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A Preliminary Protocol for Induction of Tuberculosis Spondylitis by Mycobacterium Tuberculosis Strain H37R: In-vivo New Zealand White Rrabbits Model

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ABSTRACT

Background: Proper animal model is a critical prerequisite of any experimental study, while it is a lack for tuberculosis spondylitis. This study aimed to validate a protocol for induction of tuberculosis spondylitis in an animal model.
Methods: Ten New Zealand white rabbits were used and divided randomly into four experimental groups (n = 8), with 2 rabbits in each group, and the control group (n = 2). A 0.2 mL suspension of 108 CFU/mL H37Rv strain M. tuberculosis was delivered into the drilled hole with a depth of 6-10 mm at the midpoint of the 12th thoracal corpus compared with the saline in the control group. Evaluation of imaging examination by using plain X-ray was done within the targeted time followed by euthanasia protocol.
Results: A survival rate of 100% was observed in the present study with all rabbits having stable vital signs during the incubation period. Group 3 with an incubation period of 6 weeks showed the highest success rate for inoculation of M. tuberculosis bacteria in which 7 samples were positive. This was followed by group 2 (75%, incubation 4 weeks), group 1 (37.5%, incubation 2 weeks), and group 4 (12.5%, incubation 8 weeks). The high positive rates were also reported by using culture and PCR staining, 62.5% and 75%, respectively. A slight destruction of the vertebral body was observed in both groups 2 and 3 starting at 4 weeks, postoperatively. Histopathology specimen exhibited an infiltration of numerous inflammatory cells.
Conclusions: Establishment of spondylitis tuberculosis model by using New Zealand white rabbit could be successfully formed through 0.2 mL suspension of 108 CFU/mL M. tuberculosis strain H37Rv with a direct inoculation method towards the midpoint of the 12th thoracal vertebral body. In addition, this model showed a high positive rate with a 100% survival rate in both control and 4 treatment groups

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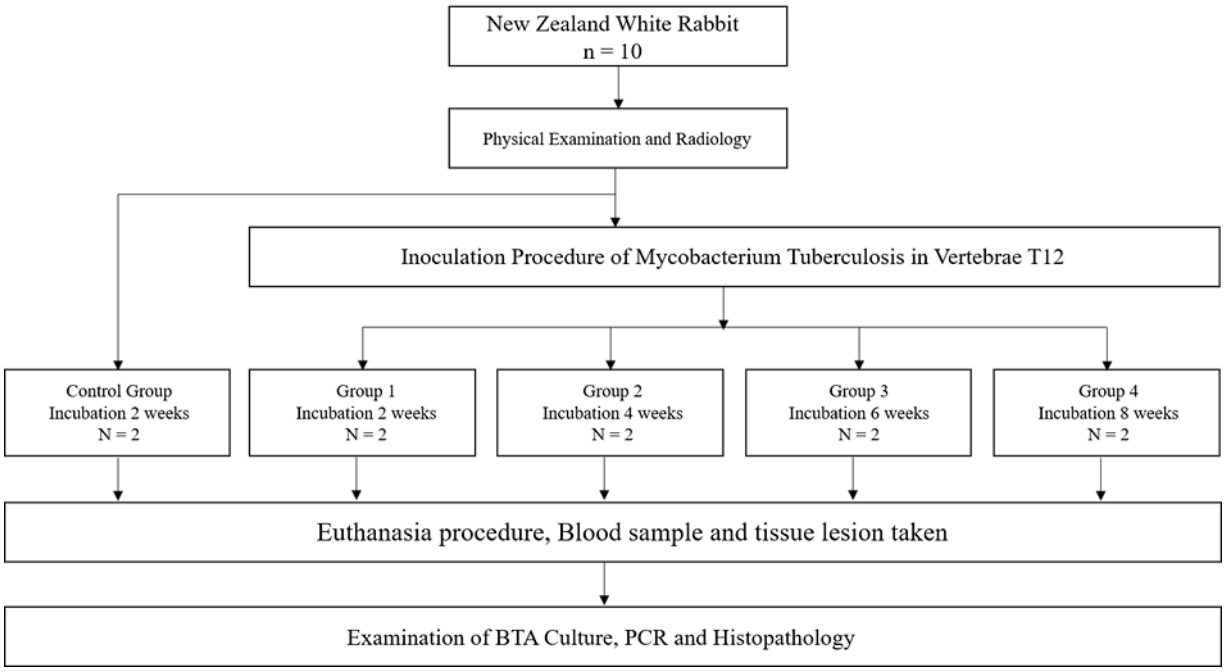
GRAPHICAL ABSTRACT



Introduction

Tuberculosis (TB) infection remains to be a global health problem in which an estimation of a third of the world's population is infected with it. From all of the cases, extrapulmonary TB (EPTB) may occur in 20% of patients with pulmonary TB. Osteoarticular TB contributes to around 35% of EPTB and the spine becomes the most commonly affected site of infection, accounting for approximately 50% of osteoarticular TB. *Mycobacterium tuberculosis* infection in the spine, also known as spondylitis TB, can cause damage to the corpus of the vertebra and its adjacent structures resulting in spinal instability [1-4]. The pathogen transmission in spondylitis TB may result from a primary infection by a direct bacterial infection to the vertebral body or secondary infection by haematogenous or lymphomatous spread from other sites to the vertebra. The pulmonary and genitourinary lesion becomes the primary site of the secondary infection spread. A rich vascular plexus of the arterial arcade and Batson's paravertebral venous plexus will further facilitate the haematogenous. In addition, central vertebral lesions may be caused by spread through the intraosseous venous system [1,2].

The animal model becomes an essential and important instrument to better comprehend the pathogenesis and pathophysiology of a disease. Furthermore, this model is prominent in investigating the effectiveness and safety of new drugs or other possible surgical treatments. Several types of animals have been used as experimental models for TB infection, including mice, guinea pigs, rabbits, and primates [5,6]. Each model represents different characteristics of TB infection in humans so the selection of the right model is essential [7]. The New Zealand white rabbit becomes the preferred model because of its relative relation in the context of disease progression and considerable susceptibility to *Mycobacterium tuberculosis* infection [8-12]. Besides, the characteristic of the rabbit spine which is not too small will make it possible to induce infection or perform other interventions in an easier way [13-15].

Making animal models of spondylitis TB can be achieved through a primary or secondary infection approach. The secondary infection approach by using a closed aerosol pump system is considered as a more ideal method since it is consistent with the more common pathogenesis. Previous studies have shown successful inoculation of *Mycobacterium tuberculosis* by using aerosol spread by 6–33 weeks of

exposure [16-18]. However, the issues of time consumption, difficult procedures, and high cost become consideration since the level of Animal Bio Safety Level 3 (ABSL3) need to be fulfilled in the facility to achieve accepted biosafety and biosecurity standard. A low positive survival rate remained to be reported. In addition, the standard dose of inoculated bacteria and the length of observation duration persist to be unclear [13-15,19]. Therefore, in the present study, we tried to establish a spinal tuberculosis model in rabbits by using direct inoculation of mycobacterium tuberculosis strain H37Rv and evaluate its efficacy based on histopathological, immunohistochemical, radiographical, and bacteriological examinations.

Previous studies had shown successful inoculation of MTB in rabbits model, whether it is pulmonary or osteoarticular TB. New Zealand white rabbit is reported to have a considerable sensitivity and susceptibility towards MTB. In addition, the spine characteristics of this kind of rabbit are not too small in size which allows for easier and more variety of interventions. However, a high mortality with subsequent low positive rates remained to be reported. In this present study, we aim to investigate and evaluate the establishment of a spinal TB model in rabbits by using the direct inoculation method into the vertebral body.

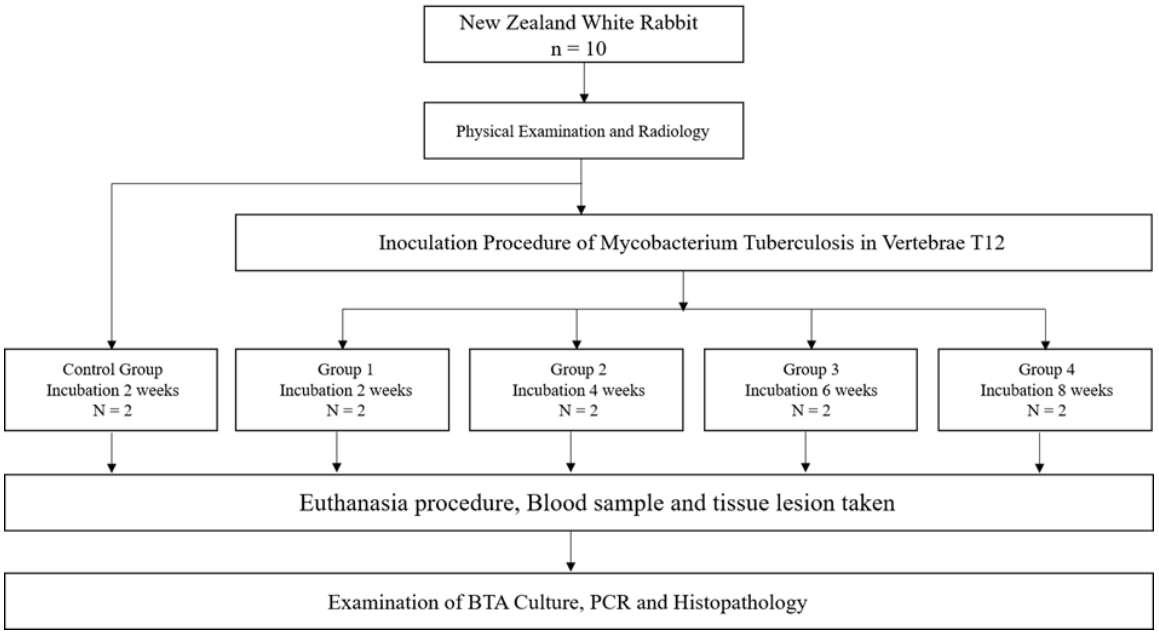
Materials and Methods

Ethics Statement

This present study was an animal study by using a rabbit model. All animals involved in this study received care and treatment in compliance with the guide of the Government of the Republic of Indonesia's Regulation No. 95. 2012 (Veterinary Public Health and Animal Welfare) and the National Guidelines on Health Research Ethics by Health Research Ethics Committee, the Ministry of Health, the Republic of Indonesia, as the guidelines followed for the welfare and treatment of laboratory animals. The experiment protocol was reviewed and approved by the Animal Ethics Committee Faculty of veterinary medicine, Bogor Agricultural University with ethic No. 003/KEH/SKE/II/2020.

Animals

This study used white rabbits of the New Zealand strain. The sample selection was based on the rabbit's body weight, bone maturity, sex, clinical, radiological, and laboratory examination. In addition, this selection process was managed by a Laboratory Animal Basic Training (in-house)-certified veterinarian with experience of more than 10 years of working with laboratory animals. The inclusion criteria were skeletally-matured rabbits weighing a range between 2500-3500 grams which were in a healthy condition. The exclusion criteria were rabbits with congenital spine anomaly and/or other spine abnormalities caused by trauma, infections, or neoplasm condition (Flowchart 1).



Flowchart 1: Research Protocol and Rabbits Grouping

M. Tuberculosis Suspension

Single-cell suspension of human *Mycobacterium tuberculosis* strain H37Rv was prepared in Middle brook's liquid medium. This bacterial suspension was further homogenized and incubated in a shaker with a speed of 150 rpm and an environmental temperature of 37°C for 18 hours. The suspension was then diluted by using sterile saline to obtain a number of bacteria of 10^8 CFU/mL (colony forming units/millilitre) or equivalent to 0.132 A (absorbance) of Optical Density (OD) by using measurement in the wavelength of 600 nm. The preparation protocols referred to the standard operating procedures implemented in the Clinical Microbiology Laboratory of the Faculty of Medicine Universitas Indonesia.

Experimental Design

Ten adults New Zealand white rabbits were divided into 5 groups by using a randomization table as follow: 8 rabbits in the 4 experimental groups and 2 rabbits in the control group in which the induction procedure was not carried out. The first experimental group was inoculated with *Mycobacterium tuberculosis* bacteria for 2 weeks, while the second experimental group was for 4 weeks, the third experimental group for 6 weeks, and the fourth experimental group for 8 weeks. Each experimental group consisted of 2 rabbits, as displayed in Figure 1. During the observation of the study, all rabbits lived separately in individual cages with regulated living environments and feeding programs in the form of pellets and rabbit feed. After the targeted duration of incubation period was achieved, a euthanasia procedure using euthal injection of 150 mg/Kg was performed, and then followed by necropsy to collect vertebral tissue samples.

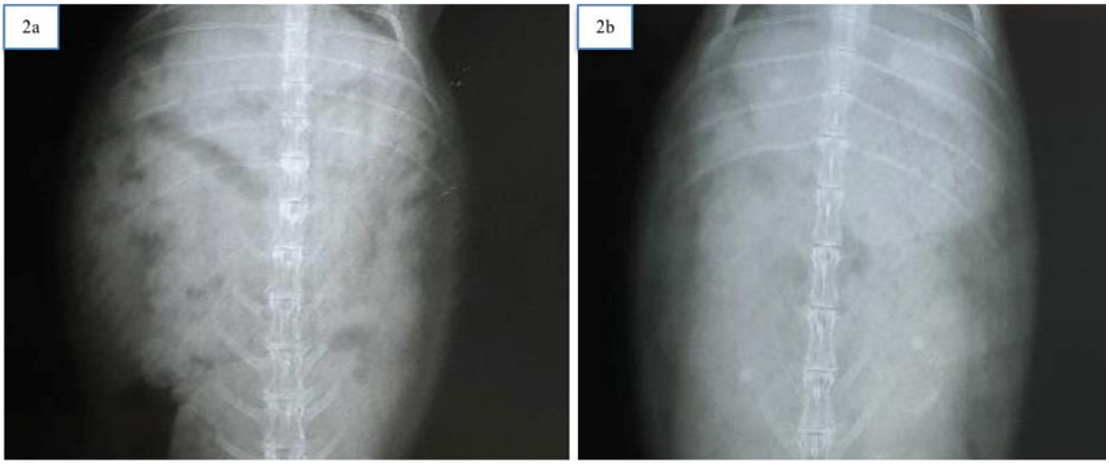


Figure 1: A slight destruction and disc space narrowing with sclerotic changes and relatively irregular bone density suggesting the presence of inflammatory reaction were observed in the plain X-ray of T12 vertebral body in the a) experimental group, compared with b) control group

Preparation of Animal Model

Anesthesia

3% of sodium pentobarbital (30 mg/kg) was administered via the auricular vein for anaesthesia. In addition, an intramuscular injection of an anaesthetic mixture of ketamine HCl (44 mg/kg) and xylazine (5 mg/kg) was delivered.

Inoculation of M. tuberculosis

After the administered anaesthetics had worked, the rabbits were placed in the right lateral decubitus position, in which the left side of the rabbit's back was faced towards the operator. A routine antiseptic and aseptic procedure by using 70% alcohol and betadine were implemented on the shaved backs of the rabbits, followed by draping with a sterile cloth with the surgical site uncovered. The 12th thoracal vertebra was identified by tracing down the 12th rib and its transverse processes. A transverse skin incision of 5 cm width was made starting from the spinous process towards the left lateral area of the shaved back. The transverse process and lamina of the 12th thoracal vertebra were exposed by separating paraspinal muscle. A hole of 6-10 mm depth was drilled at the midpoint of the 12th thoracal corpus

(+5 mm from the transverses process) by using a 1.5 mm drill bit. After haemostasis was achieved, 0.2 mL suspension of 10^8 CFU/mL *Mycobacterium tuberculosis* was administered aseptically into the hole. Subcutis fat was used to cover the hole by using a root dissector. The incision was then closed layer by layer and the surgical wound was covered by bandages. The rabbits were put back in their cages for post-surgical recovery and observation. Intramuscular ketoprofen (3 mg/kg) was administered every 12 hours for 3 days. The surgery protocols were in accordance with the principles of the standard precautions, established safety measures, aseptic technique, and animal ethics.

Study Parameters

Clinical examinations of general conditions were observed, including daily activities, the presence of wound healing, signs of infection, paralysis, appetite, and body weight. The observation was done twice a day at 8 a.m. and 4 p.m. and body weight was measured for 3 days. At the end of the 2nd, the 4th, the 6th, and the 8th week, radiological examination was performed by using plain X-ray for each treatment group followed by euthanasia protocol by intravenously euthal injection of 150 mg/Kg. Samples from the infected lesions were then harvested for further examinations. In this present study, the success of *Mycobacterium tuberculosis* bacteria inoculation in the vertebral bodies of rabbits was assessed based on 4 modalities, including Acid Fast Bacilli (AFB) staining, culture, histopathology, and Polymerase Chain Reaction (PCR) examination.

The BTA staining was mentioned to be positive if 1 or more AFB were found in 100 fields of view. A positive culture examination was declared if there was bacterial growth in the Lowenstein-Jansen medium after 9-14 days followed by positive Niacin and PNB tests. Positive histopathological examination results were stated if there was a tissue reaction to Mycobacterium tuberculosis bacterial infection finding, such as the presence of Datia Langhans cells, giant cells, caseos necrotic tissue, and so on. Staining was performed with the samples from the affected vertebral body, upper and lower endplates, or adjacent intervertebral disk. The PCR result was declared to be positive if there was a picture of the DNA band of the *Mycobacterium tuberculosis* H37RV, which is the same bacteria inoculated into the vertebral body of rabbits in this study. The assessment of these parameters was in accordance with WHO and IUATLD recommendation.

Results

Animal General Conditions

All ten rabbits, in both experimental and control groups, successfully survived until the end of the incubation period and completed the experiment. All the rabbits had stable vital signs without remarkable changes in appetite and body weight. In addition, there were not any significant adverse events, such as peritoneal rupture during surgery, postoperative paraplegia, post-operative trauma, pulmonary complications, or multi-organ failure. All the rabbits were euthanized within the scheduled time without any re-adjustment to the protocols of the experiment.

X-ray Findings

A slight destruction with sclerotic changes of the T12 vertebral body was found radiographically in the plain X-ray imaging starting at the incubation period of 4 weeks post-surgery. Likewise, the findings showed intervertebral disc changes in which the intervertebral space became slightly narrower and slightly blurrier with the new formation of osteophyte. Besides, the corpus and endplates density was relatively irregular, as demonstrated in Figure 2. In contrast, there was no sign of abnormal vertebral changes in the control group, either at the 2nd, the 4th, the 6th, or the 8th week, postoperatively.

Gross Anatomic Observation

There were different degrees of destruction across the vertebral endplates, vertebral body, and intervertebral disc observed in 5 of all the 8 surviving rabbits in the experimental group with positive histopathological findings. Besides, granulation tissue and necrotic substance were detected within a close range of the inoculated vertebra area. However, there was no obvious dissemination of *M. tuberculosis* in the liver, lung, or other organs of these rabbits. In addition, no apparent bone or intervertebral destruction and

paravertebral abscesses were found in the control group or rabbits in the experimental group with negative histopathological examination and imaging findings.

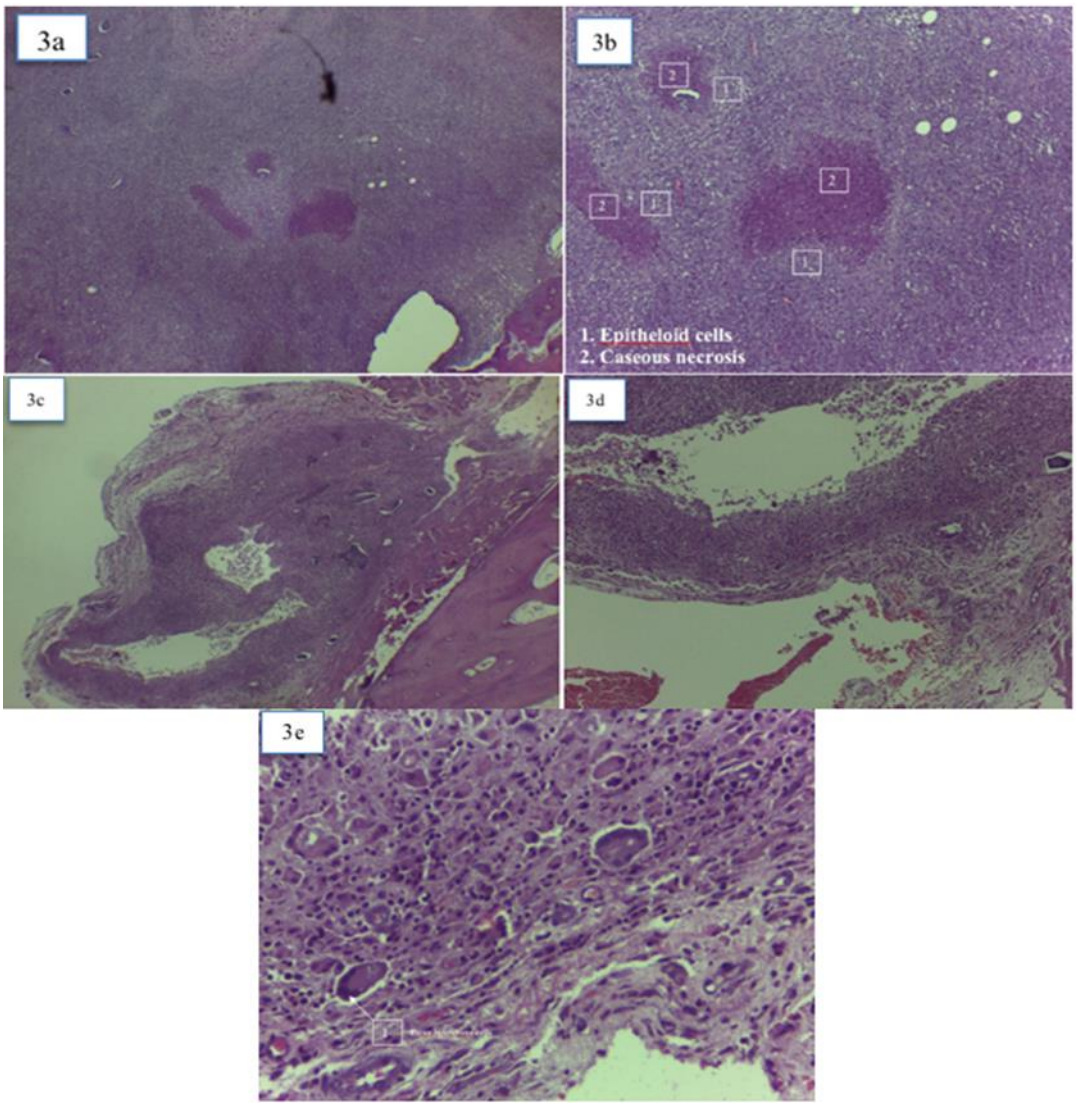


Figure 2: Histopathological examination with HE staining showed the presence of a) inflammatory cells infiltration (40x magnification), b) epitheloid cells and caseous necrosis (100x magnification), c) tubercle (25x magnification), d) macrophages (100x magnification), and e) datia langhans cells (400x magnification)

Histopathological Examination

There were 2 samples from all 8 rabbit samples in the experimental group that had tissue reactions to *Mycobacterium tuberculosis* bacterial infection. Infiltration of inflammatory cells consisting of countless lymphocytes, macrophages, limited epithelial cells, and caseous necrosis reaction was observed in the histopathological specimen in addition to the bony destruction and tubercle formation in the affected vertebral region, as displayed in Figure 3. Nevertheless, normal trabecular bone structures with neither inflammatory cell infiltration nor epithelioid cell formation were observed in these 2 specimens.

TB Culture Detection

The result of culture was positive in 5 of 8 rabbits in the experimental group. There were colonies with a slightly pale yellow appearance observed on the medium. No culture growth was found in the other 5 specimens, specifically 3 rabbits from the experimental group and 2 rabbits from the control group. In addition, there was no contamination in all the 10 specimens.

Inoculation Success Rate

The success of *Mycobacterium tuberculosis* inoculation on rabbit vertebral bodies was assessed based on BTA staining, culture, PCR, and histopathological examination. The results of these examinations and the percentage of

successful inoculation from each group are represented in Tables 1 and 2.

Table 1: The Result of Successful Inoculation of *Mycobacterium tuberculosis* Bacteria

Group	Examination	Successful Inoculation of Mycobacterium tuberculosis Bacteria
Control	BTA positive	0 (n=8)
	Culture positive	0 (n=8)
	PCR positive	0 (n=8)
	Histopathology positive	0 (n=8)
1	BTA positive	1 (n=8)
	Culture positive	1 (n=8)
	PCR positive	1 (n=8)
	Histopathology positive	0 (n=8)
2	BTA positive	1 (n=8)
	Culture positive	2 (n=8)
	PCR positive	2 (n=8)
	Histopathology positive	1 (n=8)
3	BTA positive	2 (n=8)
	Culture positive	2 (n=8)
	PCR positive	2 (n=8)
	Histopathology positive	1 (n=8)
4	BTA positive	0 (n=8)
	Culture positive	0 (n=8)
	PCR positive	1 (n=8)
	Histopathology positive	0 (n=8)

Table 2: *Mycobacterium tuberculosis* Bacterial Inoculation Success Rate

Group	*Mycobacterium tuberculosis* Bacterial Inoculation Success Rate
Control	00.00% (0/8)
1	37.50% (3/8)
2	75.00% (6/8)
3	87.50% (7/8)
4	12.50% (1/8)

Discussion

Establishing animal models become a critical step in the research of disease and its treatments. For decades, various animal models have been used to predict the body's immune response to tuberculosis infection for the purpose of diagnostic and treatment research. These models included rats, monkeys, goats, rabbits, deer, guinea pigs, and others [5-10]. In the present study by using New Zealand white rabbits, a 100% survival rate of rabbits in both the experimental and control groups was achieved and the establishment of the model succeeded in the experimental rabbits. In addition, a high success rate of 87.5% inoculation based on BTA staining, culture, PCR, and histopathological examination was observed in the rabbits with an incubation period of 6 weeks. The proper animal selection, bacterial strain, dosage, and route of infection are considered as three main factors involved in successful establishment of spondylitis tuberculosis in our model.

Thorough and comprehensive studies on TB infection by using rabbits as the primary model have been performed extensively [10,12-16,19]. A

rabbit model has been the best model to evaluate the effectiveness of drugs or other treatment regimens in bone TB, lately TB vaccine by using the attenuated form of *M. bovis* bacillus Calmette-Gu'erin (BCG) [11,20]. The rabbit itself has different characteristics from the other two animals, namely the ability to form cavities in response to the infection process so that this condition may help in understanding the factors that cause and influence the disease progression [14,21]. Previous studies in rabbits had also revealed a response of immunity for the *M. tuberculosis* [10,13,14,22,23]. Besides, the dimension of the rabbit spine which is not too small will facilitate the induction of infection or other interventions as a basic surgical procedure in manageable way interventions [13-15,19]. These factors became the considerations for using rabbits as a proper and appropriate animal model in our study.

The Mycobacterium tuberculosis strain H37RV was used in the current study. This strain has been widely utilized for laboratory research on Tuberculosis infection [24-26]. Highly invasive, infectious, transmittable, and comparable antigenicity characteristics were observed similarly with tuberculosis in the clinical setting. In addition, its moderate level toxicity caused an easier control in managing the number of bacteria compared with the wilder strains or species. Both pulmonary and osteoarticular tuberculosis models have been also successfully established by using this strain [13-16,18,19]. In addition, the dosage of H37RV *Mycobacterium tuberculosis* is considered as a critical factor in creating this spinal tuberculosis model in rabbits. A very high dose will result in excessive dissemination of bacteria, leading to a widespread infection and even death, while a lower dose will be associated with a low positivity rate in the local lesion.

A preliminary study by Liu et al. indicated the death of all rabbits involved in the experiment when 0.3 ml of a 5 mg/ml TB suspension was used. Reducing the amount of TB suspension to 0.2 ml remained resulted in half of the experimental rabbit's death [14]. In contrast, a study by Rahyussalim et al. in which 0,1 ml of 10^7 CFU/mL TB suspension was used for a period of 14 weeks incubation in a rabbit model reported positive results that were only found in 1 PCR and 1 histopathological examination [15]. Nevertheless, the use of 0.2 mL suspension of 10^8 CFU/mL in the present study was determined to be the accepted dosage since a high survival rate of 100% with a high positivity rate of 87.5% was achieved. This is in accordance with the theory that rabbit has sufficient resistance to the bacterium Mycobacterium tuberculosis H37RV so a larger dose of Mycobacterium tuberculosis (>10^3 CFU/ml) is needed to establish an effective infection [15,27].

Establishing a spondylitis tuberculosis model can be achieved by using an indirect paravertebral inoculation, vasa vasorum injection, aerosol inhalation, or direct inoculation procedure [14,18,28,29]. In inoculation through an indirect paravertebral access, the dispersed bacterial suspension towards adjacent soft tissues became the main concern. This condition was associated with the spread of *M. tuberculosis* to the sites which did not happen to be the focus of the experiment. In addition, ambiguous lesions might be observed. The most comparable route with the typical infection in clinical settings can be achieved through vasa vasorum injection. However, the small size and frail structures of terminal arteritis feeding the vertebral bodies came to be the issue during the inoculation procedure. Besides, emboli can happen in this tiny vessel due to the presence of *M. tuberculosis* itself [28-30]. Poor control and management of lesions in other sites of the body, especially in the lung, may also result in hematogenous spread of bacteria which may lead to the establishment of TB infection in the spine. The droplet nuclei formed from *M. Tuberculosis* suspension or liquefaction were dispersed through a closed aerosol pump system. Yet, there was the standard requirement of facilities with ABSL3 to perform the protocol procedures [16-18].

In this present study, we used the direct inoculation procedure toward vertebral body to introduce the bacteria in establishing a spondylitis tuberculosis model. Previous studies had successfully created osteomyelitis model in animals by using a direct inoculation to the

targeted bone [31,32]. In addition, this technique had been successfully used by previous researchers to establish a spinal TB model in rabbits [13-15]. The site of implantation in the centre of vertebral body with a distance of 5 mm from the upper or lower endplate and a depth of 5-10 mm suggested an optimal area for implantation and development of spinal TB in our study. The lateral approach was used in this study due to the more adequate and optimal exposure of vertebral body and intervertebral disc to perform the interventions. This technique can minimize accidents or complications during the surgery [33]. Moreover, the study by Liu et al. reported that lower blood loss and complication rates with higher survival rates were observed in the subject with a lateral approach compared with the posterior approach [14].

Establishing an accurate diagnosis of spondylitis TB has become a difficult issue, especially during the early stage of the disease. The specific clinical manifestations were reported to be blurry and unclear during this period [34,35]. However, in recent years, with improvements in diagnostic tools, such as polymerase chain reaction, and imaging techniques, the earlier diagnosis of *M. tuberculosis* infection in spine has become possible to establish. In addition, histopathological and culture examination remained to be essential and necessary for TB diagnosis [1,2,35]. The early diagnosis of spondylitis TB is important since it will lead to a proper and prompt treatment so that the disease progresses can be prevented towards poor conditions and complications.

Imaging examination by using the plain X-ray has become the most fundamental radiographic modality. This imaging technique is the most commonly used method due to its good availability and inexpensive cost. Destruction across vertebral body, its endplates, intervertebral disc space narrowing, and kyphotic or gibbous deformity become the common findings and typical characteristics of spondylitis TB plain X-ray [36,37]. In our study, a slight destruction towards vertebral body of T12 with a slightly narrower disc space, minimal sclerotic changes, and a relatively irregular bone density were observed within the incubation periods of 4 and 6 weeks. In addition, our results were in accordance to the previous research in which spinal tuberculosis in rabbit model had developed since the 4th week based X-ray examination [13-15]. Nevertheless, an extensive spread towards and apparent destruction of vertebral body relatively occurred late during chronic phase of the disease progression so that there might be a low positivity rate of imaging findings in the early stage of the disease [37]. This condition frequently results in a missed or wrong diagnosis and delay in the treatment which lead to complications of spine deformity and neurological deficits.

Histopathology examination becomes an essential diagnostic tool to confirm MTB infection. Histopathologic findings in spondylitis TB primarily indicated inflammatory cell infiltration, sequestrum formation, and trabecular breakage. Inflammatory reaction included an extensive number of lymphocytes, few epithelial cells, the presence of multinucleated giant cells, and caseous necrosis across the lesion. Further progression may result in the formation of typical tuberculous nodule or cavity [1,2,35]. The results of our study showed much more amount of inflammatory cells, tubercles, macrophages, and epithelioid cells with caseous necrotic tissue, datia langhans cells, and tubercle formation. However, these findings were discovered only in the 2 experimental rabbits (25%). This condition might be associated with the wrong selection of specimen, the short duration of incubation period, or the extent of destruction which was relatively still mild. Microbiological diagnosis by using the culture of *M.tuberculosis* is also important. In this study, the high positive rate of 62.5% was observed. This was similar to the study of Liu et al. in which 67% of positive rate was reported by using culture in a modified Lowenstein-Jensen medium for 4 weeks [14].

The highest success rate for inoculation of Mycobacterium tuberculosis bacteria in the vertebral body of rabbits was in group 3, namely 7 of 8 samples (87.5%) with an incubation period of 6 weeks, followed by group 2 (75%, with an incubation period of 4 weeks), group 1 (37.5%, with an incubation period of 2 weeks), and group

4 (12.5%, with an incubation period of 8 weeks. The low positivity rate in group 4 with an incubation period of 8 weeks has differed from other studies conducted by Geng et al. where 9 out of 17 rabbits with an incubation period of 8 weeks had a higher positive culture (52.9%) [13]. Imaging findings were also within the normal limit. The imaging examinations in 8th week often found further bone and intervertebral disc destruction with an uneven bone density and irregular margin of the vertebral body. Besides, scoliotic or kyphotic deformity with profound abscess or sequestrum formation might be observed [13,14,36,37]. This low positivity rate might be caused by the virulence or toxicity of bacteria which was still moderate or low in addition to the possibility of the immune system which was in a good condition. Furthermore, an inappropriate method of inoculation could result in the overflow of bacteria with bleeding during the implantation phase of surgery. Previous studies had shown an increased rate of successful creation of tuberculosis lesions by using gelfoam sponges after the drilling [13,14,38]. Moreover, the low number of subjects and sample in the group 4, 2, and 8 respectively, might also be the cause of this discrepancy

Our study has several limitations. First, the number of samples used in this study was small and limited so the involved rabbits condition might not fully represent the optimal situation that should be frequently discovered. Second, this study only used the plain X-ray to examine the radiographical changes. Imaging examinations by using CT-scan and MRI exhibit better sensitivity and specificity in diagnosing spine TB [37,39]. Bone changes in spondylitis TB, such as endplate destruction, osseous hyperplasia, sclerotic and osteolytic destruction, and osteophyte can be better observed with CT [37,39]. In addition, the early adjacent soft tissue and intervertebral disc changes due to inflammatory reaction can be revealed using MRI study [37,40,41]. Third, our duration of investigation was short with the longest incubation period of 8 weeks. Moreover, spillover of the *M. tuberculosis* suspension could be found due to the direct implantation without the use of gelfoam sponges so that this might decrease the actual amount of bacteria inoculated in the spine, leading to the lower toxicity to produce the spine TB model.

Conclusion

In conclusion, creating spondylitis Tuberculosis in New Zealand white rabbit can be successfully established using direct inoculation method of *Mycobacterium tuberculosis* suspension with a dose of 0.2 mL of 10^8 CFU/ml. Our method and technique were rather simple and uncomplicated with a high positive rate of success of 87.5%. Besides, the staining, histopathological, culture, and PCR results showed a relatively similar characteristic in the human spondylitis TB. This animal model is important and can be used to investigate the pathology, disease progression, or safety, quality, and efficacy of new drug, vaccine, or other treatments. Nevertheless, further studies with a larger sample and more appropriate inoculation procedures should be performed to strengthen the model establishment with a higher success of positive and survival rate. In addition, the use of more sensitive imaging examinations by using CT-scan and MRI with a longer period of observation and evaluation is also important.

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Authors' contributions

All authors contributed to data analysis, drafting, and revising of the article, and agreed to be responsible for all the aspects of this work.

Conflict of Interest

There are no conflicts of interest in this study.

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