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Leptospirosis in Slaughterhouse Personnel: A Seroepidemiologic Study Using Microscopic Agglutination Test

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Abstract

Background and Objective: Meat can be contaminated by *Leptospira* species. This bacterial pathogen causes severe leptospirosis disease in humans and animals. The major aims of this study were to assess seroepidemiological prevalence of leptospirosis in employees of a slaughterhouse in Guilan Province, Iran, using microscopic agglutination test and further investigate the positive samples using nested polymerase chain reaction method.

Material and Methods: In this study, 150 employees of a slaughterhouse in Guilan Province, Iran, were participated after completing written consents and personal questionnaires. This sample size was calculated based on the mean prevalence of the pathogen in the region. After assessing sera of the participants for Leptospira antibody using microscopic agglutination test, urine samples were collected from the positive participant for further assessments using nested polymerase chain reaction.

Results and Conclusion: Based on the results, microscopic agglutination test was positive for 10.7% of the participants. However, Nested-PCR was negative for the positive microscopic agglutination tests on sera collected from the participants with antibodies against Leptospira antigens. The current results demonstrate that Leptospira can occur in asymptomatic humans in slaughterhouses and highlight the high potential of the disease transmission to humans in the province. Therefore, further extended control and prevention measures for slaughterhouse workers are recommended to guarantee the food safety.

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1. Introduction

Meat can be contaminated by a variety of microbial pathogens such as bacterial *Brucella* spp., *Listeria* spp., *Escherichia coli* and *Campylobacter* spp.; viral *Orthonairovirus* Sp. (agent of Crimean-Congo hemorrhagic fever), Adenovirus, Astrovirus, Norovirus, Rotavirus, Hepatitis A virus and Hepatitis E virus; as well as fungal *Penicillium* spp., *Aspergillus* spp. and *Cladosporium* spp. as well as parasitic *Toxoplasma gondii*. One of these pathogens is *Leptospira* species, which can cause the severe disease of leptospirosis in humans and animals [1]. Leptospirosis is one of the most outbreak tropical bacterial diseases worldwide, occurring majorly in rural and urban areas of subtropical and tropical regions [2]. The disease agent belongs to Leptospiraceae family and Leptospira genus. Leptospirosis causes great economic losses since infection with this disease decreases animal reproduction and products (e.g., meats and dairies) and increases mortality in animals. Most mammals can act as renal carriers and store the bacteria in their renal tubules for some time. Leptospirosis is considered an occupational disease in humans and urine of infected animals

is the major source of the infection [3,4]. All Leptospira serotypes are able to enter the human body through damaged skin and mucous membranes. These bacteria can cause widespread clinical symptoms in humans, including flu-like syndrome, jaundice, Weil syndrome, meningitis and even death. Therefore, clinical diagnosis is difficult due to widespread and nonspecific clinical symptoms as well as lack of sufficient knowledge in the basic microbiology of *Leptospira* spp. [2,5]. Leptospirosis is reported in Iran since 1957 and several reports have shown that the disease agent threatens the public health and burdens livestock production [6]. After seven decades of research, the most common reported serotypes in Iran are Hardjo, Pomona, Icterohaemorrhagiae, Grippotyphosa and Conicola. The Conicola serotype is more likely to occur in veterinarians and dog owners, the Grippotyphosa serotype in rice farmers, shepherds and farmers and Pomona serotype in livestock workers [7].

In general, leptospirosis is an occupational disease worldwide and slaughterhouse employees are one of the high-risk groups for infection with this pathogen. Infected employees can further transfer the bacterial pathogen to meat and meat products through their contaminated hands, tools and workwear. Contamination of meats with *Leptospira* spp. may result in infection of the consumers with severe and life-threatening consequences. This is a major health concern that urges careful monitoring of the food chain hygiene. Every year, foodborne diseases cause hundreds of million infections worldwide that burden heavy budgets on governments as well as medical costs on the patients. Therefore, accurate standardization of the food processing, including slathering, transportation, refrigeration and freezing, packaging and delivery, is critical. Furthermore, leptospirosis is highly prevalent in Guilan and Mazandaran Provinces, Iran, due to the province special climate and geography and people lifestyle, which promote facilitated propagation of the bacteria. The pathogenic species of Leptospira genus is *L. interrogans* [1].

As previously, stated, incomplete knowledge of the microbiology of *Leptospira* spp. and different manifestations of its disease has made diagnosis of leptospirosis quite complex. Many patients may not demonstrate symptoms and act as the silent carriers of the bacterial pathogen. This urges health monitoring of people working in food industries as well as those providing public services to prevent further spread of the pathogen in the society. To address this, careful health monitoring of the slaughterhouse personnel is important, particularly in the highly-prevalence region of northern Iran. Therefore, the current study was carried out to seroepidemiological investigate leptospirosis in slaughter-house employees in Guilan Province, Iran, using microscopic agglutination test (MAT) [7,8]. In the present study, MAT was used, a standard method for the detection of *Leptospira* species according to the World Health Organization. Then, samples reported as positive in MAT were verified using nested polymerase chain reaction (Nested-PCR).

2. Materials and Methods

2.1 Sampling

This study was ethically approved by the Ethics Committee, Tehran University of Medical Sciences, Tehran, Iran (ethical approval no. IR.TUMS.SPH.REC.1398.327). A number of 150 employees from one of the slaughterhouses in Guilan Province, Northern Iran, participated in this study with generally 98.7% of the participants (148 from 150) were male. First, volunteer participants filled out written consents and personal questionnaires; then, blood samples were collected from these participants. Sera were immediately separated from the blood clot and appropriately transferred to Leptospira Research Laboratory, Faculty of Veterinary Medicine, University of Tehran, Karaj, Iran.

2.2 Standardization of live antigen and dilution of serum

In the present study, five common-spread serovars of *Leptospira* Sp. in Iran were used, including Hardjo, Pomona, Icterohaemorrhagiae, Grippotyphosa and Conicola. Since live bacteria were used as antigens in MAT, concentration of the bacteria should first be standardized as 2×10^8 cell per ml. In the current study, 1:50 serum dilution (the final cut-off point of 1:100) was used for the first step of MAT based on the scientific reports from various regions of Iran and the authors' long personal experiences. Microscopic slides were examined at 100× magnification for possible agglutination using dark-field microscope (Olympus BX50, Tokyo, Japan). If at least 75% of the *Leptospira* spp. Became agglutinated, samples were reported as positive and if they showed less than 50% or no agglutination, samples were reported as negative. Samples that showed 50% agglutin-ation were reported as suspicious samples [9].

2.3 Titration of the positive samples

Sera positive for agglutination at 1.100 titers were further diluted to calculate the final titers. Two-fold dilutions from 1:100 up to 1:1600 were prepared from all the positive serum samples. The highest dilution with an agglutination rate of ≥ 75% was reported as the final titer [10].

2.4 DNA extraction from the urine samples

In this study, DNA extraction kit (DNP) (Sinaclon, Tehran, Iran) was used for DNA extraction according to the manufacturer's instruction. At least 1500 bacteria per milliliter were needed for DNA extraction. The overall quality of the extracted DNA samples was investigated using Nano-Drop One spectrophotometer (Thermo Fisher Scien-tific, Waltham, USA).

2.5 Nested polymerase chain reaction

In the present study, two Metabion primer pairs [11] were synthetized (Pishgaman, Tehran, Iran) and used for the Nested-PCR, including the primer pair of A and B and the primer pair of C and D (Table 1). The A and B primers respectively belonged to nucleotides 38 to 57 and 348 to 368 of the 16S rRNA gene structure of *Leptospira* spp., generating 331-bp amplicons [11]. Moreover, C and D primers respectively belonged to nucleotides 58 to 77 and 328 to 347 of the 16S rRNA, generating 290-bp amplicons. In the first stage of Nested-PCR, 4 µl of the target DNA from each sample were used and in the second stage of the Nested-PCR, 2 µl of the target DNA from the first Nested-PCR product were used. The first stage of the Nested-PCR set was carried out to detect Leptospira DNA in urine samples. Briefly, each PCR reaction included 12.5 µl of master mix, 0.5 µl of each primer at with 10 pg concentration, 4 µl of DNA and 7.5 µl of sterile distilled water in a total volume of 25 µl (Sinaclon, Tehran, Iran). To carry out the second stage Nested-PCR set, 2 µl of the first Nested-PCR amplicons were added to the second Nested-PCR as target DNA. Amplification cycles in the Mastercycler PCR machine (Eppendorf, Hamburg, Germany) were as follows: In general, an initial denaturation of 3 min at 94 °C was used followed by 1.5 min at 61 °C and 2 min at 72 °C. Then, 30 cycles of 15 s at 94 °C, 20 s at 61 °C and 25 s at 72 °C were set. The final extension was carried out for 5 min at 72 °C. Differences between the first and the second stages included use of 63 °C in Steps 2 and 5 of the second stage instead of 61 °C of the first stage. Final amplicons of the Nested-PCR were investigated using 1% agarose gel with ethidium bromide in 1% TAE buffer visualized under UV light (UVP gel documentation system, Analytik Jena, Germany). All the experiments were rechecked [12,13]. Protocol used in this study for the detection of Leptospira-positive sera has been shown in Figure 1.

3. Results and Discussion

Assessment of the participants in this study has revealed that 44.7% of these participants were in the age range of 30–39 years, 24.7% were under 30 years old and 30.7% were over 40 years old. Furthermore, 48% had 1–3 years old, 32.7% had 3–6 years and 19.3% had more than 6 years of work experience. The MAT was positive for 10.7% and negative for 89.3% of the participants. Nested-PCR was negative in urine samples of the participants with MAT-positive results. The participants' mean age was 35.49 years with a standard deviation of 8.84 years in the age range of 20–63 years and their mean work experience was 3.83 years with a standard deviation of 2.53 years in the range of 1–12 years (Table 2). This study showed that the variables of age, gender, work experience and occupation type included no significant associations with the MAT results ($p < 0.05$). In other words, none of the qualitative variables affected the MAT results. In the current study, an assessment of the quantitative variables showed that the age of MAT-positive participants was significantly lower than that of MAT-negative ones ($p = 0.027$). In other words, the participants' age was effective in responding to MAT. Moreover, the results of this study demonstrated no evidence of the work experience effects on the MAT results ($p = 0.417$).

Studies in the country's northern cities have indicated most positive serum titers from rural areas of Guilan Province [14]. In the present study, 16 out of 150 patients had positive MAT results. Moreover, five out of these 16 people had suspicious reactions. In total, four samples included antibodies against the Hardjo serotype, one sample had antibodies against the Conicola serotype, one sample had antibodies against Icterohaemorrhagiae, eight samples had antibodies against the Grippotyphosa serotype and two samples had antibodies against Pomona serotype.

None of the individuals had clinical symptoms and their Nested-PCR results were negative [15]. Technically, Nested-PCR is more sensitive than conventional PCR. In, a study by Hassanpour et al. on leptospirosis showed that out of 30 blood samples of Khoy (Northwestern Iran) slaughterhouse employees, nearly 13.3% had a positive serum titer using MAT and the most common serotype belonged to Grippotyphosa (80%) [16]. In a comprehensive study on 2735 Iranian human blood samples referred to the Leptospirosis Research Laboratory, University of Tehran, Iran, 2001–2007, positive serum titers varied 12-53% using MAT and the highest frequency of positive serum titer belonged to Guilan and Mazandaran Provinces [7]. In a study on adults in Chile, Terrazas et al. reported the serum prevalence of leptospirosis as 4%. The most common infection was in women and the predominant serotype was Icterohaemorrhagiae [17]. A comprehensive study by Vieira et al. on leptospirosis with 4816 samples from Portugal (1986–2003) showed that 1024 cases were infected with leptospirosis. The most common serotypes were Icterohaemorrhagiae (17.3%), Pomona (12.9%) and Balum (11.3%), respectively [18].

Table 1. Primers used in the current study

No.	Oligo sequence 5'-3'	BC†	EC‡	MW* (Da§)	T†† (°C)	bp	Ref.
A	GGCGGCGCGTCTTAAACATG	20	190.0	6.158	63	331	29
B	TTCCCCCCATTGAGCAAGATT	21	194.0	6.341	59		
C	CAAGTCAAGCGGATGAGCAA	20	209.0	6.184	58	290	29
D	CTTAACCTGCTGCCTCCCGTA	21	182.0	6.293	63		

†bp, base pair; ‡, electrical conductivity; *MW, molecular weight; § Da, Dalton; ††T, temperature.

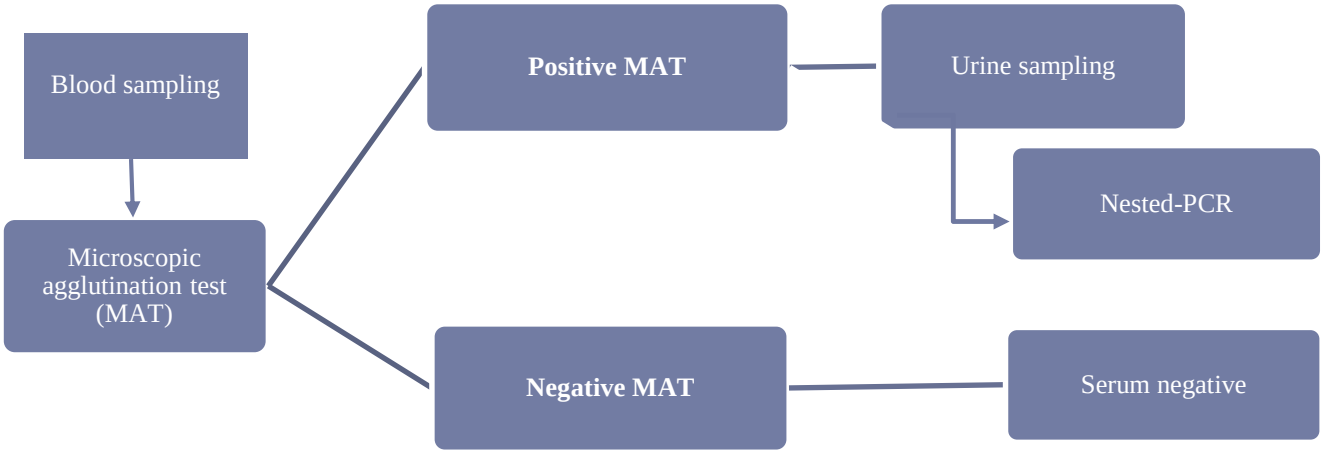

Figure 1. Protocol used in this study for the detection of *Leptospira*-positive sera

Table 2. The participants' demographic information for the seroepidemiologic assessment of leptospirosis in slaughter-house personnel.

Factor	Condition	Number	Percent (%)
Sex	Female	148	98.7
	Male	2	1.3
Age	< 20 years old	3	2.0
	20–29 years old	34	22.7
	30–39 years old	67	44.7
	40–49 years old	35	23.3
	50–59 years old	10	6.7
	≥ 60 years old	1	0.7
Work experience	1–3 years	72	48.0
	3–6 years	49	32.7
	6–9 years	27	18.0
	> 9 years	2	1.3
Job	1	8	5.3
	2	44	29.3
	3	24	16.0
	4	7	4.7
	5	2	1.3
	6	13	8.7
	7	11	7.3
	8	36	24.0
	9	3	2.0
	10	2	1.3
MAT result	Negative	134	89.3
	Positive	16	10.7
		Mean	ND†
	Age	35.49	8.84
	Work experience	3.83	2.53

† Not detected

A study in China (2005–2015) on leptospirosis reported 7763 cases of the disease. Nearly 69% of the cases belonged to men and 31% to women. Number of the people who died of leptospirosis in China in those ten years was 168, with the highest mortality in the fifth decade of life [19]. In 2021, serological studies on 250 serum samples randomly collected from the clients of various wards of Imam Reza Hospital in Amol, Northern Iran, showed that nearly 10% of the samples included positive reactions for leptospirosis using MAT, with the predominant serotype of Hardjo [20]. In general, 1.2-41.3% of sera from abattoir staff have been reported positive for *Leptospira* spp. Worldwide. These reports from various world regions of all continents, including NZ [21,22], Iran [23,24], Yemen [25], Brazil [26] and Morocco [27], reveals the global hazard of food contamination with this dangerous pathogen. However, contaminations were mostly reported from sheep abattoirs rather than cattle abattoirs. This addresses further importance of sheep in dissemination of leptospirosis. Sometimes, infection with the bacteria can cause the active disease in meat workers. For example, three leptospirosis cases were reported from 20 workers in a NZ abattoir, 2008–2009, and two of them were hospitalized [28].

Based on the recent reports of the International Center for Climate Change and Development on gradual increases in temperature leading to ice melting at the poles, submergence of regions of the earth and occurrence of adverse climate conditions such as floods and hurricanes, the importance of

leptospirosis in the next years becomes more significant. Results of studies in Leptospirosis Research Laboratory, University of Tehran, have revealed increases of the existent serotypes in Iran from eight to 15 serotypes as well as a clear necessity of further attention of the executive officials and researchers to this disease. Heavy rains and floods increase the risk of leptospirosis by transferring the pathogen to its animal hosts that have the closest contact with humans [2]. Several outbreaks of leptospirosis have been reported worldwide with geographic diversities, including India, Laos, Indonesia, Italy, Brazil, Nicaragua, USA and Australia, following bad climate conditions.

4. Conclusion

In general, food safety is one of the greatest public concern worldwide since it is directly linked to the people general health. Technically, Leptospira agent can infect consumers of contaminated foods. Results from this study and the current studies within the decade clearly show that despite improving health conditions in various dimensions, leptospirosis prevalence has increased in the country. One of the critical reasons for this increase includes that leptospirosis is a multifaceted disease and therefore, its clinical diagnosis with the available techniques is not quite reliable. Thus, it is recommended to develop specialized laboratories for the definitive diagnosis of the disease in addition to the initial clinical diagnosis. Regarding significantly young age of the serum-positive participants of this study, needs of public education and awareness of at-risk individuals in endemic areas and livestock-linked occupations seem critically necessary.

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6. Conflict of Interest

The authors declare no conflict of interest.

7. Authors Contributions

Conceptualization, R.M.N.F. and G.R.A.; methodology, A.H.; statistical analysis, A.R.F., writing-original draft preparation, A.H.; writing-review and editing, R.M.N.F. and G.R.A.

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لپتوسپیروز در کارکنان کشتارگاه: یک مطالعه سرواپیدمیولوژیک با استفاده از آزمون آگلوتیناسیون میکروسکوپی

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چکیده

سابقه و هدف: گوشت می‌تواند توسط سویه‌های مختلف لپتوسپیرا آلوده شود. این باکتری بیماری‌زا باعث بیماری لپتوسپیروز حاد در انسان و حیوان می‌شود. هدف اصلی این مطالعه بررسی شیوع سرواپیدمیولوژیک لپتوسپیروز در کارکنان کشتارگاهی در استان گیلان با استفاده از تست آگلوتیناسیون میکروسکوپی و بررسی بیشتر نمونه‌های مثبت با استفاده از روش واکنش زنجیره‌ای پلیمراز تودرتو (Nested-PCR) بود.

مواد و روش ها: در این مطالعه ۱۵۰ نفر از کارکنان کشتارگاهی در استان گیلان، ایران، پس از تکمیل رضایت نامه کتبی و پرسشنامه شخصی شرکت کردند. این حجم نمونه بر اساس میانگین شیوع بیماریزا در منطقه محاسبه شد. پس از ارزیابی سرم شرکت‌کنندگان از نظر آنتی‌بادی لپتوسپیرا، با استفاده از آزمایش آگلوتیناسیون میکروسکوپی، نمونه‌های ادرار از شرکت‌کننده مثبت برای ارزیابی‌های بیشتر با استفاده از واکنش زنجیره‌ای پلیمراز تودرتو جمع‌آوری شد.

یافته‌ها و نتیجه‌گیری: براساس نتایج به‌دست آمده، آزمون آگلوتیناسیون میکروسکوپی برای ۱۰/۷ درصد از شرکت‌کنندگان مثبت بود. با این حال، Nested-PCR برای آزمایش‌های آگلوتیناسیون میکروسکوپی مثبت روی سرم‌های جمع‌آوری‌شده از شرکت‌کنندگان دارای آنتی‌بادی علیه آنتی‌ژن‌های لپتوسپیرا منفی بود. نتایج حاضر نشان می‌دهد که لپتوسپیرا می‌تواند در انسان‌های بدون علامت در کشتارگاه‌ها ایجاد شود و احتمال زیاد انتقال بیماری به انسان در استان را برجسته می‌کند. بنابراین، اقدامات کنترل و پیشگیری گسترده‌تر برای کارگران کشتارگاه برای تضمین ایمنی مواد غذایی توصیه می‌شود.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

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واژگان کلیدی
- لپتوسپیروز،
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