Molecular and Serological Study of Pathogenic Serovars of Leptospira Interrogans in Cattle

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ABSTRACT

Bovine leptospirosis is a highly prevalent infection worldwide causing serious losses in cattle production and serving as a source for human infection. Diagnosis and assessment of prevalence of this infection in bovine herds is difficult. Early diagnosis of leptospirosis is important because severe leptospiral infection can have a fulminant course. The aim of this study was to perform a molecular and serological study of leptospirosis in 6 industrial dairy herds with previous records of leptospirosis, in Khorramabad suburbs of Iran. For this study, 100 blood and urine samples from cattle were collected. Serum samples were used for method MAT and urine samples were used for nested PCR for detection of leptospiral DNA in the samples. Serological results showed that 20 samples (20%) had a positive reaction against one or more serovars and 80 samples (80%) had a negetive reaction (Table 2). 10 samples (50%) showed serological reaction with more than one serovar

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(Table 3). The most prevalent leptospira serovars were Grippotyphosa 9(45%) and Canicola 8(40%), respectively. The least prevalent was Hardjo 3 (15%) (Table 4). All of sera were seronegative for other serovars (Table 4). The result of the present study also showed that most seropositive cases 8(40%) were observed in 3-4 years old cows (table 5). Nested PCR results showed that 19 (19%) of animals had a positive reaction and 81 (81%) had a negetive reaction (Table 2). Moreover, leptospira DNA was 31.1% in the cows with at least one clinical symptom and it was 9.1% in the cows without any clinical symptoms. With regard to these results, the difference between two groups of the cows was significant statistically (P=0.005) (table6). The comparison between nested PCR and MAT results showed that all cattle were positively regarding to nested PCR hadn't show positive serum titer in MAT test, and viceversa. The nested PCR exhibited high specificity and sensitivity for detection of pathogenic serovars in urine from cattle and this approach was much more sensitive than conventional MAT. The results of this study suggest that nested PCR could be an excellent approach for epidemiological studies and useful as a tool for early diagnosis of leptospirosis irrespective of serovar.
Key words: Leptospirosis, diagnosis, Cattle, Nested PCR, MAT.

Introduction

In the recent years, leptospirosis is identified as a global public health problem because of its increased mortality and morbidity in different countries (3, 28). Bovine leptospirosis is a worldwide zoonotic disease and is caused by pathogenic spirochaetes of the genus leptospira. The organism affects many mammalian species, including humans. Animals may become inapparent carriers and shedding of leptospires, primarily in the urine, serves as a source of infection for other animals and humans (27). The bacteria can survive in damp soil, fresh water, mud, and vegetation for a long time. Hence, the mode of transmission in human is either by contact with contaminated soil or water or with body fluid of

infected animals and may lead to potential lethal disease (3, 20). In cattle, leptospirosis is an important cause of abortion, stillbirths, infertility, poor milk production and death, all of which cause an economic loss (9). The causative agents of leptospirosis belong to the genus leptospira, which contains both saprophytic and pathogenic species (20). Laboratory diagnosis of leptospirosis is a confusing topic for treatment and surveillance because of its varied symptoms. In addition, delay in treatment of patients, due to the lack of available effective techniques for rapid diagnosis of disease may cause lethal sequel (4, 8). The clinical signs associated with bovine leptospirosis are variable and depend on the infecting serovar and the susceptibility of the animal. Clinically, bovine leptospirosis is difficult to diagnose because the signs are non-specific and easily confused with other diseases such as viral diseases (10, 39). The identification of carrier animals is therefore crucial in tackling the leptospiral infection. However, the current methods used for diagnosis are insufficient for this purpose. Traditionally, the reference method for diagnosis of leptospirosis is the microscopic agglutination test (MAT). However, this test has several drawbacks, including the requirement for a permanent stock of reference strains representing the appropriate serogroups, subjectivity involved in reading the results under dark-field microscopy, inability to differentiate titres of natural infection from vaccinal titres and the failure to identify most chronic shedders (38). Moreover, the assay is labour intensive and represents a biohazard to laboratory staff (10, 24, 36). Serologic tests do not allow early leptospiral diagnosis, especially in infections caused by serovar Hardjo type Hardjo-bovis (4, 15), as antibodies become detectable on approximately the seventh day after infection (34). Leptospira interrogans serovar hardjo is the primary causative agent of bovine leptospirosis throughout the world and responsible for most of the losses attributable to the disease (11). Leptospiral isolation is costly, very difficult, and often unsuccessful (13, 21), also is time consuming, subject to contamination and may require 4–6 months (10). It is

necessary to improve diagnostic procedures for animal leptospirosis. Molecular techniques such as PCR have the potential to improve leptospirosis diagnosis (19, 25). Recent developments in molecular biology, particularly the introduction of polymerase chain reaction (PCR) (30), are promising for the diagnosis of leptospirosis, as for that of other slow growing microorganisms such as Mycobacterium tuberculosis (7). A number of PCR assays have been applied to various clinical specimens such as urine, blood, cerebrospinal fluids and semen in order to detect leptospiral DNA (23, 41). These studies have proved that PCR is faster and more sensitive than the conventional tests. The aim of the present study was to evaluate the use of nested PCR to detect leptospires in urine samples from cattle naturally infected with leptospira spp. using primers described by Merien et al. amplify a 331 bp fragment of the rrs (16S rRNA) gene of pathogenic and nonpathogenic leptospires. The majority of the PCR primers developed so far target the 16S rRNA gene. This gene is highly conserved among leptospira species and other bacterial species (12, 15, 20, 26)

Material and Methods

Study population

According to the literature review of leptospirosis in Iran but not specially studies which had been conducted in West of Iran. Finally 6 herds were selected in Khorramabad. At the first stage, 100 blood samples were collected from all cows in case of MAT test during February 2014 till April 2014 and At the second stage, during May 2014 till July 2014, 100 urine samples were collected from the first stage cows, in case of nested PCR.

Serological procedures

Blood samples were taken from tail vein using a 10 ml glass tubes and transferred to the Laboratory Microbiology of the Faculty of Veterinary Medicine, University of Lorestan. Blood samples were centrifuged and serum separated and transferred in 2 ml micro tubes, stored at -20°C until

analysis. Then for testing MAT all samples serum transferred to the Leptospira Research Laboratory of the Veterinary Research and Teaching Hospital of the Faculty of Veterinary Medicine, University of Tehran. MAT was implemented on all serum samples, according to the standard method (44), using live 7-10 days antigens, representing the following leptospira interrogans serovars: Pomona, Grippotyphosa, Hardjo, Icterohaemorrhagiae and Canicola. All serovars were grown in specific liquid medium locally known as GRA–SINA which is produced in Leptospira Research Laboratory in Tehran University. Serial 2-fold dilution of each serum was prepared ranging from 1:50 to 1:1600 then 10 µl of each diluted test sera was added to an equal volume of antigen suspension on a microscope slide. Following incubation at 30°C for 1.5 h, the slide was examined under a dark-field microscope, using long working distance objectives at X100 or X200 magnifications. Agglutination was noted by observing clumps of leptospires. The lowest dilution that each serum was considered significant was 1:100. The end point titer was the highest titer in which 50% agglutination occurred, so that the lowest titer that was considered as positive was 1:100. The antigen that gave the highest titer was considered to be the infective serovar.

Molecular procedures

At second step, 100 urine samples, from the cattle that had been obtained the blood samples with the certain health conditions, have been collected from the mid- part of the urine stream in the sterile dark glass containers and then the urine samples have been transported to section PCR Laboratory Microbiology of the Faculty of Veterinary Medicine, University of Lorestan. In laboratory, at first step, there has been used Gerritsen method to prepare the urine samples in order to conduct PCR and nested PCR Test (14). The final sediment deposition at this stage has been suspended by 100 ml of the liquid and they have been stored in the freezer -20 °C until DNA extraction. At second step, all the urine samples, that were prepared at first step by using of Gerritsen method

and transported to the freezer, have been extracted from the freezer and there were used Acoprep DNA extraction kits (Cat. No. K-3032) by manufacturing company USA Bioneer. At the end of this step, from each urine sample, there was extracted about 6 microgram of DNA per 200 micro-liter by final detergent solution (30 ng / µl) which have been confirmed by determining A260/A280 ratio and have been stored in the freezer -20 ° C until conducting PCR and nested PCR test (Fig. 1).

Requirement materials to test PCR and Nested PCR

Positive Control: There was used leptospira Hardjo culture sample that the final amount of DNA in this sample was 1.58 ng/ml.

Negative Control: There was used distilled water with twice sterile as a negative control instead of sample DNA.

Ice: There was used a piece of ice to keep cool materials during producing the mixture Nested PCR and PCR.

Primer: The method of testing PCR and nested PCR in this present study is based on Merien (1992). There was used two primer pairs A/B in the PCR stage and primers C/D in nested PCR stage that were designed by some part of the pathogenic gen leptospira interrogans servear Icterohaemorrhagiae (Strain 140) manufactured by Synagen that their nucleotide sequence are shown in table 1. They are able to investigate all pathogenic strains of leptospira (2). The primers A and B are related to nucleotides 37-57 and nucleotides 348-368, respectively, of the structure of the first gene 16S from the bacteria leoptospira interrogans (rrs). Also, the primers C and D are related to nucleotides 58-77 and 328-347 of the gen 16S rRNA, respectively. The primers A/B has been amplified a 331 bp fragment during PCR reaction (Fig. 2). To increase the sensitivity of the technique to detect the low amount of leptospira DNA in the urine sample, the primers C/D have detected a 289 bp fragment inside the PCR products and have amplified it during nested PCR (Fig. 2). primers C/D were originally designed to confirm leptospiral origin of PCR products (26) (The product of C/D is internal to the amplicon obtained with A/B).

PCR and Nested PCR conditions

In the present study, there was used PCR kit(Cat. No. PR8250C) manufactured by Synagen Company to perform PCR and nested PCR. DNA amplification was performed in a 25 μL reaction volume consisting of 4 μL of template DNA added to a tube containing 12.5 μL Master mix, 0.5 μL primer A, 0.5 μL primer B and 7.5 μL Sterile Deionized Water.The standard conditions for PCR were as follows: 94°C for 3 min, 63°C for 1.5 min, 72°C for 2 min, followed by 30 cycles (each) at 94°C for 15 s, 63°C for 20 s, 72°C for 25 s, and a final extension at 72°C for 5 min (an additional 10 min was included at the end of the cycles to complete extension of the primers). All reaction were performed in a Perkin Elmer 2400 thermocycler (Ependorf – Master Cycler Gradient). Aliquots were analyzed by 1% agarose gel electrophoresis with ethidium bromide (0. 5 $\mu L / \mu L$) and UV transillumination (UV Transilluminator M-15). The nested PCR reaction was performed under the same conditions, using 2 μL of the first PCR reaction as template and 9.5 μL Sterile Deionized Water. The preparation of reaction mixtures, the DNA extraction (clinical samples and positive controls) and the subsequent amplification and detection of the PCR products were all performed at different locations within one building. This strict spatial partition of the different technical steps involved in the PCR was necessary to prevent contamination. In addition, tables and equipment were decontaminated periodically with HCl 10 %.

Data analysis

All data obtained from molecular and serological methods were analyzed using SPSS, version 16 software. Chi-square and Fisher's exact tests were used to detect significant differences among nested PCR and MAT results. A p value of ≤ 0.05 was considered statistically significant.

RESULTS

The results of this study indicated that 20 (20%) cows had a positive reaction against one or more serovars of leptospira interrogans (Table 2). Ten samples (50%) showed serological reaction with more than one serovar. One sample (5%) showed serological reaction with four serovars, and three samples (15%) showed reaction with three serovars. Six samples (30%) showed serological reaction with two serovars. Ten of the positive samples (50%) showed serological reaction with one serovar (Table 3). The most prevalent leptospira serovars were Grippotyphosa and Canicola with 45% and 40% respectively. The least prevalent leptospira serovar was Hardjo with 2.5% (Table 4). All of sera were seronegative for other serovars (Table 4). The most frequent titer level was 1:100 and the least frequent titer was 1:400 with 55% and 10% respectively for all serovars (Table 4). Regarding to the age groups, the result of the present study also showed that most seropositive cases 8(40%) were observed in 3-4 years old cows (table 5). The results of nested PCR test were investigated by the electrophoresis and by using of TBE 0.5X buffer on Agarose Gel 1% and coloring with ethidium bromide and then 289 bands were observed on the gel. Due to obtained results in nested PCR test on the urine samples of 100 cows related to six dairy herds at second step, leptospira DNA was investigated among 19 cows (19%) that the results were positive. Also, leptospira DNA was not investigated among 81 cows (81%) that the results were negative (table 6). Moreover, from 19 cows with positive PCR, 14 cows had at least one of the clinical symptoms of leptospira disease in their history and 5 cows had no clinical symptom and were apparently healthy. From 81 cows with negative PCR, 31 cows had some clinical symptoms and 50 cows had no clinical symptom (table 6). Based on the results obtained in this study, leptospira DNA was 31.1% in the cows with at least one clinical symptom and it was 9.1% in the cows without any clinical symptoms. With regard to these results, the difference between two groups of the cows was significant statistically (P=0.005) (table6). The comparison

between nested PCR and MAT show that all the cows with positive PCR have not positive serum titer. In another words, based on nested PCR test performed on the urine samples of the cows in this study, leptospira DNA was investigated among 19 cows (19%). Among the cows with positive PCR, 18 cows (18%) had positive serum titer and 1 cows (1%) had negative serum titer. The sensitivity and the feature of nested PCR method, compared to gold standard technique, were 90% and 98.75%, respectively showing that nested PCR is near to sensitive (94.74%) than MAT method.

DISCUSSION

The ultimate aim of veterinary and medical disciplines is to control and eradicate diseases in populations. Early and accurate diagnosis is therefore important in developing effective strategies for this purpose. Another important point is the determination of contributing factors to the disease through large-scale epidemiological surveys. However, the absence of rapid and accurate diagnostic tests has been a major hindrance in carrying out such surveys. This study employed nested PCR combined with two pair of genus-specific primers in order to investigate the presence of leptospiral DNA in the urine of cattle in west region of Iran (Khorramabad). The aim of this study was to determine whether nested PCR with primers derived from the 16S rRNA gene could be used to directly detect pathogenic leptospires in biological samples as an alternative to traditional diagnostic methods, such as leptospiral isolation and serology using the MAT. The results of this study showed that 19 (19%) of urine samples were contaminated. Also these urine samples in 1 (5.3%) of cows served as a reservoir of disease in dairy farms in Khorramabad suburbs while they were negative in their MAT samples. So it could be stated that the animal reservoirs increase the risk of potential spread of disease to other animals and especially humans and dangerous for human health, and this deserves special attention.

In a study conducted in Tehran district in 1992, 30% of the samples from cows of dairy farms were positive for leptospira. The highest and lowest serovar contamination was Hardjo and serovar Icterohaemorrhagiae respectively (1). Using MAT method, Sakhaee et al. (2007) found 14.47% of the sera samples collected from 380 cattle industrial dairy farms in Tehran district were positive for leptospira. The highest and lowest prevalence of serovar was Icterohaemorrhagiae (42.52%) and Balom(1.15%) respectively (31).

In the present study, the dominant serovars were Grippotyphosa and Canicola, that primary are hosted by mice and dog. Thus, rodents must be controlled in dairy farms for decreasing prevalence of disease in Khorramabad suburbs. Also the highest Canicola were related to with keeping dogs on dairy farms. Also In this study the low prevalence of Hardjo were. Rodrigues et al. (1999), found that Icterohaemorrhagiae and Pomona as dominant serovars in Brazilian cattle during 1996 and 1997. While previous studies had shown that Hardjo and Pomona serotypes were predominant (29). These results suggest that changes in the common serovars in the region occurred. According to studies conducted in Ahvaz, the high incidence of leptospirosis attributed to hot and humid weather of Khuzestan region and the heat temperature was reported more important than moisture (16). Due to the global warming of the earth, increases in prevalence of the disease over the time can be expected. Since serovar Pomona is related to annual rainfall, we conclude that increase in rainfall in North and central parts of Iran are a reason for higher prevalence of Pomona in this study. In a study by Durham and Colleagues during 1991-1992 have been done in Australia, Tarassovi and Hardjo serovars, respectively, were having the highest and lowest prevalence and none of this samples did not show positive reaction against Pomona, due to know low rainfall in the area. Because rainfall is very involve in serovar prevalence (16). In conclusion, although the disease is seen in tropical countries, it could also be present in cold and mountainous regions such as Khorramabad suburbs.

Considering the results of this study it should be noted that serovar changes is most common and related to weather condition. So, it is necessary to screen the serovars in every region regularly to prevent the spread of the disease.

PCR followed by Southern blotting with a probe derived from L. interrogans serovar Hardjo bovis were used by Van Eys et al. (1991) to detect leptospira spp. in bovine urine samples. Gerritsen et al. (1991) used the same primers for a semiquantitative study for PCR of L. interrogans serovar Hardjo bovis. Wagenaar et al. (2000) used nucleic acid hybridization to detect leptospira spp. in bovine urine. However, all of these experiments were performed with urine collected from animals experimentally infected with leptospira under controlled laboratory conditions. Recently, Talpada et al. (2003) used PCR to detect leptospira spp. in urine samples of naturally infected cattle using G1 and G2 primers, previously demonstrated by Gravekamp et al. (1993) to be specific for leptospira spp. The current study evaluated the use of the nested PCR as an alternative method to detection by MAT using the 16S rRNA gene coding region, which is confined to pathogenic strains of all leptospira spp. This report demonstrates that a previously described nested PCR protocol could be successfully used in diagnosis of bovine leptospirosis. Additionally, the amplification of 16S rRNA gene allowed the identification of infecting leptospiral species. The 16S rRNA sequences are conserved among members of leptospiral species. The results of this study suggest that PCR could be an excellent approach for epidemiological studies. The nested PCR possesses advantages over more traditional methods, such as MAT or isolation and culture of leptospires; it is simple, rapid, sensitive and appears to be able to differentiate between pathogenic and non-pathogenic species. Nested PCR is a rapid, specific and sensitive tool that can aid in the detection of leptospira spp. in bovine urine samples from herds with a clinical suspicion of leptospirosis. In this study, the sensitivity and the feature of nested PCR method, compared to gold standard technique, were 90% and

98.75%, respectively showing that nested PCR is near to sensitive (94.74%) than MAT method. In other words, nested PCR method, compared to gold standard method MAT, can detect 90% the infected cows and 98.75% non-infected and healthy cows. So, there has been used Positive Predictive Value (PPV) and Negative Predictive Value (NPV). There were obtained 94.74% from Positive Predictive Value of nested PCR method and 97.53% from Negative Predictive Value of nested PCR. Also, 79 (79%) cows were negative in nested PCR method and all of them had shown negative serum titer in MAT method. Therefore, from the cows that their leptospira DNA in urine samples were detected, 18% had positive results and 1% had negative results in MAT test. Based on statistical analysis, there was computed Kappa Agreement Coefficient Value between two tests (Kappa Value= 0.9) showing suitable agreement between two test methods ($P<0.001$) (Table 2). The nested PCR exhibited high specificity and sensitivity for detection of pathogenic serovars in urine from cattle. Sullivan (1972) reported that there was no correlation between MAT results and leptospirauria. Because animals shed the leptospires in urine in the early days of infection, antibody secretion may not be at detectable levels by MAT. Although MAT is recommended for use as a screening test at herd level, it has been reported to be unreliable for diagnosing infection at individual level (17). MAT lacks sensitivity also in detecting animals infected for more than two years due to the decline in agglutinating antibodies (32). The other important disadvantage of MAT is the inability to distinguish vaccinated animals from infected ones (18). The test is also laborious and time consuming. The necessity for a live antigen to carry out the test poses a risk for the laboratory workers. In spite of all these disadvantages, MAT is still widely recognized as a reference test at both individual and herd levels due possibly to the determination of serogroups involved in an infection, which has epidemiological value because different serogroups may not be associated with a particular clinical form of leptospirosis (6).

In this work, we first evaluate a polymerase chain reaction (PCR) based method for diagnosis of leptospirosis. Primers were designed to amplify a 331 bp region within the 16S rRNA gene that is conserved among pathogenic leptospira. The method was very specific for pathogenic serovars, however, it lacked sensitivity. To enhance the sensitivity, another primer pair was designed to amplify a 289 bp region within the 331 bp region of the 16S rRNA gene and was used in a nested PCR assay. This approach was much more sensitive than conventional PCR. We have described a novel nested PCR method for diagnosis of leptospirosis. This approach has the potential of allowing detection of the pathogen soon after infection, unlike most methods used, which are based on the detection of antibodies against the pathogens. The PCR results from clinical samples depend greatly on the purity of the extracted target DNA. The presence of inhibitory factors in serum or urine samples that impede amplification reactions have to be taken into account (21, 33, 42). In addition, PCR may fail when leptospires are present in very low numbers (15). The only drawback of the nested PCR approach is the high risk of contamination by the amplicon, resulting in false-positive results. To avoid contamination, sample processing and pre-PCR set-up were performed in different rooms from post-PCR manipulations. The workflow should always be unidirectional; each room should have its own dedicated set of pipettes and other equipment to avoid movement of materials and instruments between restricted areas. DNA extraction duplicates and nested PCR duplicates are valuable for PCR methods to provide the laboratory with a measure of the precision of the analysis. Negative controls, consisting of DNA extraction blanks and PCR blanks treated like samples, are especially necessary to control contamination in each step to prevent false-positive results. In conclusion, on the basis of its sensitivity and specificity, the PCR method described here should be useful as a tool for early diagnosis of leptospirosis irrespective of serovar.

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Table 1: The nucleotide sequences of the primers in PCR and Nested PCR(Merien, 1992).

Nucleotide Sequence (5'→3')	Primer Profile
GGCGGCGCGTCTTAAACATG	A
TTCCCCCCATTGAGCAAGATT	B
CAAGTCAAGCGGAGTAGCAA	C
CTTAACCTGCTGCCTCCCGTA	D

Table 2: The comparison between the results of Nested PCR and MAT tests.

Total		1:100 ≥ MAT results				Nested PCR results
		(٪)	negative	(٪)	positive	
19	19	1	1	18	18	positive
81	81	79	79	2	2	negative
100	100	80	80	20	20	Total

Table 3: Prevalence of MAT reaction with one or more serovars in 20 positive samples.

Number of serovar	Number of MAT positive(%)
One	10(50%)
Two	6(30%)
Three	3(15%)
Four	1(5%)
Total	20(100%)

Table 4: Frequency distribution of positive MAT in 100 serum samples of 6 industrial herds in Khorramabad suburbs by serovars and titers (Titers ≥ 1:100 were considered positive).

	Titer			
Serovar	1:100	1:200	1:400	Total positive (%)
Grippotyphosa	5	3	1	9(45%)
Canicola	4	3	1	8(40%)
Hardjo	2	1	0	3(15%)
Icterohaemorrhagiae	0	0	0	0(0%)
Pomona	0	0	0	0(0%)
Total	11(55%)	7(35%)	2(10%)	20(100%)

Table 5: Distribution of the MAT positive samples in 5 age groups of dairy cattle and feedlot in Khorramabad suburbs.

Age groups	MAT positive (%)	MAT negative (%)	Total
<1	1(5%)	19	20
1-2	2(10%)	18	20
2-3	5(25%)	15	20
3-4	8(40%)	12	20
>4	4(20%)	16	20
Total	20(20%)	80(80%)	100

Table 6: The absolute and relative frequencies of positive and negative results in Nested PCR test on the urine samples.

Significant level	Total		Nested PCR results				Disease clinical symptoms
	Relative frequency (٪)	Absolute frequency	Relative frequency (٪) Negative cases	Absolute frequency Negative cases	Relative frequency (٪) Positive cases	Absolute frequency Positive cases	
	45	45	68.9	31	31.1	14	Symptomatic
p=0.005	55	55	90.9	50	9.1	5	Asymptomatic
	100	100	81	81	19	19	Total

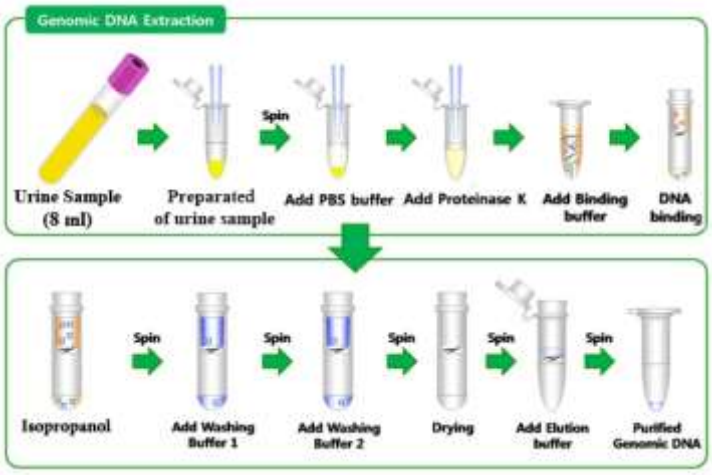

Figure 1: Extraction steps DNA from urine samples schematically

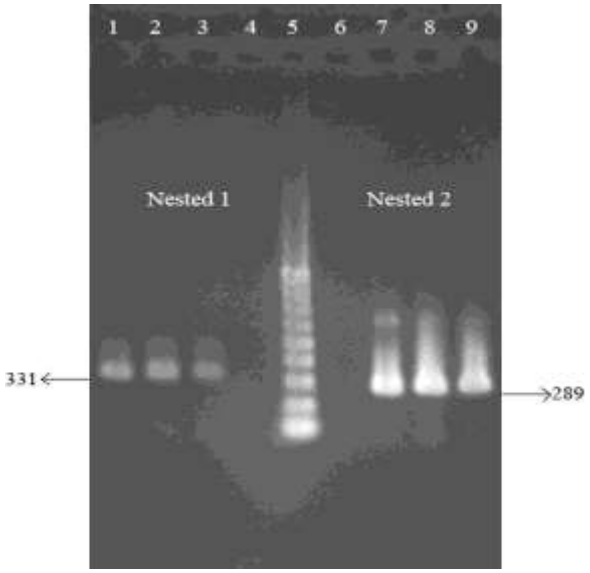

Figure 2: PCR and Nested PCR results. Order 1&2 containing positive samples PCR, Order 3&9 containing positive control (L. hardjo), Order 4&6 containing negetive control, Order 5 containing marker 100 bp ladder, Order 7&8 containing positive samples Nested PCR.