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Experimental infection with *Yersinia pseudotuberculosis* in European brown hare (*Lepus europaeus*, Pallas)

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ABSTRACT

Objective: To investigate clinicopathological, bacteriological and pathological aspects of an experimental infection with *Yersinia pseudotuberculosis* (*Y. pseudotuberculosis)* in hares to verify the efficacy of serology for the *in vivo* diagnosis. Moreover, the pathogenicity of two *Y. pseudotuberculosis* strains was investigated in order to detect potential differences.

Methods: Twelve European brown hares (*Lepus europaeus*, Pallas) were experimentally infected *per os* and *via* conjunctival mucosae with *Y. pseudotuberculosis*: six subjects were infected with a strain isolated from a naturally infected hare (YpH) and six subjects with a strain isolated from a naturally infected rabbit (YpR). Two hares were used as negative controls. All animals were subjected to clinical, bacteriological and serological examinations during 9 weeks following the infection and, at the end of the control period, subjects still alive were euthanized and submitted to a complete post mortem examination.

Results: All faecal samples collected during the control period were positive for bacteriological examinations and to a PCR for the *inv* gene of *Y. pseudotuberculosis*, while only one YpH-infected hare showed a positive haemocultures. From the 2nd to the 9th week post infection (pi), serological analysis revealed specific antibodies with titers ranging from 1:10 to 1:160 in all YpH-infected and two YpR-infected subjects. All the YpH-infected and two YpR-infected hares scored positive for *Y. pseudotuberculosis* by means of bacteriological investigations. Grossly, suppurative multifocal lesions were detected in liver, spleen, kidney and sub-mandibular lymph nodes in both YpH- and YpR-infected hares and confirmed with histopathology. Pulmonary lesions were observed only in YpH-infected subjects. Immunohistochemistry confirmed the presence of bacterial antigen in all infected animals.

Conclusion: Results of this study revealed that YpH strain is more pathogenic for hares than the YpR strain; moreover the serological test performed in this study could be used for the diagnosis of pseudotuberculosis in hares, whereas post mortem diagnosis should be confirmed by means of bacteriological examination, PCR, histopathology and immunohistochemistry.

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1. Introduction

Yersinia pseudotuberculosis (*Y. pseudotuberculosis*) is a Gram negative bacillus, non-spore forming, non-lactose fermenter, facultative anaerobe, catalase-positive and oxidase-negative belonging to the family Enterobacteriaceae. It has a worldwide distribution in wild and domestic animals and can cause sporadic and epidemic human enteric diseases [1–4]. Wild mammals and birds are the principal reservoirs [5,6]. *Y. pseudotuberculosis* has been isolated from carnivorous, herbivorous and omnivorous animals. Recent surveys refer to pigs in Europe as a primary reservoir of human pathogenic *Yersinia enterocolitica* (*Y. enterocolitica*) and, to a lesser extent, of *Y. pseudotuberculosis* [7,8]. Recently, a human case of *Y. pseudotuberculosis* septicaemia and septic arthritis has been reported [9].

Usually, *Y. pseudotuberculosis* is responsible for subclinical enteric disease in wild and domestic animals, and abortions in cattle [10], sheep [11] and goats [12] have been reported. A septicaemic form, called pseudotuberculosis, can occur in lagomorphs, rodents, including laboratory rodents, and caged birds [13]. Post mortem examination of infected subjects reveals numerous white necrotic foci in the liver; mesenteric lymph nodes are enlarged and may show caseous necrosis [5].

Recently, outbreaks of pseudotuberculosis have been observed in brown hare (*Lepus europaeus*, Pallas) populations [14], also living in Central Italy and the infections have been confirmed by bacteriological cultures on fecal and tissue samples (personal communications – unpublished data).

A study performed in Germany on 230 European brown hares highlighted a seroprevalence of 89.6% for pathogenic *Yersinia* (*Y. pseudotuberculosis* and *Yersinia entoerocolitica*) and concluded that subclinical yersiniosis should be further investigated to explain the European brown hare populations decrease in Germany [15].

Currently, *in vivo* diagnosis of *Y. pseudotuberculosis*-infection in hares is performed through bacteriological examinations of fecal samples, but this method has a low sensitivity. The purpose of the present study was to investigate clinicopathological, bacteriological and pathological aspects of an experimental infection with *Y. pseudotuberculosis* in hares with particular attention to the antibody kinetics, in order to verify the efficacy of serology for the diagnosis *in vivo*. Furthermore, we tried to investigate the potentially different pathogenicity of two different *Y. pseudotuberculosis* strains isolated from a naturally infected hare and from a naturally infected rabbit.

2. Materials and methods

2.1. Studied animals

Fourteen 6–8 months old female captive European brown hares were challenged. Before the experimental infection, fecal samples from each animal were submitted to bacteriological investigation for *Yersinia* sp. and scored negative. Each subject was marked using an ear tag and was kept in a separate cage located in five separated pounds: subjects H1, H2 and H3 in pound A, H4, H5 and H6 in pound B, H7, H8 and H9 in pound C, H10, H11 and H12 in the pound D, and C1 and C2 in the pound E. All pound had 16–18 °C controlled room temperature and 65%–70% humidity. The animals were fed with vegetable pellets, hay and fresh water daily.

2.2. Experimental design

Subjects were infected with two different *Y. pseudotuberculosis* strains: a YpH strain previously isolated at the bacteriological Laboratory of the Department of Veterinary Science, University of Pisa from a naturally *Y. pseudotuberculosis*-infected hare (animals H1−H6); and a YpR strain isolated by the Istituto Zooprofilattico Sperimentale di Lazio e Toscana, section of Pisa, from a naturally *Y. pseudotuberculosis*-infected rabbit (animals H7−H12). Both strains were typed by API System 20E (bioMérieux, Marcy l'Etoile, France), PCR and, subsequently, submitted to serotypization and tested for their biochemical reactions towards raffinose, Simmons' citrate agar and melibiose in order to determine the biovar. Both strains belonged to O:1a serotype, biotype 1. After typing tests, the strains were stored in Brain Heart Infusion Broth (Difco, Becton, Dickinson and Company, Sparks, MD, USA) added with 14% of glycerol at −80 °C until used. In order to prepare the inocula, the two strains were cultured on Tryptose Agar (Oxoid, Basingstoke, Hampshire, England) tubes and incubated at 30 °C for 48 h. Bacteria were harvested with sterile normal saline solution and passed through a sterile gauze. Serial dilutions were passed onto Tryptose Agar plates. After 48 h incubation at 30 °C the colonies were counted and the number of viable bacteria/mL of suspension was calculated.

Six hares were infected *per os* with 0.5 mL of a solution containing 10^9 CFU/mL of *Y. pseudotuberculosis*, three subjects (H1, H2, H3) using YpH, while three subjects (H7, H8, H9) with YpR. The remaining six hares were infected intraconjunctivally with 0.5 mL (0.25 mL/eye) of the same solution, three subjects (H4, H5, H6) with the YpH strain and three (H10, H11, H12) with the YpR strain. After instillation, the eyelids were manually closed for 60 s to increase conjunctival adsorption. The last two hares (C1 and C2) were kept as uninfected controls in a separate cage.

Post infection controls were carried out on each hare to verify rectal temperature, antibody presence, bacteraemia and *Y. pseudotuberculosis* fecal shedding. Rectal temperature, blood and fecal samples were taken before the infection and weekly from the 1st to the 9th week post infection (pi) after the hares were anesthetized with intramuscular injection of 10 mg/kg pv of chloridrate tiletamine and chloridrate zolazepam. Blood samples for serology and bacteriology were collected by venipuncture from an auricular vein (Vacutainer system, Belliver Industrial Estate, Plymouth, UK). The blood samples were allowed to clot, centrifuged at 1200 g for 15 min, and the serum stored at −20 °C until serological tests. The animals still alive after 63 days (9 weeks) pi underwent deep anesthesia and were euthanized (intracardiac Tanax® injection; 1.2 mL containing embutramide 200 mg/mL, mebezone iodure 50 mg/mL, tetracaine chloridrate 5 mg/mL; Hoechst Roussel Vet Gmbh, Munich, Germany).

2.3. Gross pathology, histopathology and immunohistochemistry

All the subjects that died or were sacrificed at the end of the control period underwent a complete post mortem examination. Representative portions of liver, spleen, kidney, intestine, lungs, mesenteric and sub-mandibular lymph nodes were sampled for bacteriological, molecular and pathological examinations. Tissue samples were fixed in 10% buffered formalin (pH 7.4) and

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underwent histopathological and immunohistochemical examinations. Four-micrometer serial sections were stained with hematoxylin and eosin. Gram stain and phosphotungstic acid haematoxylin stain (PTAH) were performed in order to detect bacterial colonies in tissues and areas of fibrinoid necrosis, respectively. Immunohistochemistry for the detection of *Y. pseudotuberculosis* antigen was carried out with a murine monoclonal antibody directed against *Y. enterocolitica* (mouse anti-*Y. enterocolitica* O:9, US biological, Swampscott, Massachusetts, USA) incubated 1 h at 37 °C, because of the lack of a specific commercial antibody to *Y. pseudotuberculosis* at the time of our investigation. Antigen retrieval was performed with 10 mM/l citric acid (pH = 6) in a microwave oven. The slides were dried at room temperature and then washed with running tap water. Endogenous peroxidases were blocked and the slides were incubated with the primary antibody diluted 1:1000 in a buffer solution. A commercial streptavidin-biotin-peroxidase kit (Vector Laboratories, Inc., Burlingame, CA, USA) was used to detect specific antibody binding. previously applying a 1:200 dilution of A biotinylated goat antimouse immunoglobulin was applied as a secondary antibody (AO433; DakoCytomation, Denmark) with a dilution of 1:200 for 45 min at room temperature. The enzymatic reaction was developed with the use of 3-1-diaminobenzydine (DAB; Sigma Chemical Co., St. Louis, Missouri, USA). The slides were subsequently counter-stained in hematoxylin and permanently mounted. As positive control, the liver of a hare naturally infected with *Y. pseudotuberculosis* and PCR confirmed was used. Negative controls were obtained both by omitting the primary antibody and by using an unrelated primary monoclonal antibody.

2.4. Serology

Serum samples were tested for antibodies to *Y. pseudotuberculosis* with a slow agglutination at low temperature micromethod assay, using a lipopolysaccharide (LPS) antigen, prepared with *Y. pseudotuberculosis* ATCC 29833 strain and stained with a 0.1% crystal violet solution, following the procedure previously described by Franzin and Curti [16]. Dilutions from 1:10 to 1:1280 of each sample were tested in wells of 96 U-shaped microtiter plates, which were incubated at 37 °C for 1 h, then at 4 °C overnight.

2.5. Bacteriology

Direct and cold enrichment culture methods were performed in order to isolate *Y. pseudotuberculosis* and their results were

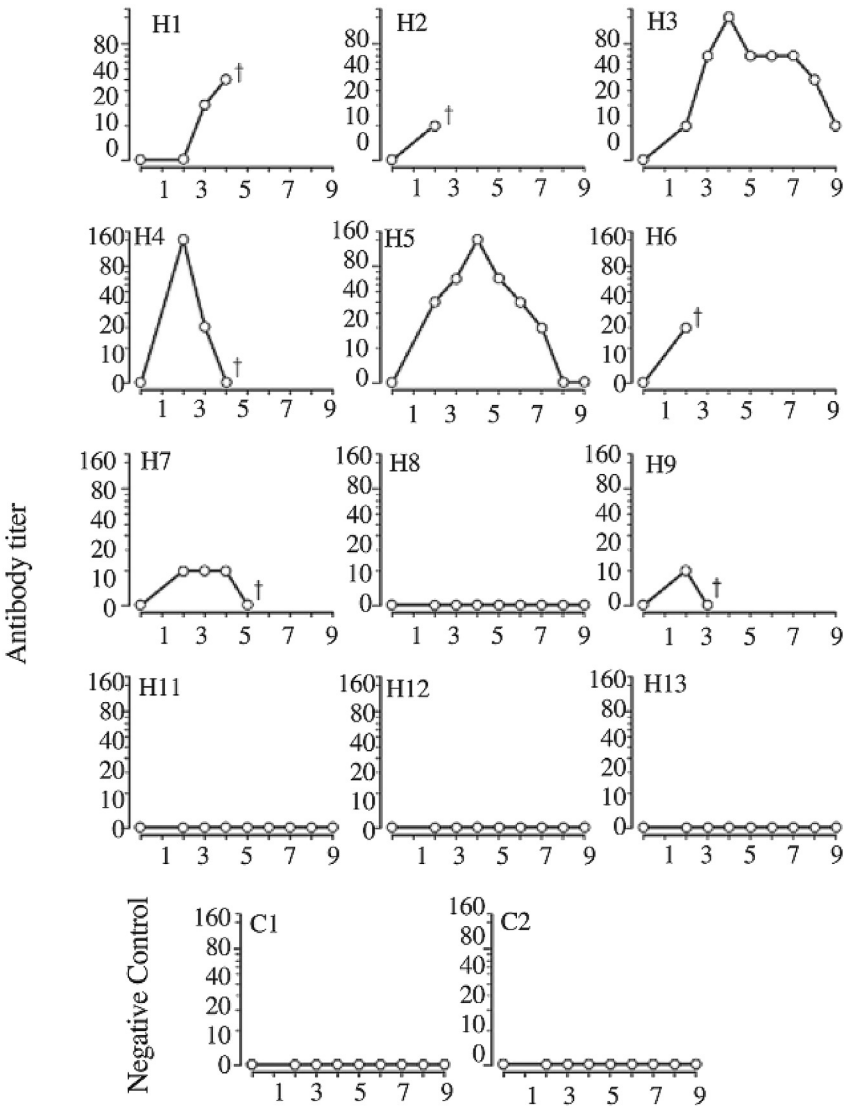

Figure 1. Trend of anti-Yersinia pseudotuberculosis antibody titres in hares infected with *Yersinia pseudotuberculosis* strains isolated from a naturally infected hares (H1-6) and a naturally infected rabbit (H7-12) and controls (C1-2); († Death of the subject).

Table 1
Gross lesions observed and results of bacteriological investigations and PCR analysis carried out on European brown hares tissues.

Animal	Liver			Spleen			Sub-mandibular lymphnodes			Intestine			Lung			Kidney		
	Lesions	Culture	PCR	Lesions	Culture	PCR	Lesions	Culture	PCR	Lesions	Culture	PCR	Lesions	Culture	PCR	Lesions	Culture	PCR
YpH-infected hares	6/6	6/6	6/6	6/6	6/6	6/6	3/6	6/6	6/6	0/6	6/6	6/6	6/6	6/6	6/6	3/6	6/6	6/6
YpR-infected hares	2/6	2/6	2/6	2/6	2/6	2/6	2/6	2/6	2/6	0/6	2/6	2/6	0/6	1/6	1/6	1/6	1/6	1/6
Controls	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2

YpH = *Yersinia pseudotuberculosis* isolated from naturally infected hare; YpR = *Yersinia pseudotuberculosis* isolated from naturally infected rabbit.

compared. Regarding the direct method, 1 g of each fecal material was homogenized in 5 mL of sterile saline solution, then a loop of each sample was cultured onto a CIN (cefsulodin-irgasan-novobiocin, Oxoid) Agar plate and a Yersinia Agar (Yersinia Selective Agar Base + Yersinia Selective Supplement, Oxoid) plate. All plates were incubated at 30 °C for 48 h.

For the second method, 1 g of fecal material was cultured in 9 mL of Yersinia broth and incubated at 4 °C for 21 days. Subcultures on CIN Agar plates were performed after 7, 14 and 21 days; all the plates were incubated at 30 °C for 48 h. Suspect colonies underwent Gram staining. In order to verify the presence of bacteremia, three drops of citrated whole blood, aseptically collected from each hare, were added to 2 mL of Trypticase Soy Broth (Oxoid) and cultured on tubes containing Trypticase Soy Agar (Oxoid); the tubes were incubated in a slanting position at 30 °C for one week. Portions of tissues were homogenized in 10 mL of sterile physiological solution (pH 7.0) by stomacher (PBI); 1 mL of each sample was cultured by direct and cold enrichment methods.

2.6. PCR assays

DNA was extracted from tissues and fecal samples using the DNeasy Tissue Kit (Qiagen, GmbH, Hilden, Germany) according the manufacturer's recommendations. The DNA samples were stored at 4 °C. A PCR, specific for a 183 bp fragment of the *inv* gene of *Y. pseudotuberculosis*, was performed on all DNA samples, using the primers invF and invR and reaction conditions previously described by Thoerner *et al* [17]. *Y. pseudotuberculosis* ATCC 29833 strain and distilled water were used as positive and negative controls, respectively. PCR products were analyzed by electrophoresis on 1% agarose gel at 100 V for 45 min; gel was stained with ethidium bromide and observed at the UV transilluminator. Gelpilot 100 bp Plus Ladder (Qiagen) was used as DNA molecular weight marker.

2.7. Statement of animal right

The present study has been approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

3. Results

No changes in body temperature were detected in all hares infected with both YpH and YpR strains. Six animals died during the experimental period: four infected with YpH strain and two infected with the YpR strain. Among the hares infected with YpH, H4 subject died the 38th day pi showing mucopurulent keratoconjunctivitis and cachectic status, while hares H1, H2 and H6 died the 34th, 16th and 12th day pi respectively presenting moderate weight loss only. Subjects H3 and H5 did not develop clinical signs related to pseudotuberculosis during the study. Among the hares infected by YpR subjects H7 and H9 died the 38th and 21st day pi respectively.

Y. pseudotuberculosis was isolated through cold enrichment cultures, from the fecal samples of all the YpH-infected hares (H1−H6), and from subject H7 and H9 infected with YpR. No isolation was achieved from fecal samples of controls and YpR-infected hares H8, H10, H11 and H12. All direct bacteriological

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examinations gave negative results. Haemocultures resulted positive the 10th day pi only in hare H6.

The results of microagglutination tests are reported in Figure 1. Antibodies against *Y. pseudotuberculosis* were detected inconstantly from the second week pi until the ninth week pi (Figure 1). The antibody titers ranged between 1:10 to 1:160 and were observed in all the six hares infected with YpH strain and in two subjects (H7 and H9) infected with YpR strain. Hare H3 still showed a minimum positive titer (1:10) at the end of the study when it was put to sleep.

In all YpH-experimentally infected hares, multifocal pyogranulomatous round lesions, ranging from few mm to 3 cm in diameter, often bulging on the surfaces of the organs were observed in liver and spleen independently from the route of infection. Only 2 YpR-challenged hares (both infected *per os*) showed similar hepatic and splenic lesions. Pyogranulomas, multifocal to widespread were observed in sub-mandibular lymph nodes only in YpH-hares infected *via conjunctiva*. Pulmonary lesions were noticed in all YpH-infected hares independently from the route of infection but not in YpR-challenged subjects. Multifocal to coalescing renal pyogranulomas, on the surface of the organs and in the deep medulla, were observed in 4 subjects infected *per os* (2 YpH infected *per os*, 1 YpH *via conjunctiva*, and 1 YpR *per os*). No macroscopical lesion in the intestine and mesenteric lymph nodes were recorded in any subject except a mild diffuse vascular congestion of the small intestine in all subject infected *per os*. Control animals showed no gross lesions.

YpH strain was re-isolated by direct culture from all tissue collected from hare H2, from liver, spleen, lung and intestine of hare H4 and from liver and spleen of hare H6. YpH strain was also re-isolated by cold enrichment culture from all tissues of hares H1, H3, H5 and H6.

When tissue from YpR-infected animals were tested, both with direct and cold enrichment methods, all the organs collected from hare H9 scored positive while only liver, spleen, intestine and sub-mandibular lymph nodes from hare H7 showed a positive result. Bacteriological cultures carried out on tissues from subjects H8, H10, H11, H12, C1 and C2 resulted negative.

All DNA samples obtained from the tissues of YpH-infected hares resulted PCR-positive while, among YpR-infected group, only the subjects H7 and H9 resulted positive by PCR. The remaining subjects, including the controls, were PCR-negative. The results of pathological gross findings, bacteriological and molecular investigations are summarized in Table 1.

The distribution of microscopic lesions was consistent, in all subjects, with the gross appearance with the exception of the intestine and mesenteric lymph nodes of all hares infected *per os*, in which, microscopic pyogranulomas were noticed in the mucosa and transmurally in the muscular layers with a multifocal pattern. Lesions were characterized by a necrotic center containing hyperbasophilic cellular debris, degenerated neutrophils and abundant polymerized palely eosinophilic proteinaceous material (fibrin deposition confirmed with the PTAH stain-Figure 2a). Periferically, necrotic foci were surrounded by dense aggregates of macrophages, neutrophils and small to medium sized lymphocytes and bacterial colonies of hyperbasophilic cocco-bacillar elements interspersed among them. Few multinucleated giant cells were also present at the periphery of pyogranulomas. Bacteria tested Gram negative by means of

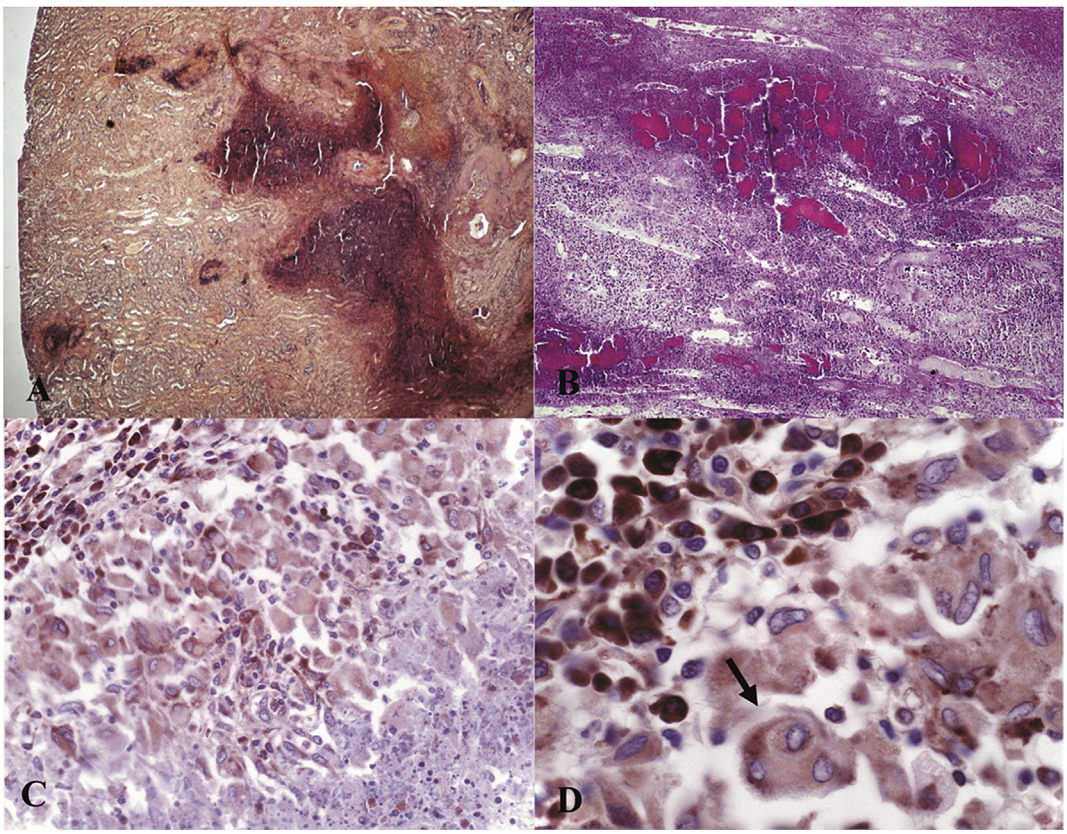

Figure 2. A) Kidney, multifocal to coalescing foci of fibrinoid necrosis in the cortex and in the deep renal medulla. PTAH stain; ob. 20×; B) Kidney, gram negative bacterial colonies within a pyogranulomatous renal lesion. Gram stain; ob. 40×; C) Liver, cytoplasmic positive immunostain for *Yersinia* antigen in macrophages surrounding a pyogranulomatous lesion. IHC stain; ob. 40×; D) Liver, cytoplasmic positive immunostain for *Yersinia* antigen in a multinucleated giant cell (arrow) and in macrophages within a hepatic pyogranuloma. IHC; ob. 100×.

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Gram stain and were often found in the cytoplasm of macrophages and giant cells (Figure 2b). No microscopic lesions were observed in controls and all the organs were unremarkable.

The distribution of immunohistochemical positive results were in accordance with the gross and microscopic distribution of the lesions and the bacterial antigen was strongly and intensely demonstrated in the cytoplasm of macrophages and giant cells (Figure 2C and D). Bacterial colonies also immunostained strongly positive, while no immunostain was obtained in the necrotic center of pyogranulomas and in all the organs collected from the control animals.

4. Discussion

The present study experimentally reproduced *Y. pseudotuberculosis* infection in hares and some differences were observed concerning the two strains used. Infection was readily established in all six hares with YpH strain previously isolated from a naturally infected subject regardless the route of infection, as demonstrated by the results of serology, bacteriology, molecular and pathological investigations. Four of the six YpH-infected subjects died within the end of the study period, while the *Y. pseudotuberculosis* strain previously isolated from a naturally infected rabbit succeeded to induce infection and a serological response only in two of the six challenged subjects and only when they were infected *per os*. Furthermore, these two animals developed macroscopic and microscopic lesions and bacterial antigen was demonstrated by IHC. The presence of bacteraemia has been demonstrated only in one YpH-infected hare, possibly because the blood samplings were obtained weekly to lower the stress in the animals and this timeframe could be too wide to detect the bacteraemic phase.

Direct bacteriological cultures were not sensitive as the etiological agent was re-isolated from tissues in three hares only and it was never detected in fecal samples. The cold enrichment method showed better results and allowed *Y. pseudotuberculosis* isolation from all the samples of YpH experimentally infected hares (possibly due to inhibition of competitive contamination bacteria).

Molecular analysis showed good results and could represent a valid and confirmatory support to use together with histopathology/immunohistochemistry and bacteriological cultures, (PCR detected YpH strain DNA in all tissue and fecal samples within 2–3 days).

The microagglutination test detected anti-*Y. pseudotuberculosis* antibodies in blood sera of YpH-infected hares from the second week pi. Four YpH hares had specific antibodies, even if at low titer, when deceased. One hare (H5) became seronegative eight weeks after the infection, whereas another subject (H3) still showed a low antibody titer when was sacrificed. With rabbit strain infection, only two animals seroconverted and antibodies persisted for few weeks pi with minimum titer of 1:10. These results suggest *Y. pseudotuberculosis* infection does not stimulate a strong humoral response and therefore serology alone is not considered a fully reliable method to the detect yersiniosis *in vivo*.

Fecal-oral transmission is the main route of natural *Y. pseudotuberculosis* infection and the agent has been isolated from soil and stream water, suggesting that preying upon infected animals and ingesting environmental contaminated substances is important for the transmission of the disease [18,19] and intestinal lesions are characteristics of the natural infection. Moreover, in the experimental infection the transmission mode has been shown to affect the distribution of the lesions. In fact, microscopic intestinal changes were found to be closely related to the digestive route of infection as well as the submandibular lymph node involvement that has been observed only in the infection *via conjunctiva*.

On the contrary, hepatic and splenic lesions have been reported in all YpH-infected subjects independently from the route of infection, suggesting a key role of liver and spleen in the diagnosis of pseudotuberculosis in hares at necropsy and in field sampling. Pulmonary lesions were present only in animals infected with the YpH strain suggesting a higher virulence for hares than the strain isolated from rabbit. Renal lesions were not correlated with either route of infection or bacterial strain.

Our study demonstrated that the antibody used for the immunohistochemistry, although raised against *Y. enterocolitica*, showed a good cross-reactivity with *Y. pseudotuberculosis*, as confirmed using positive controls. This antibody could be useful in pseudotuberculosis diagnosis when a specific antibody to *Y. pseudotuberculosis* is not commercially available.

In conclusion, our study demonstrated that YpH was able to infect all the challenged subjects, whereas it was possible to induce the disease in a reduced number of hares when YpR was used. These results suggest that the spread of the infection could be partially species specific. The strains able to infect rabbits and hares could be different from those responsible of infection in large animals (pigs, cattle, sheep, goats) that could have a low pathogenicity for lagomorphs and this could potentially explain the variation of gross pathological changes observed among different host species [2].

Serological diagnosis may be useful when a large number of animals must be tested, in order to verify the health status of a hare population. In case of the detection of seropositive subjects, direct methods, in particular PCR should be used. The bacteriological cultures with the cold enrichment method resulted much more sensitive than the direct cultures. PCR gave the same results obtained by the cold enrichment method, but it is much faster (2–3 days). Moreover, the specific PCR used in this study allows detecting bacterial strains which encode for the *inv* virulence gene, thus the molecular method, unlike bacteriology, is potentially able to discriminate between pathogenic and non-pathogenic strains.

Conflict of interest statement

All authors gave their informed consent prior to their inclusion in the study. None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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