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Review Article

The potential of sheep in preclinical models for bone infection research – A systematic review

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ABSTRACT

Background: Reliable animal models are critical for preclinical research and should closely mimic the disease. With respect to route of infection, pathogenic agent, disease progression, clinical signs, and histopathological changes. Sheep have similar bone micro- and macrostructure as well as comparable biomechanical characteristics to humans. Their use in bone research is established, however their use in bone infection research is limited. This systematic review will summarise the key features of the available bone infection models using sheep, providing a reference for further development, validation, and application.

Method: This systematic review was designed according to the PRISMA guidelines and registered with PROSPERO. Quality was assessed using SYRICLE's risk of bias tool adapted for animal studies. PubMed, MEDLINE, Web of Science and EMBASE were searched until March 2022.1921 articles were screened by two independent reviewers, and 25 were included for analysis.

Results: Models have been developed in nine different breeds. *Staphylococcus aureus* was used in the majority of models, typically inoculating 10^8 colony forming units in tibial or femoral cortical defects. Infection was established with either planktonic or biofilm adherent bacteria, with or without foreign material implanted. Most studies used both radiological and microbiological analyses to confirm osteomyelitis.

Conclusions: There is convincing evidence supporting the use of sheep in bone infection models of clinical disease. The majority of sheep studied demonstrated convincing osteomyelitis and tolerated the infection with minimal complications. Furthermore, the advantages of comparable biology and biomechanics may increase the success for translating *in vivo* results to successful therapies.

The Translational potential of this article: In the realm of preclinical research, the translation to viable clinical therapies is often perilous, and the quest for reliable and representative animal models remains paramount. This systematic review accentuates the largely untapped potential of sheep as large animal models, especially in bone infection research. The anatomical and biomechanical parallels between sheep and human bone structures position sheep as an invaluable asset for studying osteomyelitis and periprosthetic joint infection. This comprehensive exploration of the literature demonstrates the robustness and translational promise of these models. Furthermore, this article underscores the potential applicability for sheep in developing effective therapeutic strategies for human bone infections.

1. The translational potential of this article

In the realm of preclinical research, the translation to viable clinical therapies is often perilous, and the quest for reliable and representative animal models remains paramount. This systematic review accentuates the largely untapped potential of sheep as large animal models, especially in bone infection research. The anatomical and biomechanical parallels between sheep and human bone structures position sheep as an invaluable asset for studying osteomyelitis and periprosthetic joint infection. This comprehensive exploration of the literature demonstrates

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the robustness and translational promise of these models. Furthermore, this article underscores the potential applicability for sheep in developing effective therapeutic strategies for human bone infections.

2. Introduction

Infections in orthopaedic surgery including fracture management can have serious consequences, causing prolonged hospital admissions and increased patient morbidity and mortality [1]. This is particularly relevant in prosthetic joint infections (PJI) where patients typically undergo two stage revision and require extended periods of intravenous antibiotic therapy, causing long hospital admissions [2,3]. Despite advances in perioperative care, the failure rate for these operations remains high and the mortality associated with PJI is worse than some cancers [4]. Similar complications can be seen in surgery for open fractures, where bacterial contamination can necessitate more complex external bone fixation or the failure of internal fixation and subsequent removal of osteosynthesis material. Collectively these complications represent a significant burden on both the patient and healthcare systems worldwide. It is estimated that the overall annual cost of PJI in America alone will reach $1.85 billion by 2030 [5]. Furthermore, in the context of increasing antibiotic resistance, there is a significant potential for further deterioration in the outlook for this patient group.

As focus on this problem grows, increasingly more research is being carried out to develop novel therapeutic approaches and improve surgical strategies. *In vivo* preclinical animal models have been critical in evaluating and understanding disease pathophysiology, biocompatibility and the development and testing of novel antimicrobial therapies. However, the success for translating promising preclinical results into viable clinical therapies, as in many areas of research, remains challenging [6]. Generally, the closer animal models resemble the human biology and disease, the greater the external validity, and the more likely *in vivo* animal results will successfully translate to clinical practice. Accordingly, in orthopaedic research there is increasing focus on the development of large animal models for therapeutic evaluation.

Large animal models offer various advantages in orthopaedic research including more similar bone dimensions, and biomechanics. Indeed, bone size approaching human equivalence provides the opportunity of studying standard implants in the experimental setting. Dogs have been used as large animal models and although denser, share remarkably similar bone structure to humans [7], however the ethical concerns surrounding their use has rendered them increasingly unpopular. Pigs have relatively similar bone microstructure and macrostructure to humans and are established as successful osteomyelitis models [8]. Their main disadvantage is their rapid growth rates during adolescence and their unwieldly size at maturity which can reach over 150 kg. This can limit some experimental designs, in particular assessment over longer time periods. This can be compensated by the use of mini pigs, although the advantages associated with larger bone size is then diminished. Sheep are becoming popular as large animal models. Their docile nature, relatively low cost and comparable bone structure are beneficial in bone research. Compared to humans, sheep have slight denser bone composition and relatively fewer harversian canals, otherwise bone microstructure and macrostructure are broadly similar to humans [9]. Sheep are ruminants, which precludes the use of oral antibiotics, however, this limitation can be circumvented with intramuscular or intravenous therapy. More generally, one challenge working with large animals can be the availability of commercial kits for biomarker analysis and sheep are no exception, nevertheless standard blood analyses are available. While sheep have been used in infection research in a range of organ systems, their use in bone infection research is limited.

In the following systematic review, we have gathered the accumulated literature where sheep have been utilised as an animal model in bone infection research. Our aim is to provide an overview of the experimental designs and procedures, thereby establishing a comprehensive reference and basis for future protocol design. Ultimately, we wish to establish if there is sufficient evidence suggesting sheep are a viable, relevant and useful pre-clinical large animal model for this area of research.

3. Methods

Protocol: The study was planned, conducted, and reported with reference to the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analysis) guidelines. However, as this review assess experimental animal studies without meta-analyses some aspects of the guidelines were deemed inappropriate. The protocol was registered with the PROSPERO International Prospective Register of Systematic Reviews, prior to the literature search (2022 CRD42022316218). Quality assessment was performed using SYRCLE's risk of bias tool adapted for animal studies.

Study identification: The Search strategy was developed in consultation with a scientific research librarian from the University of Southern Denmark and tested ad-hoc by testing for the inclusion of known articles. The resulting search strategy *((bone infection) OR (osteitis) OR (osteomyelitis) OR (joint infection) OR (infectious arthritis) OR (bacterial arthritis) OR (peri-implant infection) OR (peri-prosthetic infection) OR (periprosthetic infection)) AND ((sheep) OR (ovine*))* was used to interrogate Pubmed, MEDLINE, Web of Science and EMBASE until 31st March 2022.

We included all studies introducing bacteria with the purpose of establishing *in vivo* infection in sheep bone. This includes bacteria introduced alone or in combination with implanted foreign material. In vitro and ex-vivo studies were excluded as were non-bacterial inoculations or studies without the explicit aim of establishing bone infection. Thus, studies where bacteria were deliberately introduced in, or around ovine bone were included. Whereas studies on ovine bone where bacterial contamination occurred as an unintended complication were excluded.

All studies were included regardless of publication age or language in the initial results, however in the full text screening, studies where a reliable English language translation was not achievable, were subsequently excluded. As a result, only English, Scandinavian and German language articles proceeded to data extraction. Systematic reviews, meta-analyses of experimental studies and case studies were excluded.

Study screening: The search results were initially imported to endnote, where duplicates were removed, and the remaining studies imported to covidence. Covidence software identified further duplicates which were manually assessed and removed. In stage one studies were screened for title and abstract by two reviewers working independently, conflicts were resolved by a third reviewer. In stage two, the full text was assessed for eligibility by the same two reviewers working independently and studies excluded according to the exclusion criteria, conflicts were resolved by a third independent reviewer.

Quality assessment and data extraction: The first author quality assessed the resulting articles and performed data extraction using custom extraction templates in covidence. All articles were assessed twice by the same reviewer with a minimum of a one-week period between primary and secondary data extraction. The data extracted considered the article characteristics, study design, animal characteristics, inoculation method and dose, antibiotic therapy, presence and character of implanted foreign material, study duration, and methods of analysis. Where data was missing, contact to the corresponding author was attempted twice.

Statistical analysis: Statistical analysis was performed using Pearson's chi-squared test using STATA software. A p value of under 0,05 was considered significant.

4. Results

4.1. Search results

In stage one, 1921 studies were screened for title and abstract. 1856 studies were excluded corresponding to the selection criteria. Accordingly, the full text of 64 articles was assessed for eligibility in stage two and a further 39 excluded. The resulting 25 articles proceeded for data extraction (Fig. 1).

4.2. Article characteristics

A total of 25 articles were included for data extraction (Table 1) with publication dates ranging from 1974 to 2021. Overall, the number of articles published annually, using sheep as bone infection models have increased markedly during the past 50 years. With the exception of two German articles the remining were written in English. Three articles were excluded at the full text stage (two Chinese and one French) as English translations were not available. All articles scored low on the majority of quality scores according to the SYRICLE risk of bias tool adapted for animal studies.

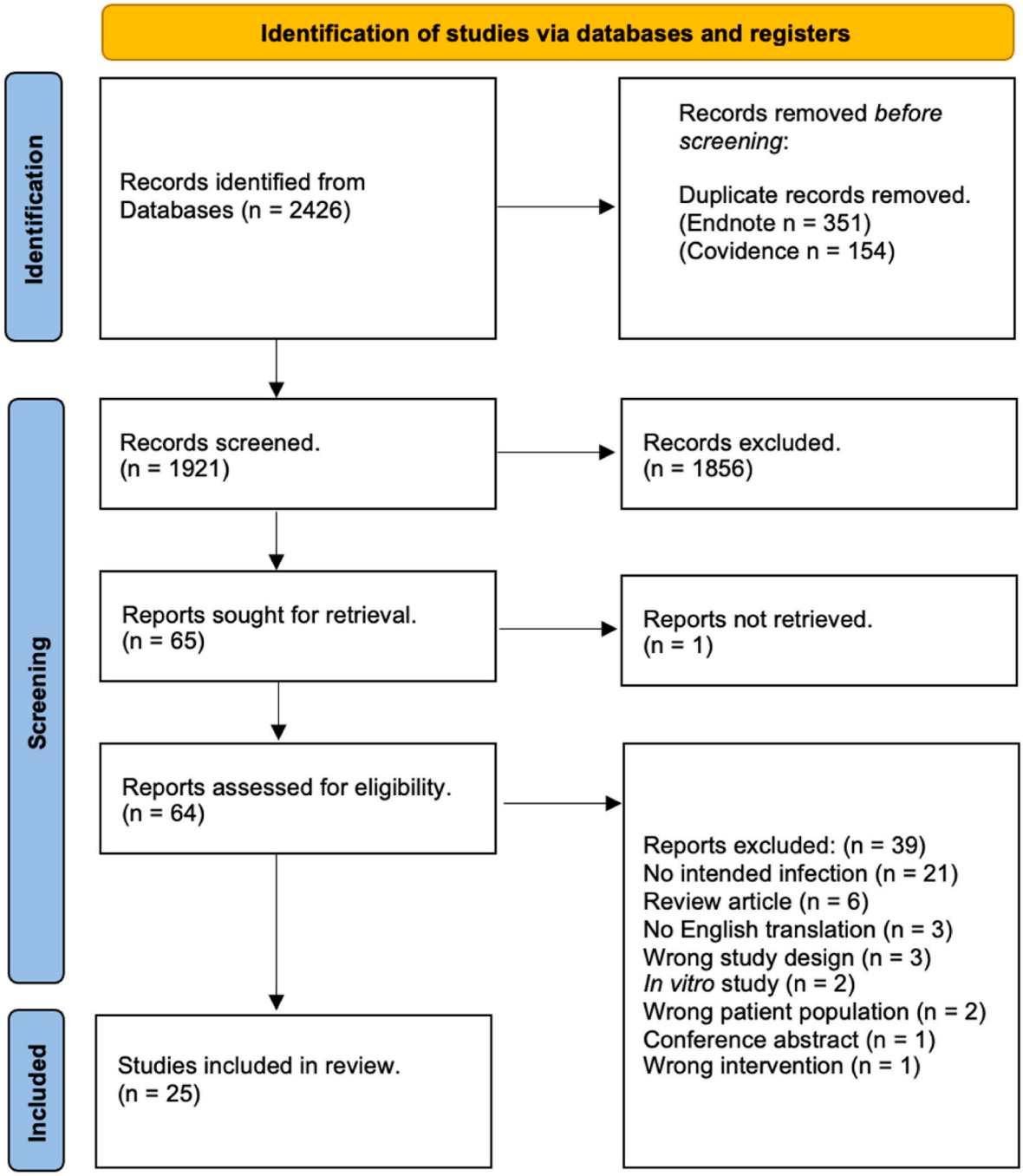

Figure 1. Prisma chart with studies included and excluded during stages 1 and 2. Reasons for excluding in stage 2 include: no intended infection where infection was an unintended complication, wrong study design, wrong intervention, and wrong patient population where the studies considered sheep flocks in non-experimental based settings, non-osseous aspects of the skeletal system, and veterinary diseases associated with animal husbandry, respectively.

Table 1
List of articles included for data extraction with publication date, PubMed ID and language.

Study ID	PMID	Publication date	Lead Author	Country of origin	Language	Reference
Boot 2021	33526492	March 2021	W. Boot	Switzerland	English	[10]
Clasper 2001	11382422	January 2001	J.C. Clasper	UK	English	[11]
Clasper 1999	10632463	October 1999	J. C. Clasper	UK	English	[12]
Clasper 2001	11332613	April 2000	J.C. clasper	UK	English	[13]
Collinge 1994	8,036,188	May 1994	C.A. Collinge	USA	English	[14]
Ferrell 2019	30957870	April 2019	Z. Ferrell	USA	English	[15]
Foster 2021	33305875	December 2020	A. L. Foster	Switzerland	English	[16]
Gimeno 2018	28976634	October 017	M. Gimeno	Spain	English	[17]
Gimeno 2013	23651643	May 2013	M. Gimeno	Spain	English	[18]
Hill 2002	12168650	October 2001	P. F. Hill	UK	English	[19]
Kaarsemaker 1997	9186226	November 1996	S Kaarsmaker	Netherlands	English	[20]
Klein 2021	34124751	June 2021	K. Klein	Germany	English	[21]
Laure 2008	17600319	June 2007	B. Laure	France	English	[22]
McLaren 2014	24908426	June 2014	J. S. McLaren	UK	English	[23]
Moriarty 2017	28853767	August 2017	T. F. Moriarty	Switzerland	English	[24]
Qu 2014	25102546	August 2014	H. Qu	USA	English	[25]
Schaer 2012	22082621	November 2011	T. P. Schaer	USA	English	[26]
Schenck 1974	4420219	January 1974	R. D. Schenck	Germany	German	[27]
Sinclair 2013	23564717	June 2012	K. D. Sinclair	USA	English	[28]
Stewart 2012	22854994	August 2012	S. Stewart	USA	English	[29]
Wannske 1976	962682	October 1976	M. Wannske	Germany	German	[30]
Watson 2020	32599360	June 2020	E. Watson	USA	English	[31]
Williams 2019	30710710	January 2019	D. L. Williams	USA	English	[32]
Williams 2012	22940221	November 2012	D. L Williams	USA	English	[33]
Williams 2012	22492534	June 2012	D. L. Williams	USA	English	[34]

4.3. Sheep characteristics

A range of different breeds were used, Swiss Alpine, Columbia-Cross, Ile de France, English mule, Dorset-Cross, Suffolk-Cross, Black-Head, Dorper and Ramboullet. Six (24%) articles did not report the precise breed of sheep used. The age of the sheep ranged from 0,5 to 9 years old. Three studies (12%) did not specify the age, instead reporting the sheep as either adult or skeletally mature. Four (16%) articles reported using sheep one year old or younger. The weight of animals ranged between 44 and 100 kg None of the articles reported individual weights. Six articles (24%) did not report the weight of the sheep and the remainder presented the weight as a mean ± standard deviation or range. Except for one neutered ram, used as a control; all studies reporting the sex used female sheep. Six studies (24%) did not report the sex (Table 2).

Table 2
Sheep characteristics, breed, age, weight and sex.

Study ID	Breed	Age (years)	Weight (kg)	Sex
Boot 2021	Swiss Alpine	2–4	58.6 (55.5–66.5)	Female
Clasper 2001	Mixed	Unspecified	75 (60–94)	Female
Clasper 1999	Mixed	Unspecified	81(70–93)	Female
Clasper 2001	Mixed	Skeletally Mature	70 (65–76)	Female
Collinge 1994	Unspecified	Adult	Unspecified	Unspecified
Ferrell 2019	Columbia-cross	2–4	60–100	Female
Foster 2021	Swiss alpine	3–7	69–98	Unspecified
Gimeno 2018	Unspecified	Adult	35–40	Female
Gimeno 2013	Unspecified	Adult	40–45	Female (1 neutered ram)
Hill 2002	Suffolk-cross	Unspecified	65 kg (55–80)	Female
Kaarsemaker 1997	Texel crossbreed	3–5	55.4 ± 8.7	Unspecified
Klein 2021	Swiss Aline	2–3	75 (61–83)	Female
Laure 2008	Ile de France	4–9	Unspecified	Unspecified
McLaren 2014	English Mule	4–5	53–74	Female
Moriarty 2017	Swiss alpine	2–4	75 (54–91)	Female
Qu 2014	Dorset-cross	3–4	Unspecified	Female
Schaer 2012	Dorset-cross	Skeletally Mature	Unspecified	Female
Schenck 1974	Black-Head	1	70 kg	Unspecified
Sinclair 2013	Suffolk-cross	2–3	90 ± 12 kg	Female
Stewart 2012	Dorset-cross	3–4	Unspecified	Female
Wannske 1976	Black-head	1–2	70	Unspecified
Watson 2020	Dorper	0.5–0.8	28.6 ± 1.5 kg	Female
Williams 2019	Rambouillet	1–3	Unspecified	Female
Williams 2012	Columbia Cross	2–3	75 ± 25 kg	Female
Williams 2012	Columbia Cross	2–3	90 ± 20 kg	Female

4.4. Study design

None of the studies had a blinded design and only two mentioned random allocation of animals to the intervention, though without further clarification. Seven studies (28%) did not include a control group. The remaining 18 studies (72%) had on average 7 animals (1.7–10.2) in the control group and most controls were in a separate group of animals. One study had both control and intervention in the same animal, using one hindleg as the intervention and the contralateral hind leg as the control. There were on average 8.9 animals (4.5–13.3) in the intervention groups. The average duration of the studies was 53.4 days (70.1–36.8) (Table 3). In 56% of the studies some animals were euthanised early due to complications. The majority (95.1%) were secondary to complications related to the infection; however, two sheep (3.3%) developed a fracture and one (1.6%) developed oesophageal obstruction (Table 4).

4.5. Bone infection

The majority of studies inoculated with planktonic *Staphylococcus aureus* suspensions (88%), two studies inoculated with *S. aureus* biofilms (8%) and one study used *Staphylococcus epidermidis* (4%). The tibia was the most common site for inoculation (72%). The femur was used in five studies (20%) and the final two studies infected the mandible (4%) and iliac crest (4%) respectively. All studies introduced infection with some form of bone defect. Defects in the bone cortex accounted for 56%, with the remainder medullary (24%) and trabecular bone (20%). The method for forming the defect included drilled hole (32%), reamed tract for intramedullary (IM) nail (16%), Pin tract (16%) osteotomy (12%) screw tract (8%) The remining 16% did not clearly describe the method for

Table 3
Use of control, number of animals in control and intervention groups.

Study ID	Control group (none = no control, same = control in same animal, Separate = control in separate animal)	Number of animals in control group.	Number of animals per intervention	Duration of study (days)
Boot 2021	Separate	6	6	56
Clasper 2001	None	0	3.7 (3/3/5)	1 (hour)
Clasper 1999	None	0	14	14
Clasper 2001	Separate	6	6	28
Collinge 1994	Separate	3	3	19
Ferrell 2019	Separate	14 (7 per group)	7	84
Foster 2021	None	0	5	112
Gimeno 2018	Separate	3	6	7
Gimeno 2013	Separate	1	4	10
Hill 2002	Separate	6	6	42
Kaarsemaker 1997	Same	52	52	84
Klein 2021	None	0	4	21
Laure 2008	Separate	12 (4 per group)	6	45
McLaren 2014	Separate	6	6	14
Moriarty 2017	None	0	6	98
Qu 2014	Separate	3	3	28
Schaer 2012	Separate	6	6	28
Schenck 1974	None	0	25	35
Sinclair 2013	Separate	3	5	84
Stewart 2012	Separate	4	5	84
Wannske 1976	None	0	24	44
Watson 2020	Separate	3	3	63
Williams 2019	Separate	7	7	168
Williams 2012	Separate	9	9	84
Williams 2012	Separate	5	5	84
Mean (95% CI)		7.0 (1.7–10.2)	8.9 (4.5–13.3)	53.4 (70.1–36.8)

Table 4
Number of animals euthanised early due to complications, number of days completed and reason for euthanasia.

Study ID	Number of animals euthanised early	Average number of days completed prior to early euthanasia	Reasons for euthanasia
Clasper 2001	6	16.5	swollen stiff joints, unable to weight bear
Collinge 1994	0	N/A	N/A
Ferrell 2019	7	6–11	Grade III infection
Gimeno 2018	1	4	Unspecified
Gimeno 2013	3	7	Reached humane endpoint
Hill 2002	2	28	Excessive weight-loss
Kaarsemaker 1997	14	Unspecified	Sepsis, fracture, viral pneumonia
Moriarty 2017	3	84	Oesophageal obstruction, weight loss, lameness, closed fracture
Sinclair 2013	4	10.5	Soft tissue infection
Williams 2019	14	2–44	Soft tissue infection
Williams 2012	5	Unspecified	Grade III infection, fracture
Williams 2012	2	21	Grade III infection

creating the defect. The defect size varied according to method and included both critical and non-critical defects. The bacterial inoculation was injected directly into the defect, or around an implant (60%). Some studies (24%) inoculated with bacteria injected onto collagen or gelatine sponges, serving as a bacterial reservoir. Two studies (8%) used a cotton gauze soaked in bacterial suspension. The remining two (8%) used biofilms, as mentioned previously. The mean inoculum was 5.6×10^8 colony forming units (CFU), with a range from 1×10^4 to 5.24×10^9 CFU. In broad terms, the most frequently used concentration was 10^8 CFU (32%).

All except one study incorporated a foreign material in the model (96%). The majority comprised metal implants of either stainless steel 38% or titanium 25%. Five studies (21%) did not explicitly define the type of material, however as external fixator pins, they are likely to be stainless steel. Two studies used polymers (8%) and two used the same cotton gauze as the initial inoculation (8%) (Table 5).

4.6. Systemic antibiotics

Most studies (60%) did not use systemic antibiotics either preoperatively or postoperatively. Of those that administered preoperative antibiotics (12%) ceftiofur was used in all cases. One study (4%) co-administered penicillin. Eight studies (32%) administered antibiotics postoperatively. No association between the addition of pre- or postoperative antibiotics and the number of animals euthanised early was found ($p = 0.88$, $p = 0.13$ respectively). The specific antibiotic used varied, and the average duration was 16 days (range 1–56) (Table 6).

4.7. Analysis

The sheep in all studies were monitored clinically for signs of infection. Ten studies (40%) measured inflammatory cell changes during the study. Two studies (8%) also monitored serum fibrinogen, an acute phase reactant. Of the studies monitoring systemic inflammatory markers including white blood cells (WBC) leucocyte count, full blood count (FBC) or fibrinogen, only one (4%) found a significant rise during the experiment. Bone imaging *in vivo* was carried out in 64% of the studies and in all cases plain radiographs were used. *Ex vivo* imaging was carried out in 72% of studies and included Plain radiographs (56%), Micro CT (28%), Scanning electron microscopy (SEM) (17%), and magnetic resonance imaging (MRI) (6%). A number of studies (28%) did not conduct histological analysis, choosing to focus specifically on microbiological outcomes. Of the studies including histological analysis (72%), Hematoxylin-Eosin (H&E) staining of bone or surrounding soft tissue was most common (64%). 18% conducted Sanderson's rapid bone stain (SRBS), 6% Methylene blue-basic fuchsin staining, 6% modified tetrachrome staining, and 6% Gram staining. The histological results showed varying degrees of inflammatory cell infiltration, proloferation of fibroblasts, and collagen deposition, varying with the severity of infection, duration, and antibiotic use. Notably, areas of haemorrhage and necrosis were often present, and the cellular infiltration was typically time dependant and dominated by neutrophils and macrophages. The majority of studies (72%) reported the spatial localization of bacteria which typically involved all aspects of the infected area, including bone marrow, osteocyte lacunae and surrounding soft tissue (Fig. 2). Two studies (12%) supplemented with Brown and Benn staining for bacterial localisation. One study (6%) included immunohistochemistry with calcein labels to quantify mineralizing surfaces around a resorbable bone filer; and two studies (12%) utilized von Kossa stain to further characterize the tissue morphology within the osteotomy region. All studies reported radiological, microbiological or histological evidence of established osteomyelitis in control animals, regardless of inoculation dose or pre- or postoperative antibiotics (Table 7). Furthermore, the bacterial localisation did not appear to be related to the inoculation dose.

Table 5
Methods of bone infection. Location, presence of defect and foreign material. Infective organism, method of inoculation and inoculation dose.

Study ID	Specific bone	Bone defect	Gross location in the bone	Position in bone tissue	Foreign material	Foreign material description	Foreign material type	Bacterial Strain	Bacterial identification number	Inoculation dose (CFU)	Inoculation method
Boot 2021	Tibia	Tract from IM nail	Whole bone	Medullary	Yes	IM nail	Titanium	*S. aureus*	EDCC 5443	1.0 × 10^7	Inoculated on a collagen sponge
Clasper 2001	Tibia	Pin tract	Whole bone	Cortical	Yes	External fixation pin	Unspecified	*S. aureus*	ATCC 29213	2.5 × 10^8	suspension placed in the wound around the pin
Clasper 1999	Tibia	Pin tract	Whole bone	Cortical	Yes	External fixation pin	Unspecified	*S. aureus*	ATCC 29213	2.5 × 10^5	Suspension placed in the wound around the pin after 7d
Clasper 2001	Tibia	Pin hole from external fixation pin	Whole bone	Medullary	Yes	IM nail and External fixation pin	Unspecified	*S. aureus*	ATCC 29213	2.4 × 10^8	Placed in the wound around the pin
Collinge 1994	Iliac crest	Pin tract	Whole bone	Cortical	Yes	Pin	Stainless steel ± silver coating	*S. aureus*	ATCC 25923	1,0 × 10^5	Injected onto the surface of the pin underneath the skin
Ferrell 2019	Femur	7.0 × 7.5 × 9.0 mm defect anterior to MCL insertion	Distal	Cancellous (trabecular)	Yes	Amorphous	Antibiotic-eluting bone-void filler	*S. aureus*	ATCC 49230	5,0 × 10^5	Dispensed from sterile pipet
Foster 2021	Tibia	Tract from IM nail	Whole bone	Medullary	Yes	IM nail	Stainless steel coated in PMMA	*S. aureus*	Unspecified	2,0 × 10^7	Inoculated on a collagen sponge
Gimeno 2018	Tibia	Drill hole 6 mm Ø, 10 mm deep	Mid	Cortical	Yes	25 mm long, 6 mm Ø with 1.6 mm wall thickness, hollow tube.	Stainless steel	*S. aureus*	ATCC6538	6.6 × 10^5	Placed in surgical wound
Gimeno 2013	Tibia	Drill hole 6 mm Ø, 10 mm deep	Mid	Cortical	Yes	25 mm long, 6 mm Ø with 1.6 mm wall thickness, hollow tube.	Stainless steel	*S. aureus*	ATCC6538	5.5 × 10^7	Inoculated around the implant which protruded from the skin
Hill 2002	Tibia	Chevron osteotomy	Mid	Medullary	Yes	Modified humeral nail (smith and Nephew Richards)	Unspecified	*S. aureus*	ATCC 29213	3.0 × 10^8	3 × 30mm bovine type 1 collagen
Kaarsemaker 1997	Tibia	Drill hole 4.2 mm Ø, 5 mm deep	Mid	Cortical	No			*S. aureus*	PS 8368 Phage type 1	4.0 × 10^8	Injected gelatine sponge strips
Klein 2021	Tibia	10 × 10 × 5mm defect in cortex	Proximal	Cortical	Yes	28.4 × 12 × 4mm plate screwed in place	Stainless steel	*S. aureus*	ATCC 25,293,	1.0 × 10^4	Injected into defect
Laure 2008	Femur	7 mm diameter 45 mm deep reamed defect	Distal	Cancellous (trabecular)	Yes	Cylinder 7 mm Ø, 40 mm long	316L stainless steel Uncoated/coated med HA	*S. epidermidis*	Unspecified	2.0 × 10^8	Injected into defect
McLaren 2014	Femur	Drill hole 8 mm Ø, 15 mm deep	Distal	Cancellous (trabecular)	Yes	Amorphous	Polymer scaffold ± gentamycin and clindamycin	*S. aureus*	F2789	4.0 × 10^4	Inoculated into the defect
Moriarty 2017	Tibia	Reamed medulla	Whole bone	Medullary	Yes	Tibial nail	Titanium	*S. aureus*	EDCC-5443/ ATCC 25923	2.0 × 10^7	Added to a 36 mm × 18 mm collagen sponge
Qu 2014	Tibia	Reamed medulla	Whole bone	Medullary	Yes	IM nail 6 mm Ø, 140 mm long	i6Al4V Titanium alloy,	*S. aureus*	Unspecified	3.0 × 10^8	Injected into medullary space
Schaer 2012	Tibia	Mid-diaphyseal osteotomy	Mid	Cortical	Yes	8- or 9-hole LCP	Stainless steel	*S. aureus*	ATCC 25923	2.5 × 10^6	Inoculated via temporary indwelling silastic catheter
Schenck 1974	Tibia	Drill hole 10 mm	Mid	Cortical	Yes	Gauze	Cotton	*S. aureus*	ATCC 6538-P	6.0 × 10^8	Gauze soaked in bacterial suspension
Sinclair 2013	Femur	Drill hole 8 mm Ø, 35 mm deep	Distal	Cancellous (trabecular)	Yes	Porous cylinder 8 mm Ø, 25 mm long	Titanium	*S. aureus*	Unspecified	5.0 × 10^8	Contained in cylinder
Stewart 2012	Tibia	Osteotomy	Whole bone	Cortical	Yes	LCP plate	Titanium	*S. aureus*	ATCC 25923	2.5 × 10^6	14G Indwelling catheter

(*continued on next page*)

Table 5 (*continued*)

Study ID	Specific bone	Bone defect	Gross location in the bone	Position in bone tissue	Foreign material	Foreign material description	Foreign material type	Bacterial Strain	Bacterial identification number	Inoculation dose (CFU)	Inoculation method
Wannske 1976	Tibia	Drill hole 10 mm	Mid	Cortical	Yes	Gauze	Cotton	*S. aureus*	ATCC 6538-P	7.0×10^{8}	Gauze soaked in bacterial suspension
Watson 2020	Mandible	Full thickness bone defect	Mid	Cortical	Yes	Space maintainer	Unspecified	*S. aureus*	Xen36	1.0×10^{6}	Added to circular collagen sponge 10 mm Ø
Williams 2019	Femur	Drill hole 9.5 Ø, 20 mm deep	Distal	Cancellous (trabecular)	Yes	Porous plug	Titanium	*S. aureus*	Unspecified	2.0×10^{8}	Injected into implant
Williams 2012	Tibia	Plates were screwed to the bone	Proximal	Cortical	Yes	Plate	Stainless steel	*S. aureus*	Unspecified	5.2×10^{9}	In biofilms
Williams 2012	Tibia	Plates were screwed to the bone	Proximal	Cortical	Yes	Plate	Stainless steel	*S. aureus*	Unspecified	5.1×10^{9}	In biofilms

Table 6
Preoperative and postoperative systemic antibiotic use.

Study ID	Preoperative antibiotics	Systemic antibiotic therapy:	Duration of antibiotic therapy
Boot 2021	None	Vancomycin then rifampin-co-trimoxazole	Day 5–14 Day 14–42
Clasper 2001	None	Co-amoxiclav IV + IM sustained release + Gentacol in medulla and wounds	5
Foster 2021	Ceftifur 2.2 mg/kg IV 1 h before surgery	Vancomycin then Rifampicin + trimethazole	Day 14–28 Day 28–70
Gimeno 2018	None	Penicillin 1000 IU/kg	Day 0–4
Gimeno 2013	None	Penicillin 1000IU/kg	Day 0–4
Hill 2002	None	600 mg benzylpenicillin 500 mg Flucloxicillin	Day 0–7
Kaarsemaker 1997	None	Streptoprocpen 600 mg Penicillin 600,000 IU	Single dose 1 h after surgery
Moriarty 2017	Ceftiofur 2.2 mg/kg	Amoxicillin clavulanic acid	Day 56–70
Schaer 2012	Ceftiofur sodium 5.0 mg/kg, and procaine penicillin 22,000/IU	None	N/A
Stewart 2012	Ceftiofur sodium 5 mg/kg and 22,000IU/kg procaine penicillin 22,000IU/kg	None	N/A

5. Discussion

Overall, this systematic review suggests that, despite their relatively limited use thus far, sheep may provide a useful preclinical model for bone infection. All models analysed in this review successfully established local osteomyelitis and furthermore, the infection was well tolerated by the majority of animals over periods of up to 16 weeks. The animals, which were diverse with respect to breed and age, tolerated a range of different implants, defect types and anatomical locations. While inoculation dose varied according to experimental design, infection was successfully established with relatively low doses of bacteria. Furthermore, osteomyelitis was demonstrated both with and without the use of systemic antibiotics.

There is no perfect human bone equivalent amongst animal models, thus all species require compromises. The significant advantage with sheep is their comparable bone size and biomechanics permitting the use of larger orthopaedic implants. This along with their slower bone growth and bone turnover are the main advantages of the sheep model compared to pig, it's closest comparator. Indeed, a number of the models in this review successfully used standard human intramedullary nails [10,16,19,24,35,36], external fixators [11–13] and osteosynthesis plates [26,29]. Moreover, in Schaer et al. [26], and Stewart et al. [29] the use of compression plates was combined with bone osteotomy, thereby providing a model for the analysis of healing or infection prevention in the context of an infected internal fixation. While the majority of studies included in this review utilised skeletally mature animals, the success of the model across a wide range of ages, including potentially skeletally immature animals [37], suggests this model could be further developed and validated to assimilate paediatric osteomyelitis. Conversely, given the well-established osteoporotic sheep bone models [38], there is also encouraging scope to develop models assimilating osteomyelitis towards the upper end of the age spectrum.

The predominant bones for establishing infection were the tibia or

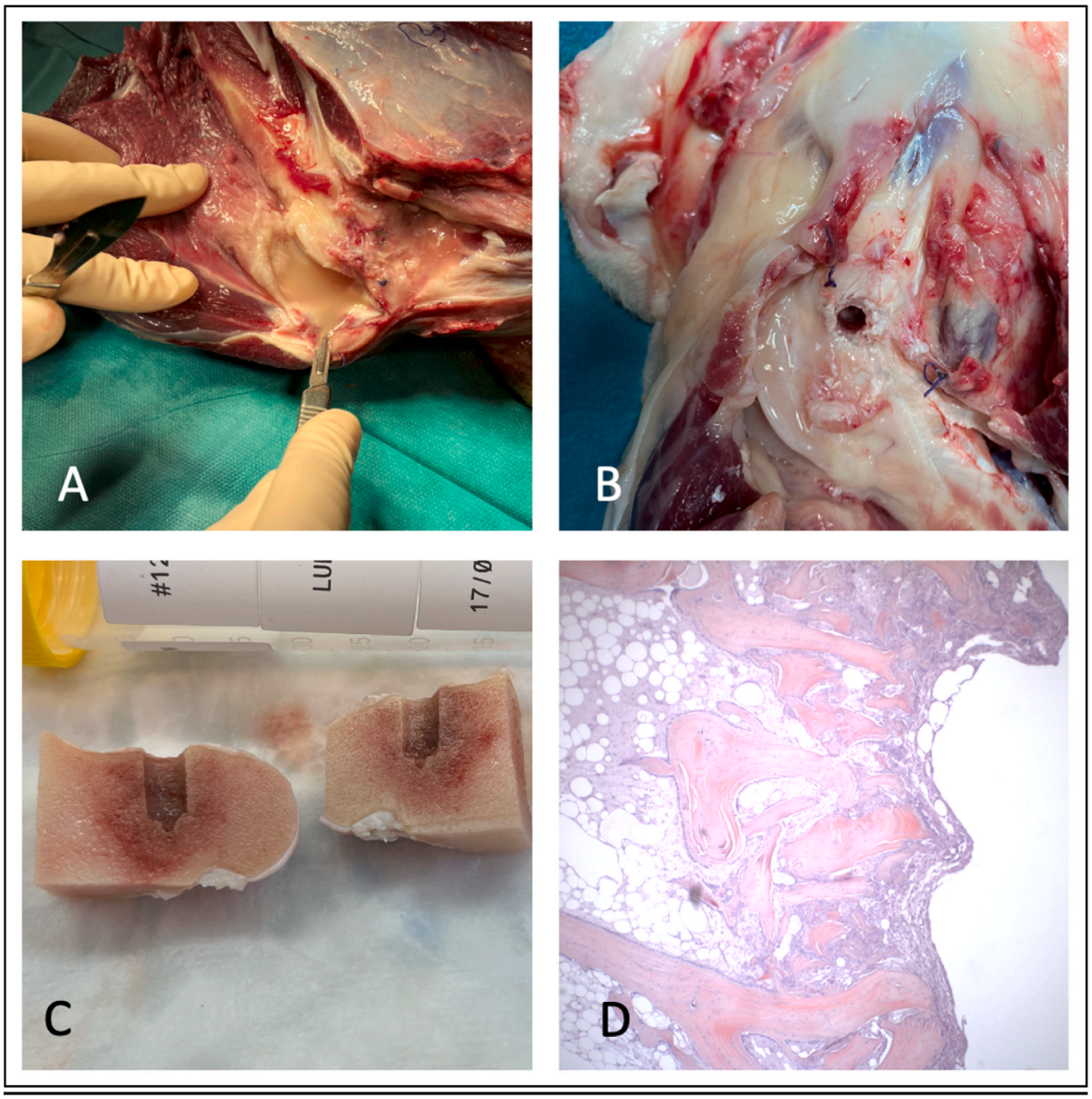

Figure 2. Representative images from ex vivo analysis of infected sheep femur following inoculation with *S. aureus*. Image A shows gross pathology with purulent soft issue infection. Image B shows the exposed femoral bone defect after removal of the implant. Image C shows the bisected femoral bone defect. Image D shows histological (H&E) section of bone adjacent to the femoral defect.

femur, this is likely due to their accessibility and representative loading as well as the predominance of limb surgery in orthopaedic surgery. Nevertheless, in the literature there are also examples of infections being established in ovine spine [39]. However, in these instances infection was targeted to the cartilaginous intervertebral disc rather than bone, thereby excluding these articles from our analysis. The lack of rise in systemic inflammatory markers, suggest the infection remains localised. An important alternative explanation is that the assays used are not sensitive enough to detect the associated changes. The infection was typically introduced in conjunction with cortical or medullary defects of varying sizes, of which the majority were considered critical. *S. aureus* was used almost universally as the infective organism, undoubtedly due to its dominance in clinical osteomyelitis and peri-prosthetic joint infection [40]. Importantly, we did not find evidence suggesting alternative bacterial strains were unviable, thus further validation of this model would not necessarily need to be restricted to *S. aureus*.

The inoculation dose and method varied significantly between studies, as did the methods for analysis. Bacterial inoculation predominantly involved direct injection into the defect or around an implant, within a range of 1×10^4 to 5.24×10^9 CFU. There was no discussion of the rationale behind the choice of inoculum dose and the substantial variations in study designs precludes a systematic assessment of related histopathological variations. Study design naturally varies according to the specific aims and is also typical where there are limited examples of the model. Furthermore, there was marked variation in the use of postoperative antibiotics. Some authors specifically identified the risk of eradicating the infection as the reason for not administering antibiotics, while others found that without some antibiotic protection the sheep became unwell and required euthanasia during pilot studies. In our experience at lower doses of infection antibiotics are unnecessary and indeed their use can lead to early clearance of the infection (unpublished data). Importantly, the lack of complications reported in these studies, despite the absence of antibiotic therapy is potentially a significant advantage over shorter study periods. In such cases, the potential of interventions targeting early stages of infection, may otherwise be obscured by systemic antibiotic therapy. The majority of studies

Table 7
Methods used for analysis *in vivo* and after euthanasia.

Study ID	Clinical analysis	Blood tests	Rise in systemic Inflammatory markers	*In vivo* bone imaging	Histological investigations	Microbiology	*Ex-vivo* bone imaging	Evidence of local osteomyelitis
Boot 2021	Weight, clinical scoring, temp	WBC	No	Radiographs	Bone biopsy	Bone marrow hardware biopsy, bolt holes and inoculation point	None	Yes
Clasper 2001	Visual inspection	None	N/A	None	None	Non-quantitative culture	None	Yes
Clasper 1999	Visual inspection	None	N/A	Radiographs	None	Medulla and pin	None	Yes
Clasper 2001	Visual inspection	None	N/A	Radiographs	None	Non-quantitative analysis of proximal and distal metaphysis, the IM nail, fixator pin tracts and joint.	None	Yes
Collinge 1994	Visual inspection	None	N/A	None	None	Quantitative analysis of bacteria on pins	None	Yes
Ferrell 2019	Modified Checketts scoring	Systemic Turbomycin Concentration	N/A	None	Sanderson's rapid bone stain (SRBS)	Quantitative culture, Soft tissue, Bone surface	Micro-CT	Yes
Foster 2021	Visual inspection	Systemic antibiotic concentrations	No	Radiographs	None	Quantitative culture, bone, soft tissue, IM nail	Radiographs	Yes
Gimeno 2018	Temperature	FBC	No	None	Gram staining	Skin, bone of implant area. lung, heart, brain, liver, spleen, kidney GI for gram staining. Swabs from implant site	Radiographs	Not specifically assessed
Gimeno 2013	Checketts criteria	FBC	No	Radiographs	Histopathology H&E stain of the soft tissues around implant and lung, liver, kidney, spleen, GI tract	Swabs from the surgical wound	Radiographs	Yes
Hill 2002	Visual inspection	Flucoxicillin assay	N/A	Radiographs	None	Swabs and screws	N/A	Yes
Kaarsemaker 1997	Temp, visual inspection	Leukocyte count	Yes	Radiographs	Bone marrow H&E staining	Tissue samples	MR scans of two sheep	Yes
Klein 2021		None	N/A	Radiographs	Bone from defect, H&E staining	Sonicated steel plate and screws	Radiographs	Yes
Laure 2008	Visual inspection	None	N/A	Radiographs	H&E stain joint synovium, interposition tissue, and bone samples	Joint fluid and cement samples	Radiographs	Yes
McLaren 2014	Visual inspection	Blood cultures	N/A	None	Modified tetrachrome of surrounding bone, H&E soft tissue	Swabs, blood cultures	Micro CT	Yes
Moriarty 2017	Visual inspection	WBC, ESR, CRP	No	Radiographs	None	Nail, bolt bone marrow, cement, cortical bone	Radiographs	Yes
Qu 2014	Visual inspection	None	N/A	Radiographs	None	Swab of IM nail, medullary cavity and screw	None	Yes
Schaer 2012	Visual inspection	FBC and Fibrinogen	No	Radiographs	H&E tibial osteotomy region	Region of interest swab	Micro CT	Yes
Schenck 1974	Visual inspection	Full blood count	No	Radiographs	Bone around defect, method unspecified	Swab from bone	Radiographs	Yes
Sinclair 2013	Visual inspection	None	N/A	None	H&E	Skin, subcutaneous, intramuscular, and bone swabs. implant	Radiographs	Yes
Stewart 2012	Visual inspection	FBC and Fibrinogen	No	Radiographs	Bone osteotomy region	Swab from osteotomy and tissue sample	Micro CT	Yes
Wannske 1976	Visual inspection	FBC	No	Radiographs	Bone antibiotic content	Bone swab	Radiographs	Yes
Watson 2020	Visual inspection	WBC	Yes	None	Methylene blue-basic fuchsin staining, Histomorphometry	Swabs from defect	Micro CT	Yes
Williams 2019	Visual inspection	None	N/A	Radiographs	Light microscopy	Swabs and tissue sample from synovium. bone around defect and implant, bone marrow sample	SEM	Yes

(*continued on next page*)

Table 7 (*continued*)

Study ID	Clinical analysis	Blood tests	Rise in systemic Inflammatory markers	*In vivo* bone imaging	Histological investigations	Microbiology	*Ex-vivo* bone imaging	Evidence of local osteomyelitis
Williams 2012	Visual inspection	None	N/A	None	Soft tissue over plate, Sanderson's Rapid Bone Stain	Swabs form region of interest	SEM, Radiographs	Yes
Williams 2012	Visual inspection	None	N/A	None	H&E	Swabs incision site,	SEM	Yes

included histological analysis, primarily using H&E staining. These analyses revealed varied inflammatory responses and bacterial localization. Some studies utilized Brown and Benn, calcein labeling, and von Kossa staining for further histological characterisation.

In the studies considered in this review a range of different breeds and age ranges were used. Although differences in breed have not been objectively examined, it is likely there are differences in bone macro- and microstructure between different genetic lineages. An advantage of the more established porcine model is the availability of inbred and pathogen free strains. However, with increasing utilisation of sheep as preclinical models, one would expect the development of more standardised strains. In these studies, there are a wide range of ages utilised, with limited explanation. The effect of age on bone metabolism is well documented across diverse animal models [41–43]. Accordingly, with further development of the model, the age-related metabolic changes in bone and its relevance to the human disease modelled, should be carefully considered. With the exception of one study, female animals were used exclusively in these models. While there is no explanation given, we speculate that this is predominantly due to availability, as the overwhelming majority of male lambs are castrated shortly after birth in traditional animal husbandry (personal communication with our animal supplier).

The success in developing osteomyelitis even with diverse protocols, suggests sheep offer a flexible basis for a preclinical model. However, this heterogeneity in methodology may preclude comparisons of results between distinct sheep models, and in our case prevented reliable meta-analysis. The culture in preclinical research is increasingly moving towards the more stringent protocol designs seen in clinical research. Furthermore, there are increasing calls for meta-analyses of animal data, to support the transition to clinical research [44]. Accordingly, working towards a consensus in protocol design will likely augment the success of data derived from ovine osteomyelitis models. In future, a greater understanding of the pathogenesis of *S. aureus* infection in sheep bone may help support a consensus of model parameters, or even a standardised model, thereby providing more widely comparable analyses. This may be particularly relevant to research into implant related infection and its prevention, where an understanding of typical periprosthetic bone destruction and cellular response could greatly advance research into future prevention and treatment strategies.

6. Future perspectives

Based on the findings of this review, we tentatively propose the following sheep model for research into adult osteomyelitis and PJI. Importantly, there are diverse successful models in this review; however, we present areas of greater collective agreement, as a potential scaffold for future consensus. We suggest using female adult ewes, with a relatively uniform bodyweight of approximately 70 kg. Infection should be established in the femur or tibia either intramedullary or cortically. For practical purposes the distal femur and proximal tibia are easily accessible. Unless specific bacteria are being assessed *S. aureus* is a clinically relevant infectious agent. An inoculation dose of 1×10^4 to 5.24×10^9 is evidently viable, however, pilot studies may provide more precise doses suitable for the specific aims of the study. Planktonic bacteria should be injected directly into a bone defect with or without implanted foreign material, avoiding soft tissue contamination. In short term studies (days-weeks) preoperative or postoperative antibiotics are probably unnecessary, however in studies over the longer term (weeks-months) systemic antibiotics should be considered, potentially, after osteomyelitis or PJI is established. For clinical monitoring during the study, the modified *Checketts scoring system* is widely used [28,45]. Blood tests are unlikely to show rises in systemic inflammatory markers and therefore may not provide sufficient cost/benefit either economically or in terms of animal welfare. Acute osteomyelitis can be confirmed microbiologically and, in chronic cases, characterised radiologically. More detailed results can be achieved with histology and immunohistochemistry. We suggest the study is designed and reported according to the guidelines proposed by Jensen et al. [46].

While our focus is on the viability of sheep as a large animal bone infection model, and thus detailed evaluation of the treatments assessed is essentially beyond the scope of this review. It is important to note that 12 of the 25 studies, utilised sheep in the evaluation of interventions to prevent peri-implant infections with remarkably encouraging results. The overwhelming focus was on antibiotic eluting implants, which overall, demonstrated histocompatibility as well as microbiological and histological evidence of bacterial clearance, reduced inflammatory infiltration and improved bone healing. In addition, similarly promising results were found with the Ceragenin CSA-13, and Micron-Thin Sol–Gel Film coating of implants respectively. Collectively these results show significant promise, and moreover, their success in large animal model suggests potentially viable clinical therapies.

7. Strengths and limitations

This systematic review included 25 articles using sheep as an infection model. We feel confident our search strategy has captured the majority of articles available partly as the total included is significantly greater than other systematic reviews which have included sheep models. Unfortunately, we were unable to reliably extract data from Chinese and French articles resulting in their exclusion which represents a limited selection bias. We have chosen not to exclude older articles for completeness, and as examples of this model are limited. However, we recognise that as reporting of animal studies has evolved to be more comprehensive, the information available from earlier articles is limited. Nevertheless, key data regarding the inoculation, bacterial stain and confirmation of bone infection was available thereby justifying their inclusion. Notably a number of these articles are from the same research groups utilizing the sheep model in different experimental settings. While this can be seen as a limitation to the diversity of models assessed, one can also argue that once the model is established within the research group, its continued use suggests satisfactory results.

Overall, the studies included in this review scored poorly on the SYRCLE risk of bias tool, largely due to the absence of randomisation and blinding. This is in part due to the age of the studies included, with some published at a time when there was perhaps less emphasis on these aspects. However, we also note that the score of more contemporary studies was not remarkably better. One could argue that as we are focusing on the experimental designs, and neither conducting meta-analyses nor extrapolating therapeutic results of animal-based experiments to humans, the risk of bias scoring is not strictly necessary. Nevertheless, although controversial, there is increased scrutiny on animal studies, and a move towards implementing the same rigorous

scientific methods expected in human trials. Accordingly, we concluded that in principle the risk of bias scores should be included, though we also contend that the poor scoring does not substantially detract from our results.

8. Conclusion

With this review we have gathered the available information on sheep bone infection models and provided an overview for future development and utilisation. As the study designs thus far are diverse, we have been unable to evaluate in depth the pathogenic effect of bacteria in sheep bone with varying inoculation doses or antibiotic concentrations. However, as more research using sheep models are published a greater understanding will develop. Ultimately, our hope is that the data presented in this review will serve as a reference and inspiration for future protocol design. Furthermore, with increasing awareness, sheep models will become more prevalent and potentially lead to a valuable standardised preclinical model for the development of viable therapeutic strategies. Collectively we surmise that these studies demonstrate a reliable and adaptable model with significant further potential.

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Declaration of competing interest

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