a higher decomposition temperature[23].

One of the most important factors to consider in the leather processing industry is microorganism contamination. The

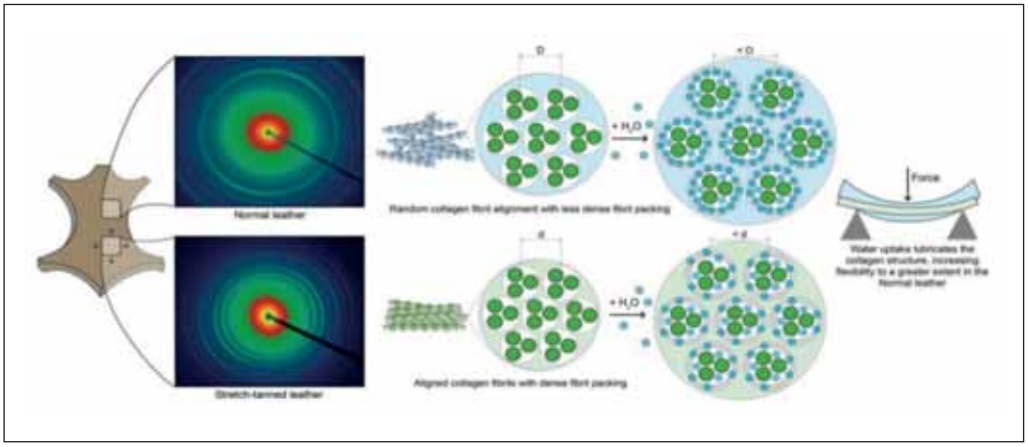

Figure 2
Description of the difference in collagen fibers in the skin due to penetration of the number of water molecules[21].

growth of microorganisms such as fungi is very easy to develop in skin products. Giving antimicrobials especially those that are volatile such as volatile organic compounds must be careful. This is because these phenolic and heterocyclic compounds are carcinogenic and toxic[24].

Blood

Anaerobic fermentation system model is one model that can be applied in treating blood waste from poultry slaughterhouses. The use of microorganisms Methanobrevibacter and Methanobacterium beijingense is used as a bio-carrier. Biocarrier is used as a medium to eradicate microflora and increase cell residence time in a digester. This model is able to reduce COD levels from waste[25].

Animal by-products are animal parts or products that come from animals that are not intended for human consumption. By-products are divided into three categories based on the risk or potential risk associated with domestic animals, communities, or the environment including wild animals. The process of removing blood from the pelagic fishing industry increases environmental pollution[26].

The process of removing blood from cattle cutting activities has been reviewed by previous researchers. The process of removing blood from the body of the animal is carried out through halal (traditional) cutting or using Electric Head-Only Stunning (EHOS). The result is no real difference from the two methods[27].

Bone

The bone is by-product that has high calcium and phosphorus compounds. At present, bone has been widely used by the animal feed industry as a source of mineral feed in the form of bone meal. The resulting bone weight is on average 15% of the carcass weight. However, these weights vary according to animal breeds, feed types and age. In addition, bone has been used also as a mixture in making organic fertilizers[28]. Bone meal is rich in calcium and phosphorus compounds which are needed in plant growth. In health research, bone from animal has been developed as a raw material for producing hydroxyapatite (HAp). In the medical world, HAp is widely applied to bone and dental objects[29,30,31]. The use of bone from animal as a catalyst has been also developed. Catalysts are used to accelerate the occurrence of a chemical reaction process in a system[32]. In relation to design art, bone has been developed as a basic material in producing accessories and handicrafts.

In Europe, there are approximately 17 million tons of by-products derived from livestock produced every year. The process of by-product processing was done through the rendering method. This method aims to process by-products into meat bone meal (MBM). In the slaughterhouses industry, when producing 1 kg of meat for human consumption, it also produces 1 kg of animal by-products. It is no longer consumed by humans but is used as animal feed ingredients. The process of producing MBM has stopped since the outbreak of bovine spongiform encephalopathy (BSE). This certainly results in an increase in the number of animal by-product[33].

Fat/Tallow

Animal fat has a solid form and contains a lot of sterols (cholesterol). Animal fats are known as tallow and lard. The term tallow is usually used for fats derived from cows and goats, while the lard for fat is extracted from pigs. Animal fats are widely used in the food sector to increase palatability and flavor enhancers. The potential of animal fat as a raw material for energy sources in the form of biodiesel has also been widely studied. One type of fat that was developed as a source of biodiesel raw material is mutton tallow through the transesterification system[34].

Tallow has a low value. If it can be converted into biodiesel, it will provide benefits as an energy source, environmental improvements and economic benefits[35]. Animal fat has also been used as a mixture in animal feed to increase the palatability of feed and bind particles of feed ingredients.

For environmental reasons, various countries are trying to find alternative energy sources. One of them is the use of biodiesel by utilizing tallow as raw material. Some of them have also mixed tallow with waste cooking oil as an energy source. This is an effort to reduce the production of waste cooking oil[36].

Brazil is a country that ranks second in the world in producing biodiesel. In the Central-West region of the country is the largest part in producing biodiesel (44.4%) and livestock slaughter activities (37.5%)[37]. Biodiesel production from tallow waste can be increased through the use of the enzyme Candida antarctica Lipase B (CALB). The results of the analysis using chromatography showed that biodiesel production reached 85.6 ± 0.08%[38].

Biodiesel consists of several components such as low alkyl fatty acids (chain length C14-C22), short chain alcohol esters, especially, methanol or ethanol. The use of biodiesel fuel is a consideration to replace fossil fuels. Making biodiesel can be

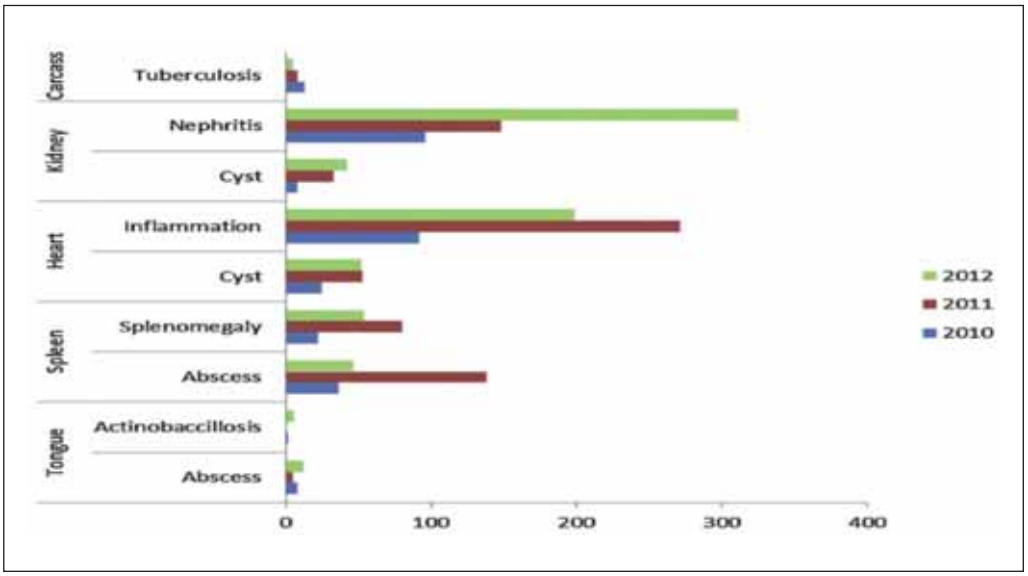

Figure 3
Description of the number of organs and carcass lost in 2010-2012[45].

done using several methods such as mixing, microemulsification, pyrolysis, and transesterification. The transesterification process is a method that is widely applied in industry. Biodiesel fuel can be produced from vegetable or animal fats[39].

Offal

Offal are internal organs and the stomach contents of animals are slaughtered without muscles and bones. Offal consist of red and white offal. Red offal consist of parts that are immediately edible, can be consumed and are considered delicious food in certain parts of the world. Red innards are used in mixed processed meats and additives in sausages. White offal are parts that can be consumed but still need further processing, such as the stomach and intestines[40].

Lately, offal have been developed as food ingredients. In certain countries use pig innards as a mineral source. Consideration of high mineral content (iron and zinc) causes the innards to be widely used[41]. Improving the quality of processed products can be improved by applying liquid smoke. This process can extend the shelf life of processed products from livestock[42].

The use of offal as food is growing rapidly along with the development of culinary variants. Offal is a collection of several organs such as the liver, heart, kidney, brain, pancreas, and tongue. Offal consists of 20% of the live weight in each animal. Offal is not much favored by consumers even though it is a very good source of protein, vitamins and minerals. Meat forgery activities are often carried out by the community on the grounds of gaining profits. This is very contrary to religion, economics, and ethics. One technique that has been developed to identify cases of counterfeiting is to use laser induced breakdown spectroscopy (LIBS)[43].

Increasing the use of offal as food is actually an option. This is due to the high price of meat. Based on the price, offal is much cheaper than the price of meat. However, some countries have not recommended using offal as human food. one reason is an impact factor on human health. The low price of offal has increase in the use as an alternative food. Several types of food have been developed by replacing tallow beef with de-fatted bovine heart. As in frankfurter processed meat products. This replacement is done for health reasons such as obesity and chronic diseases[44].

The rate of loss due to offal damage such as the tongue, spleen, heart and kidneys is experienced by many abattoirs. A study on 3 abattoirs namely: Adeliade (Ad), Queenstown (Qu) and East London (EL). The results of the study showed that the causes of tongue organ damage were caused by abscesses (0.08%, 0.03% and 0.05%) and actinobacillosis (0.02%, 0% and 0.02%) respectively. In the spleen organs caused by abscesses (0.35%, 0.94% and 0.17%) and splenomegaly (0.21%, 0.55% and 0.2%). The heart is caused by cysts (0.24%, 0.36% and 0.2%) and inflammation (0.9%, 1.85%, and 0.75%). Whereas in kidney organs caused by cysts (0.08%, 0.23% and 0.16%) and nephritis (0.94%, 1.01% and 1.18%). An overview of the number of organs and carcasses lost in the 2010–2012 period at 3 abbattoir locations is presented in Figure 3[45].

Feather

The feather waste is one type of by-product produced by the poultry slughterhouses (PSh) industry[46]. Globally, the poultry industry produces around 6 million tons of fur as a by-product every year. The main constituent of fur is protein (80-90%), where the type of protein dominated by keratin[47]. Feather waste is a product that needs to utilize to reduce PSh waste production. The use of chicken feather waste as a protein source for animal feed carried out. However, chicken feather waste still has weaknesses. One of them is the level of digestibility that is still very low[48]. Therefore, several studies developed to increase the digestibility of fur products. One of them is by using pressurized, chemical heating techniques and using the help of bacterial microorganisms (*Bacillus subtilis* sp)[49].

Effect of Animal waste

The therapeutic and sub-therapeutic application of antibiotics in the livestock industry has long been practiced in several parts of the world. Bioactive organic compounds have short retention periods and partial uptake into the livestock industry cycle. Livestock manure containing biosolid and antibiotics has the potential to affect the health of animal. Antibiotic response can be phytotoxic, hormetic and mutational. Remnants of antibiotics can contaminate animal waste which will cause animal to become resistant. The cycle of antibiotic use and its effect on livestock is presented in Figure 4[50].

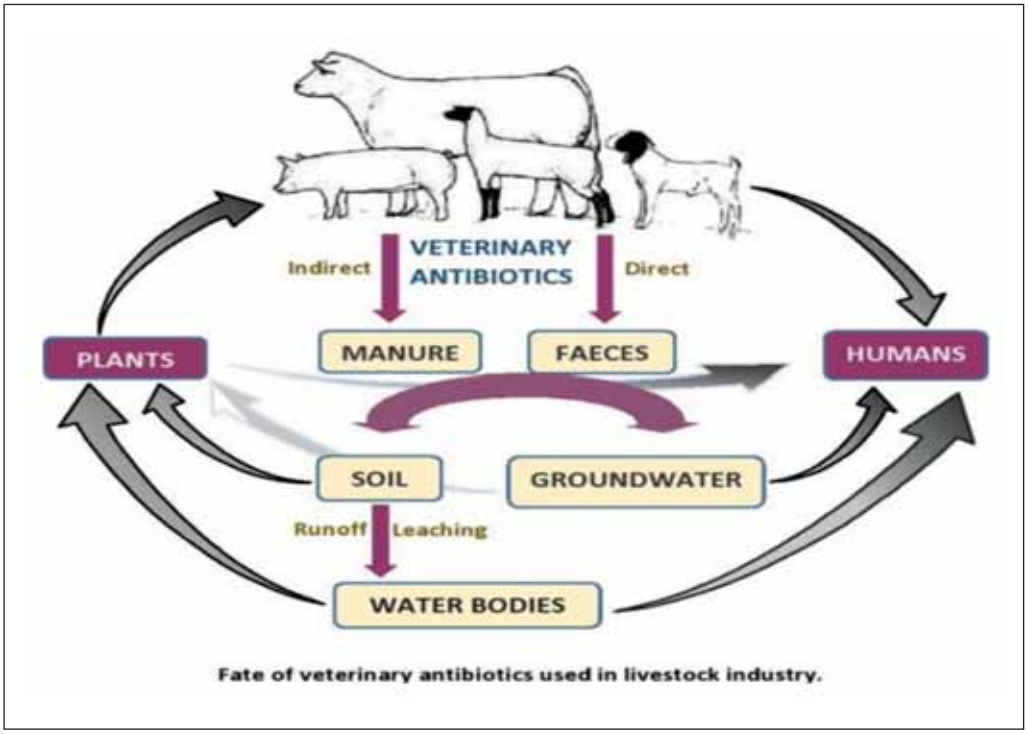

Figure 4
The process cycle of the use and spread of antibiotics through animal waste and its effect on animal health.

CONCLUSION

In animal, approximately 1/3 of its body is used as a food source in the form of meat, while the remaining 2/3 is non-meat. Actually, the economic potential of the non-meat portion can be maximized if it receives technology input. Livestock by-product applications can be used in a variety of industries, both large and small scale industries. Along with the government's efforts to increase the livestock population, of course the potential of by-products will also increase. Animal skin as one type of livestock product exported. The quality of leather exported is strongly influenced by the pattern of livestock maintenance in the cultivation process. The quality of feed, management and management of maintenance and handling of livestock in slaughterhouses (RPH) greatly determine the quality of the final skin.

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Cyclopia, cerebral aplasia and hydrocephalus in an equine foetus

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SUMMARY

Congenital anomalies of the central nervous system are quite common in domestic animals and can result from heredity or in utero exposure to teratogenic chemicals or to infectious agents. Among those is cyclopia, a congenital disease condition characterized by severe anomalies of the central nervous system and by striking skeletal malformations. Cyclopia is frequently observed in ruminants and pigs. In particular, epidemic outbreaks of cyclopia can occur in lambs in the western United States of America, due to the ingestion of *Veratrum californicum* during the early stage of pregnancy. On the other hand, cyclopia very rarely occurs in other animal species. The present report aims to describe the main pathological features of cyclopia, which has been recently observed in an equine foetus, aborted during the 8th month of gestation. At the external inspection, the foetus showed evident cranial and facial malformations, with a prominent maxillary brachygnathism. Remarkably, a single large median eye was present. After opening the cranial cavity, the brain hemispheres were totally absent, while a sketch of the cerebellum was still evident. The remaining part of the skeleton and all the internal organs appeared normally developed. Likewise, the foetal membranes were apparently healthy, prominent autolytic changes being only observed. Based on these pathological findings, a diagnosis of cyclopia associated with hydrocephalus and cerebral aplasia was made. To the best of our knowledge, this is the third case of equine cyclopia so far described worldwide, such malformation to be considered an extraordinary event in this animal species. The aetiology of sporadic cases of cyclopia remains often obscure; in human beings, it is heterogeneous with a high prevalence of chromosomal abnormalities, mainly trisomy 13. No data is currently available about the aetiology and pathogenesis of cyclopia in equids, incidental genetic defects representing the most plausible cause of such a rare disease condition.

KEY WORDS

Horse; congenital diseases; cyclopia; cerebral aplasia; hydrocephalus.

INTRODUCTION

Congenital anomalies of the central nervous system (CNS) are quite commonly observed in domestic animals, due to the slow development and maturation of the CNS, its complex and highly organized structure, as well as its sensitivity towards a wide range of harmful stimuli (Mandara *et al.*, 2011). It is always difficult to make a reliable estimate of the prevalence of congenital malformations in veterinary medicine. In a large study conducted in the USA, craniofacial malformations and hydrocephalus accounted for 4.3% and 3% of total malformations in the equine species, respectively (Crowe *et al.*, 1985). CNS malformations can result from heredity or in utero exposure to teratogenic chemicals or to infectious agents; in this respect, the recent epidemic caused by the *Schmallenberg* virus in ruminants can be considered paradigmatic (Lievaart-Peterson *et al.*, 2015). However, the aetiology of CNS malformations is complicated, multifactorial and often remains unexplained. Likewise, it is difficult, sometimes questionable, to classify such malformations on the basis of their pathogenesis, because different pathogenetic mechanisms can act together, thus contributing to the occurrence of the congenital defect (Mandara *et al.*, 2011).

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We describe herein the main pathological features of cyclopia, which has been recently observed in an equine foetus.

CASE DESCRIPTION

Materials and methods

A 9-year-old, Italian Heavy Draft breed mare aborted during the 8th month of gestation. The mare lived in a mountain area in Abruzzi region (Italy), inside a paddock provided with a roof, along with other four horses. The mare was apparently healthy and did not show any "warning" symptoms before the abortion. The foetal membranes and the aborted foetus were both referred to the Veterinary Teaching Hospital of the Faculty of Veterinary Medicine of Teramo (Italy) and therein carefully inspected. Selected nervous tissue samples were fixed in 10% neutral buffered formalin and routinely processed for histopathological investigations (haematoxylin and eosin stain).

Results

At the external inspection, the foetus (male) appeared of normal size, taking into account the stage of pregnancy and the standard of the breed. However, an evident anomaly of the head was noted (Figure 1a). The skull was globoid, with severe facial malformation and prominent maxillary brachygnathism. Remarkably, a single large median eye was present (Figure 1b). After opening the skull, the cranial cav-

Figure 1 - Equine foetus. The foetus appeared well developed, the only evident anomaly affecting the skull **(a)**. On frontal view, the cranium was severely malformed, a single large median eye being present **(b)**. After its opening, the cranial cavity was almost completely filled by yellowish-reddish fluid. A severe malformation of the nasal, maxillary and mandibular bones was also evident **(c)**.

ity appeared filled with abundant, yellowish-to-reddish fluid. The brain hemispheres were totally absent, while a sketch of the cerebellum was still evident (Figure 1c). Caudally, the macroscopic appearance of the medulla oblongata and of the spinal cord was normal. The remaining part of the skeleton and all the internal organs were regularly developed.

The foetal membranes were apparently healthy, prominent autolytic changes being only observed. Likewise, severe autolytic changes affected also the remnant of the CNS, thus preventing a suitable microscopic examination of those samples.

The above described congenital defects typically characterize cyclopia, in this case associated with hydrocephalus and cerebral aplasia.

Discussion

Cyclopia is a very complex congenital disease condition, in which striking skeletal malformations are always associated with severe anomalies of the CNS (i.e. failure of the separation of the optic vesicles, holoprosencephaly and arhinencephaly). Cyclopia is quite commonly seen in pigs and occasionally detected in other animal species. In particular, epidemic outbreaks of cyclopia can occur in lambs in the western United States of America, as a result of *Veratrum californicum* poisoning of pregnant sheep on day 14th of gestation. It has been demonstrated that *Veratrum californicum* contains the steroidal alkaloid cyclopamine, a plant-derived teratogen inhibiting the hedgehog signalling pathway, which plays a key role during the embryonic development (Lee *et al*., 2014).

On the contrary, the aetiology of sporadic cases of cyclopia remains often obscure (Mandara *et al*., 2011); in humans, it is heterogeneous with a high prevalence of chromosomal abnormalities, mainly trisomy 13 (Orioli *et al*., 2011).

To the best of our knowledge, two cases of cyclopia have been so far described in the horse (Hughes and Dransfield, 1940; Wilkens and Neurand, 1974), such congenital anomaly to be considered an extraordinary and impressive event in this animal species. Overall, the craniofacial deformities previously reported in equine cyclopia are quite similar to the present case description, no "proboscis" being observed above the orbit. The case described by Hughes and Dransfiel (1940) occurred in a Shire breed foal, which was born alive and died a short time after birth. This foal showed two eyes in a single orbit, the CNS malformations resembling those reported herein. In fact, the pons and the medulla appeared normal; on the contrary, the cerebellum was compressed and twisted, while the forebrain, the lateral hemispheres and the olfactory bulbs were undeveloped. The case report by Wilkens and Neurand (1974) was observed in a new born foal and was associated with arhinencephaly, information lacking about its gender and breed.

No data is currently available about the aetiology and pathogenesis of cyclopia in equids. However, the epidemiological features of equine cyclopia make the exposure to teratogenic chemicals or viral aetiologies unlikely, while arguing in favour of incidental genetic defects as the cause of such a very rare disease condition.

AUTHORSHIP

All named authors equally contributed to the collection and interpretation of the data, as well as to the drafting of the paper. All authors critically reviewed its content and have approved the final version submitted for publication.

CONFLICT OF INTEREST

All Authors disclose any potential sources of conflict of interest.

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