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Epidemiological study of Lumpy Skin Disease Outbreaks in North Western Iran

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Abstract

Lumpy skin disease (LSD) is a highly contagious transboundary disease of cattle with major economic losses. The present study was undertaken to address the emergence and epidemiological features of LSD in four north western provinces of Iran. These provinces have extensive borders with others country including Iraq, Turkey, Azerbaijan and Armenia. A population of 683 cattle from 91 farms were examined during LSD outbreak in Iran during 2014-2016. The information of the farms including the population size, gender, age, vaccination status, clinical signs and the number of death because of LSD were recorded in the designed questionnaires. A number of 234 blood samples were collected randomly from animals with and without clinical signs of LSD. DNA was extracted from blood samples and they were used for amplifying a fragment of 434 bp in size coupled with restriction fragment length polymorphism (RFLP) for molecular detection of lumpy skin disease virus (LSDV). The estimated prevalence, cumulative mortality and case fatality were 17.9%, 3.5% and 19.7% respectively. There was no significant difference in occurrence of the disease between male and female cattle. LSD occurrence in age groups above five years old and below six months old showed highest and lowest relative frequencies, respectively. Vaccination was significantly decreased the occurrence of clinical disease. The developed PCR-RFLP technique was able to differentiate between LSDV, sheep pox virus (ShPV) and goat pox virus (GPV). It was concluded that LSD was entered into

Iran probably from Iraq via uncontrolled animal movements along common land borders between two countries. Developed PCR-RFLP could be used as a rapid and inexpensive method for differentiating *Capripoxviruses* (CaPVs).

Key words: Lumpy skin disease, LSDV, epidemiology, PCR-RFLP, Iran

Introduction

Lumpy skin disease (LSD) is an economically important viral disease of cattle which is caused by a *Capripoxvirus* (CaPV) belonging to the *Poxviridae* family (Tuppurainen and Oura, 2012, Woods, 1988). LSD is a disease with high morbidity and low mortality rate (Barnard et al., 1994, Kitching and Taylor, 1985) affecting cattle of all ages and breeds; however, the young animals and cows in the peak of lactation are more severely affected (Tuppurainen et al., 2010, Weiss, 1968). The disease occurs and extends its boundaries in different ecological and climatic zones including Africa, Asia, Middle East and Europe (Alkhamis and VanderWaal, 2016, OIE, 2010, Tasioudi et al., 2016). The season of the year (mostly wet seasons), entrance of new animals to the herd and communal grazing and watering points have been considered as risk factors for the occurrence of LSD (Gari et al., 2011, Tuppurainen and Oura, 2012).

Due to the potential risk for rapid spread and ability to cause severe economic losses, LSD was considered as a notifiable disease by the Office International des Epizooties (OIE) (OIE, 2010). The clinical picture of the disease varies from acute to subclinical forms (Davies, 1982, Woods, 1988). Fever, inappetance, generalized skin nodules, enlarged lymph nodes, emaciation, low milk production and abortion are characteristic features of the clinical forms of the LSD. In severe cases, ulcerative lesions may develop in mucous membrane of mouth, trachea, larynx and esophagus (Radostitis et al., 2006).

The lumpy skin disease virus (LSDV), belongs to the genus CaPV, which includes sheep pox virus (ShPV) and goat pox virus (GPV). These viruses are transmitted via skin abrasions, contaminated environment and mechanically by biting arthropods (MacLachlan and Dubovi, 2011); however, it was speculated that LSDV mainly transmitted by the latter route and transmission of the virus by contact is less likely (Carn and Kitching, 1995). LSDV infects cattle,

water buffalo and wild ruminants and it does not infect and is not transmitted between sheep and goats (OIE, 2010, El-Nahas et al., 2011, Lamien et al., 2011a). LSDV is very stable in environment remaining up to six months in a suitable environment such as shaded animal pens; however, the virus is susceptible to a range of disinfectants and sunlight (Davies, 1981).

Virus isolation, electron microscopy, serological and molecular techniques have been used for LSDV detection (OIE, 2010), among them molecular techniques such as conventional and real time PCR were proved to be more accurate, reliable and faster than other methods for LSDV detection (Stubbs et al., 2012). PCR and restriction fragment length polymorphism (RFLP) has been used for differentiating ShPV and GPV (Hosamani et al., 2004) and also differentiating virulent LSDV from vaccine strain (Menasherow et al., 2014).

Prevention and control of LSD has been achieved using commercially available attenuated vaccines in countries where LSD is endemic (Tuppurainen et al., 2015, OIE, 2010). In Iran, ShPV and GPV vaccines have been used successfully for vaccination against LSDV infection. In the present study, epidemiological features of the recent LSD outbreaks in the north west of Iran, which have extensive land borders with other countries including Iraq (1599 km), Turkey (534 km), Azerbaijan (432 km) and Armenia (44 km), have been investigated. In addition, molecular methods including PCR and PCR-RFLP were employed for LSDV detection and its differentiation from ShPV and GPV.

Materials and Methods

Study areas and sampling procedure

During outbreaks of LSD in north west of Iran in 2014-2016, a number of 683 cattle belong to 91 farms from four provinces (Gilan, Kurdistan, East and West Azerbaijan) mostly with traditional husbandry system were screened for infection with LSDV (Table 1). Whole blood samples were randomly collected from a number of 234 cattle (122 cattle with clinical signs and 112 cattle without clinical signs). Blood samples were collected in EDTA contained tube, immediately transferred beside the ice to the central laboratory at the Faculty of Veterinary Medicine, Urmia University and stored at -20°C until DNA extraction.

Farms and sampled animal information

A questionnaire was designed in order to acquiring the required information of the farms and animals. The farm's population, location and its husbandry system, and the age, sex and vaccination status of animals were recorded. Examined animals were categorized into five age groups including <6 months, 6-12 months, 1-2 years, 2-5 years and greater than 5 years. The information of the animals with clinical signs including date of the onset of the disease, the number of the clinically affected cattle, clinical findings and the mortality rate were also recorded.

Viral DNA extraction

Extraction of viral DNA from blood samples were performed using NucleoSpin® Blood Kit (Macherey-Nagel, Germany). An amount of 200 µl of whole blood sample was used for DNA extraction according to instructions form kit manufacturer. Extracted DNA was quantified using NanoDrop 2000c (Thermo Scientific, USA).

Amplification of thymidine kinase (TK) gene

For detection of LSDV in blood samples, PCR was used in order to amplifying a conserved region of the thymidine kinase (TK) gene. The primer pair used for virus identification is amplifying a 434 bp fragment between positions 56698–57132 of the CaPVs genome which described previously by Tageldin et al. (2014). The PCR reaction was prepared in a 25 µl mixture containing 50-100 ng genomic DNA, 0.5 µM of each primer, 50 µM of each ATP, CTP, GTP and TTP, 2 mM $MgCl_2$, 2.5 unit SmarTaq DNA polymerase (SinaClon, Iran) and 2.5 µl 10X PCR buffer. The PCRs were performed using a QB Cycler® gradient thermo cycler (Quanta Biotech, England) with the following thermal condition: initial denaturation for 90s at 95°C, followed by 35 cycles of denaturation (45 sec at 95°C), primer annealing (45 sec at 56°C) and strand extension (60 sec at 72°C), ending with a final strand extension step for 7 min at 72°C. The resultant PCR products were separated in 1.5% (w/v) agarose gel containing SimplySafe (EURx, Poland) 2.5 µl/50 ml gel for 1 h at 75 V and visualized under UV transilluminator.

Restriction fragment length polymorphism (RFLP) analysis of PCR products

Amplified PCR products were double digested using *Rsa*I *and Hae*III endonucleases (Fermentas, Canada) according to the manufacturer's instructions. Digestion reaction was carried out in a

total volume of 15 µl containing 5 µl of PCR product, 0.5 µleach of FastDigest *RsaA*I and *Hae*III endonucleases and 1.5 µl of 10X reaction buffer. Digested fragments were separated on 2% (w/v) agarose gel stained with SimplySafe (EURx, Poland) 2.5 µl/50 ml gel for 1 h at 75 V and visualized under UV transilluminator.

Statistical analysis

Statistical analysis of obtained data was performed using Chi-square test and risk analysis in SPSS statistical program ver. 22 (SPSS IBM Corp, USA).

Results

LSD occurrence

Out of 683 cattle screened for clinical findings of LSD, including fever, inappetance, reduced milk production, skin nodules and enlarged lymph nodes, 122 cattle showed clinical manifestations of the disease, with the estimated prevalence of 17.9%. The number of cattle examined for LSD in four north western provinces and the frequency of the clinical disease has been shown in table 1. Based on the presented data, there was a significant difference in the relative frequency of the disease between provinces ($P<0.001$). The estimated overall cumulative mortality and case fatality for LSD were 3.5% and 19.7% respectively. The frequency of clinical LSD in male and female cattle were 17.1% and 18.1% respectively, revealing no significant difference between two genders. The percentage of clinically diseased animals were significantly different among age groups ($P<0.05$). Animals in the age groups above five years old and below six months old showed highest (73.3%) and lowest (39.3%) frequency of the disease, respectively.

The vaccination status of the examined cattle against LSD in four provinces were different (East Azerbaijan, 22/48; 45.8%), (West Azerbaijan, 54/64; 84.3%), (Gilan, 26/75; 34.6%) and (Kurdistan, 45/47; 95.7%). Among vaccinated and non-vaccinated cattle, clinical disease was

observed in 40.8% and 71.3% of animals respectively, which differed significantly with odd ratio of 0.27 (95% CI: 0.16-0.49) (Table 2).

Molecular detection of LSDV in blood samples

Examining 234 collected blood samples for LSDV using PCR method, CaPV was detected in a number of 80 (33.1%) examined animals (Fig 3). Among 122 cattle showing clinical signs and 112 cattle without clinical manifestations of LSD, 45 (36.9%) and 35 (31.2%) cattle were positive in PCR respectively. The percentage of positive samples in East Azerbaijan, West Azerbaijan, Gilan and Kurdistan were 27.1%, 39.0%, 40.0% and 25.6%; respectively, which were not significantly different from each other (Table 3). RFLP analysis of positive field samples using *Hae*III and *Rsa*I endonucleases was able to differentiate between three CaPVs (ShPV, GPV and LSDV) (Fig 4). All animals with positive PCR were showed identical RFLP pattern of LSDV, confirming that all PCR positive cattle were infected with LSDV.

Discussion

LSD known as a transboundary animal disease due to its severity of losses and ability to spread to other countries and its significant impact on trade and food security (Rossiter and Al Hammadi, 2008). The present study is the first published report of the occurrence of LSD in Iran. The first case of the disease which was observed in Kurdistan province reported to OIE in May 2014 by Iranian Veterinary Organization (IVO). After the emergence of the disease in Kurdistan province, LSD spread rapidly through north western and western provinces (West and East Azerbaijan, Gilan, Kermanshah and Khozestan) of Iran. Before the emergence of LSD in Baneh (a city from Kurdistan province, located near the border of Iran and Iraq), the disease was reported from Iraq in 2013 which despite the uncertainty about the origin of LSD outbreak in Iran, it is possible that the disease was introduced into Iran by the uncontrolled movements of infected animals between two countries.

Based on clinical signs of LSD, the estimated prevalence, cumulative mortality and case fatality of the disease in four examined provinces were 17. 9%, 3.5% and 19.7% respectively. These data are different from previously reports from Turkey (morbidity 12.3% and mortality 6.4%) (Sevik and Dogan, 2016), Oman (morbidity 13.6-29.7% and mortality 15.4-26.3%) (Tageldin et al.,

2014), Jordan (morbidity 26% and mortality 1.9%) (Abutarbush et al., 2015). However, the estimated overall cumulative mortality for LSD was 3.4% which was in accordance with LSD mortality reported previously (Barnard et al., 1994, Kitching and Taylor, 1985). Since the main mode of transmission of LSDV is via blood-feeding insects and the distribution of these disease vectors are heavily depending on climate, season, environmental temperature, humidity and vegetation, this may be the reason for this discrepancy in disease prevalence and mortality among different countries (Davies, 1982, Davies, 1991).

The age distribution of the disease was significantly different, showing that LSD was occurred more frequently in the older cattle which is not in agreement with the previous reports indicating the higher frequency of the disease in the younger than older animals; although, the difference was not statistically significant (Sevik and Dogan, 2016, Ayelet et al., 2013, Salib and Osman, 2011). In the present study, there was no significant difference in LSD prevalence between male and female cattle. However, in a report by Ayelet, et al. (2013) disease morbidity was higher in females than male animals.

According to IVO regulations, control of LSD is based mainly on vaccination of animals with sheep pox and goat pox vaccines coupled with other measures consisting of prevention of spread by suitable hygienic precautions, implementation of regulations for adequate quarantine, prevention of the introduction of animals into herds, destruction of cadavers, restriction of the movement of animals inside the country and also at the land borders with neighboring countries. Similar measures apparently are also undertaken in neighboring countries (Turkey, Iraq and Azerbaijan) (Zeynalova et al., 2016, Sevik and Dogan, 2016, Al-Salihi and Hassan, 2015). The efficacy of LSDV, sheep pox and goat pox vaccines compared in two recent studies. The protection efficiency of these vaccines is still a matter of controversyin these studies. Ben-Gera et al. (2015) reported that the efficacy of LSDV vaccine was superior when compared with the RM65 SPPV (10X) vaccine. Gari et al. (2015) showed that the Ethiopian Neethling and KSGP O-180 (Kenya sheep and goat pox vaccine) vaccines failed to provide protection in cattle against LSDV; whereas, the Gorgan GTPV vaccine protected all the vaccinated calves from development of clinical signs of LSD. According to OIE (2016), since all strains of capripoxviruses examined so far, whether of bovine, ovine or caprine origin, share a major neutralising site, animals recovered from infection with one strain are resistant to infection with any other strain.

Based on our findings, 40.8% of vaccinated cattle with sheep pox and goat pox vaccines and 71.3% of non-vaccinated cattle showed clinical disease revealing that vaccination significantly decreased the occurrence of clinical disease in the vaccinated animals with odd ratio of 0.27, which means that the chance of being clinically diseased was decreased by more than three times in vaccinated animals. This finding is in contrast with the results of Hailu, et al. (2014) which reported no association between vaccination and LSD occurrence.

In the present study the sampling was initiated immediately after the first report of LSD by OIE (from Baneh), and after that the region encountered the first outbreak of the disease. Since, there was no vaccination against LSD before the occurrence of the disease, the animals were immunologically naïve and therefore, the disease spread too rapidly in the country. The sampling was initiated in late spring which was concurrent with peak activity of insects in the region. From 60 vaccinated cattle with clinical manifestation of the LSD, blood samples in 25 cases (41.6%), were collected in a short time after initiation of the vaccination, i.e. just 1-10 days before the appearance of the clinicl signs. Therefore, the high frequency of clinical signs in vaccinated animals may be due to the short time between sampling and vaccination. According to Hunter and Wallace (2001) immunity to LSD starts developing 10 days after vaccination and reaches its peak after 21 days. Thus, the short time between vaccination and sampling may be the reason for high percentage of clinical signs in vaccinated animals.

Based on our findings 31.3% (35/112) of animal without clinical signs were positive in PCR for LSDV. The most probable reason for the high frequency of positive samples in animals with no clinical signs is that the blood samples were collected from animals in their incubation period. Furthermore, according to Barnard, et al. (1994) about 40–50% of experimentally infected animals exhibit the clinical signs. All our experiments have been performed at the central laboratory of the Faculty of Veterinary Medicine with high standard biosecurity measures. All laboratory equipment's, samplers, buffers, reagents, tips etc. checked several times in order to avoiding DNA contamination and false positive results.

The presence of clinical signs of LSD is the main criterion for tentative diagnosing the disease. However, the diagnosis of LSD sometimes become difficult because only transient fever or few skin lesions are present. In the present study PCR-RFLP examination of collected blood samples form cattle with and without clinical disease showed that 36.9% and 31.2% of samples were

positive for LSDV, respectively. The low detection power of PCR for detecting LSDV in the blood of clinically affected animals which has been reported in previous studies (Babiuk et al., 2008, Awad et al., 2010), is reflecting this fact that the LSDV is present at low infective titers in blood during the first days following infection.

Since ShPV and GPV vaccines are routinely used for vaccination against LSD in Iran and other countries, rapid and accurate detection and differentiation of CaPVs is essential for surveillance and confirming the disease following outbreaks in either endemic or disease-free countries. PCR-RFLP technique used in the present study was able to accurately discriminate LSDV, ShPV and GPV from each other. While the developed PCR-RFLP technique is not as reliable as real time PCR assay employed for differentiating CaPVs by Lamien et al. (2011b); however, it can be used as a relatively rapid, reasonably acceptable and inexpensive method in less equipped diagnostic laboratories.

In conclusion, the present study reported some epidemiologic features of LSD and detection of its causative agent using molecular methods for the first time in Iran and its quick spread among different provinces, causing considerable economic losses in the traditional system of animal husbandry. The epidemiological aspects of LSD in north western provinces of Iran and the importance of vaccination for prevention and control of the disease were investigated. Strict monitoring on animal movements between neighboring countries should be employed by veterinary authorities.

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Figure captions

Fig 1. The map of north west of Iran and neighboring countries showing the provinces where LSD outbreaks occurred.

Fig 2. Cattle with clinical signs of LSD. The skins nodules distributed through the body of the affected animals (A: East Azerbaijan; B: Gilan).

Fig 3. Agarose gel image of amplified PCR products of 434 bp in size from affected animals with LSD. Lanes 1 & 8: 50 bp molecular marker, Lanes 2-4: Positive field samples for LSDV, Lane 5: ShPV vaccine, lane 6: GPV vaccine, lane 7: negative control.

Fig 4. Agarose gel image of RFLP patterns of amplified PCR products. Lane 1: 50 bp molecular marker, lanes 2-4 & 7: RFLP patterns of LSDVs from field samples, lane 5: RFLP pattern of amplified PCR product from ShPV, lane 6: RFLP pattern of amplified PCR product from GPV, lane 7: Undigested PCR product.

	Provinces				Total
	East Azerbaijan	Gilan	West Azerbaijan	Kurdistan	
Number of screened cattle	99	263	257	64	683
Number of farms investigated	15	42	11	23	91
Number male animals	33	77	48	6	164
Number of female animals	66	183	204	66	519
Number of clinically diseased animals	28	58	9	27	122
Number of deaths	11	2	2	9	24

Table 1. Frequency distribution of examined cattle in four provinces

Table 2. The gender, age and vaccination status distribution of examined cattle

		Animal's health status for LSD		Total
		Clinical	Non-clinical	
Gender	Female	94	97	191
	Male	28	15	43
Total		122	112	234
Age	< 6 months	11	17	28
	6-12 months	19	20	39
	1-2 years	14	18	32
	2-5 years	41	31	72
	Greater than 5 years	33	12	45
Total		118	98	216*
Vaccination status	Vaccinated	60	87	147
	Non-vaccinated	62	25	87
Total		122	112	234

* The age of 18 examined animals were unknown.

Table 3. The results of PCR examination of 234 collected blood samples from cattle for LSDV

			Provinces				Total
			East Azerbaijan	West Azerbaijan	Gilan	Kurdistan	
Clinical	PCR	Positive	10	4	23	8	45
		Negative	18	5	35	19	77
	Total (%)		28 (22.9)	9 (7.4)	58 (47.5)	27 (22.1)	122
Non-Clinical	PCR	Positive	3	21	7	4	35
		Negative	17	34	10	16	77
	Total (%)		20 (17.8)	55 (49.1)	17 (15.2)	20 (17.9)	112
Total	PCR	Positive	13	25	30	12	80
		Negative	35	39	45	35	154

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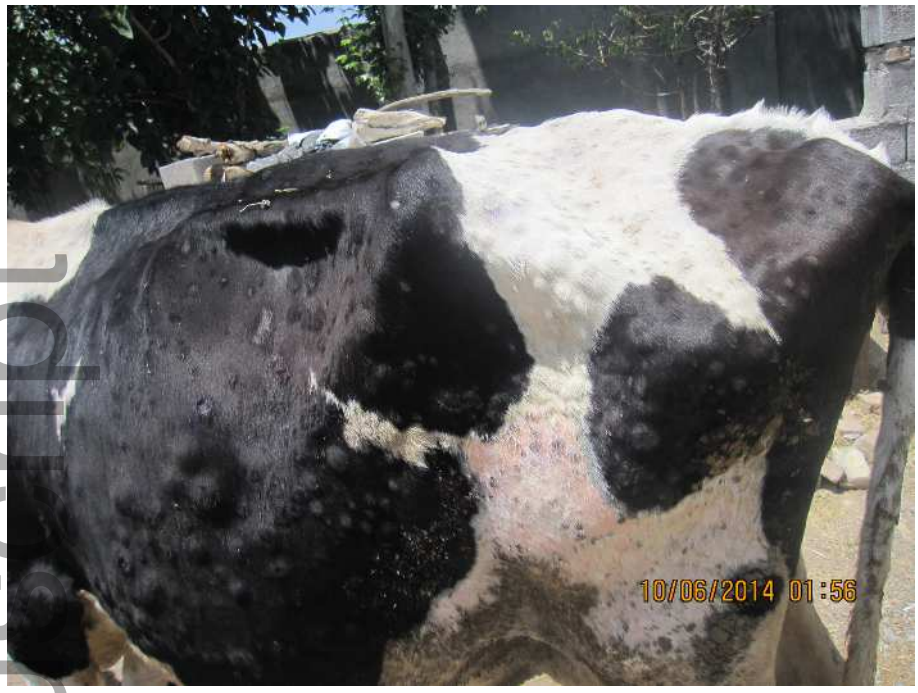

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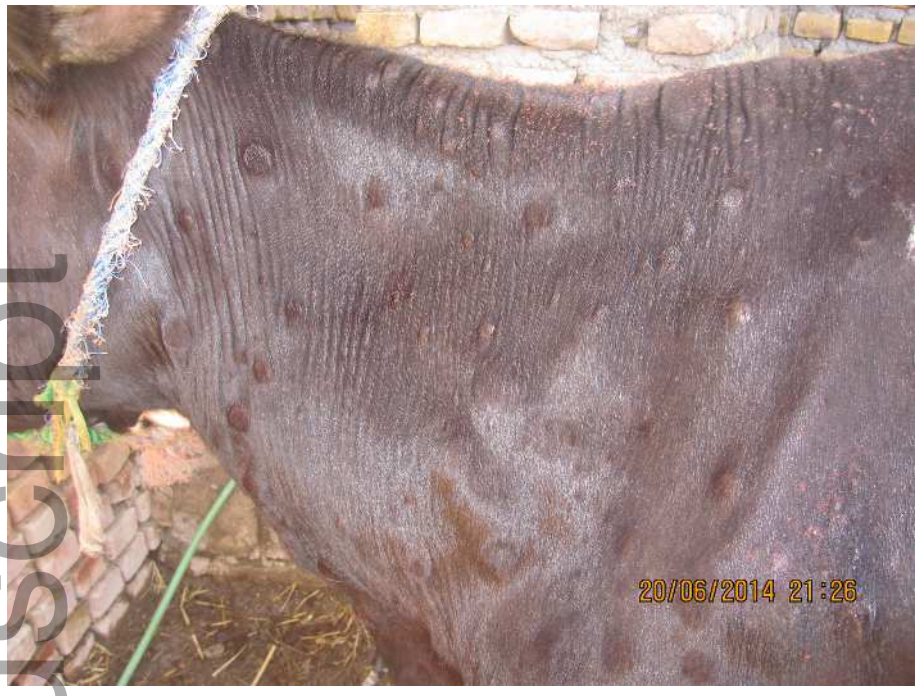

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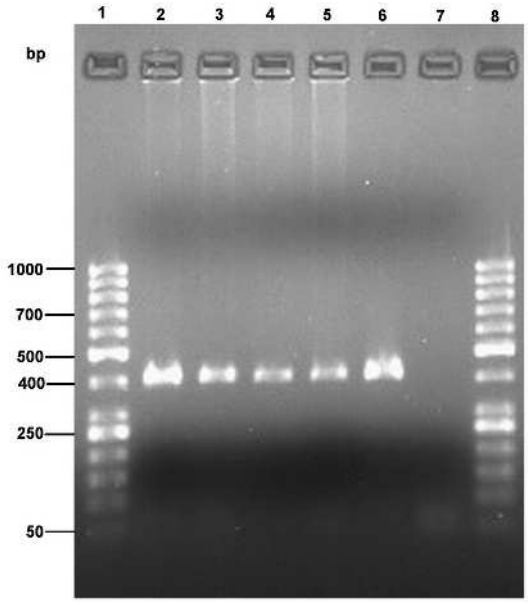

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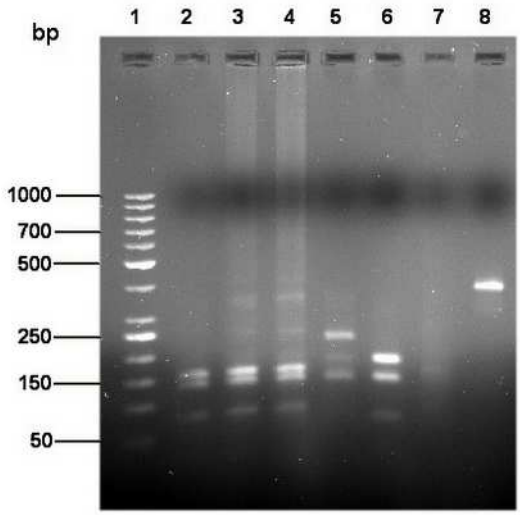

tbed_12565_f4.tif

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