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Bacteria causing endometritis and abortion in Arabian mares

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Abstract

The aim of the present study was recording the type and rate bacteria causing abortion in Arabian horse. Study the presence of leptopira in stallion`s semen by isolation serodiagnosis and molecular identification in semen samples.

A total number of 171 samples collected, Samples were cultured for isolation of bacteria as well as leptospira species. 53 different isolates (40.2%) were obtained from different samples (132), 5.3% of samples showed mixed infection (2 from uterine washes and 5 from fetal samples).. The rate of different isolates in different samples were recorded, where *S.equi* subsp. *zooepidemicus* and *P.aeruginosa* showed the highest rate of isolation (4.5%) followed by *klebsiella pneumonia* subsp *pneumonia* and *Streptococcus equi* subsp. *equi. negative isolates from the equine uterus. Burkholderia cepacia complex (BCC) and S.equi subsp. equi* were isolated in a rate of 3.8%. *Rhodococcus equi and S. enterica* subsp. Arizonae were isolated in a rate of 3%.. *Listeria monocytogenes (L.monocytogenes)* was isolated in a rate of 2.3%*; Arcanobacterium haemolyticum (A.haemolyticum)* and *Staphylococcus aureus (S.aureus)* were isolated in a rate of 1.5%. This was the first report on isolation of *A. haemolyticum* from aborted mares. *Enterobacter aerogenes (E.aerogenes); Hafnia alvei (H.alvei) S. enterica* subsp. *enterica Typhimurium (S.Typhimurium) and Staphylococcus haemolyticus (S. haemolyticus*) were isolated in a lowest rate 0.8%.

Keywords: Abortion in Arabian equine, A.hemiloticum, H.alveae, BCC

1. Introduction

Abortion occurs in a range of 10–15% of equine pregnancies and may have infectious and noninfectious causes **(Williams 2012).** Infections with viruses, fungi or bacteria cause inflammation to the placenta and affect the placental function **(Williams 2012)**. The most common bacterial causes of equine abortion include *Streptococcus zooepidemicus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Leptospira sp*. and *Nocardia sp*. (**Williams 2012).** Several other bacterial species have been implicated as cause of equine abortion but occur less commonly **(Williams 2012).** The aim of the present study recording the type and rate bacteria causing abortion in Arabian horse. Study the resence of leptopira in stallion`s semen by isolation serodiagnosis and molecular identification in semen samples.

2. Materials and methods

A total number of 171 samples collected, where 22 uterine washes; 13 vaginal washes and 97 internal organs from aborted feti with placenta and still birth (84 fetal internal organs and 13 placenta). Most of collected samples were with full history regarding loss was required (from the referring veterinarian or, if directed, from stud personnel or the owner or the lab) (Table 1). Also, 40semen samples were collected from stallions

One gram or 1ml of each sample was inoculated on 9ml buffer peptone water (BPW) and incubated for 37ºC for 24hr

Isolation of Gram positive bacteria:

All inoculated BPW were subcultured on Blood agar and specific media of each suspected microorganism as mannitol agar, tinsidal media, Alloa media and each plate incubated at suitable temperature and duration (UK standards 2014 IDs 2&4; UK standards 2018 ID3 and UK standards 2020 ID7). The suspected colonies were subjected to further identification biochemically and SRO GP24.

Isolation of Gram negative bacteria:

All inoculated BPW were subcultured on Blood agar and specific media of each suspected microorganism as pseudomonas specific media, eugenic media, macConkey agar, and eosin methylene media but in case of isolation of campylobacter, the samples were inoculated on thioglycollate broth as enrichment media then subcultured on campylobacters specific media. All plates were incubated at suitable temperature and duration according to (UK standards 2015 IDs 16&17 and UK standard 2018 ID23). The suspected colonies were subjected to further identification biochemically and SRO GN24. OIE (2018 a & b)

Isolation of salmonella species bacteria:

One ml of inoculated BPW was inoculated on 9ml RV broth and incubated for18-20hr at 37C, then one ml of each inoculated RV was subcultured on XLD or S.S. Media and incubated at 37°C for 24hr. The suspected colonies were subjected to further identification using SRO GN24 according to (OIE 2018) and confirmed by serotyping at serology laboratory in Animal Health Research Institute (accredited Lab) (using slide agglutination test (Sifin).

Isolation of Leptospira species:

The Ellinghausen, McCullough, Johnson and Harris – EMJH (Himedia) semi-solid medium was prepared in two formulations, one without antibiotics (non-selective) and the other, with the addition of 5-fluoruracil (300 mg/L) and nalidixic acid (20 mg/L), named as the selective medium The concentration of leptospires was adjusted to contain 20 to 30 live spirochetes when observed under the dark field microscopy with 100x objective. 2mL of leptospires media with 1,0 mL of semen diluted egg yolk-citrate extender pH 7, these mixtures were incubated at 28° to 30ºC for 30 min. Four ten -fold serial dilutions (10-1 to 10-4) were performed in EMJH, without agar and antibiotics. Each dilution was cultured in five tubes with selective EMJH in proportion at 1:10 (v/v). After 24h incubation at 28º to 30ºC, these dilutions were sub cultured in the same proportion in EMJH, without antibiotics, and then incubated at 28º to 30ºC for six weeks. 3 repetitions were carried out. Cultures were examined weekly under dark field microscopy and tubes showing contaminants were discarded.

Serological identification of Leptospira using horse leptospira ELISA kits (Sunlong Biotech):

According to the manufacture of kit: 50µl of negative and positive control were added (2wells for each) and one empty well as a blank control, 10µl of each sample was diluted with 40µl of sample dilution buffer and loaded into their corresponding wells mixed well and incubated at 37oC for 30 minutes, then washed for 5 times using diluted washing buffer.

50µl of HRP- conjugate (horse radish peroxidase) was added to each well except the blank one. Then, the plate was incubated at 37oC for 30 minutes, followed by 5 times of washing. 50µl of chromogen solution A and 50µl of chromogen solution B were added to each well, mixed by gentle shaking and incubated at 37oC for 15 minutes in a dark place. 50µl of stop solution was added to each well to stop the reaction. The absorbance OD was read at 450nm. The OD value of the blank well was set as zero.

Molecular identification of Leptospira in semen samples:

PCR primers LA/LB ([5¢-GGC GGC GCG TCT TAA ACA TG-3¢] and [5¢-TTC CCC CCA TTG AGC AAG ATT-3), which were objective the 16S rDNA gene at 331bp, were used to confirm the genus Leptospira. The cycling conditions consisted of an initial denaturation at 94°C for 3 minutes, 35 cycles each of 94°C for 1 minute, 57°C for 1 minute, and 72°C for 2 minute using thermocycler (Kyratec) , and additional extension at 72°C for 10min. PCR products were submitted to agarose gel electrophoresis using 1%agarose.

.3. Results and Discussion

A total of 53 different isolates (40.2%) were obtained from different samples (132), 7 samples (5.3%) showed mixed infection (2 from uterine washes and 5 from fetal samples). Only 5 samples (3.8%) showed no bacterial isolates (Table 2). These results revealed that bacterial infection play an important role in causing endometritis which cause infertility and abortion in mares **(Albihn, et al. 2003 and Ricketts et al. 1993)**. Also it was observed that the age of abortion occurred at late stage of abortion (7-9 months) only one aborted at 4th month of gestation and infected by *S.zooepidemicus* and 3 cases showed abortion at 5 months (one infected by S.enterica subsp. Arizona; one infected by *L.monocytogenes* and one by *P.aeruginosa*). **Swerczeck and caudal (2007)** reported that abortions occur early in gestation, between conception and 90 days, often go undetected and are frequently confused with infertility, while the highest incidence of bacterial abortions occurs between the fifth and tenth months of gestation.

Table (3) and Figure (1) illustrated the rate of different isolates in different samples, where *S.equi* subsp. zooepidemicus and *P.aeruginosa* showed the highest rate of isolation (4.5%) followed by *klebsiella pneumonia* subsp pneumonia and *Streptococcus equi* subsp. equi. These results agree with **LeBlanc & Causey 2009, Davis et al. 2013, Christoffersen et al. 2015)**. Also, **Ferris et al. (2014), Beehan et al. (2015), Brock et al. (2017)** recorded *P.aeruginosa* and *Klebsiella pneumoneae*) are the most Gram negative isolates from the equine uterus.

Burkholderia cepacia complex (BCC) and S.equi subsp. equi were isolated in a rate of 3.8% Bcc or *Burkholderia cepacia* Table (3), **Attili, et al. (2013)** isolated Bcc from uterine swabs of horses and cattle. Bcc is a group of catalase-producing, lactose-nonfermenting, Gram-negative bacteria composed of at least 20 different species, its distribution in animal species and associated infections are not widely documented **(Berriatua et al., 2001)**. *S.equi* subsp.equi mainly cause Strangles in foals. **Albihn et al. (2003)** and **Christoffersen et al. (2015)** reported that S.equi has also been isolated from the equine endometrium.

Rhodococcus equi and *S. enterica* subsp. *Arizonae* were isolated in a rate of 3%. Table (3), these results disagree with **da Silva et al. 2020** who isolated *R. equi* in a rate of 1.9%. *S. enterica subsp*. arizonae have not previously reported as a cause of abortion in pregnant mares and uncommonly isolated from equids in the literature, it may cause late-term abortions in susceptible animals **(Mayhew, et al. 2021)**.

Listeria monocytogenes (L.monocytogenes) was isolated in a rate of 2.3%; Arcanobacterium haemolyticum (A.haemolyticum) and *Staphylococcus aureus (S.aureus)* were isolated in a rate of 1.5% Table (3). L.monocytogenes is most commonly associated with encephalitis, septicemia, and abortion in veterinary species **(George 2009)**; however, clinical disease in the horse is rare. Previous reports of disease caused by this organism in horses ranging from 6 days to 6 years old include multi systemic infections, septicemia, pneumonia, hepatitis, abortion, and neurologic disease **Welsh (1983)**.the presence of L. monocytogenes may be due to feeding animals on silage which has a well-known risk factor for listeriosis, especially when used the silage of poor quality (pH > 5.5). **Rütton et al. (2006) and Gudmundsdottir et al. (2004)**.

A.haemolyticum, formerly known as Corynebacterium haemolyticum was first described in 1946 as cause of nasopharynx and skin infections in humans. **Hassan et al. (2009)** characterized phenotypically and genotypically seven A. haemolyticum isolated from infections of six horses. However, no data were given about the route of infection and about the zoonotic importance of these strains **(Hassan et al., 2009).** This was the first report on isolation of *A. haemolyticum* from aborted mares. These results nearly agree with **da Silva et al. 2020** in isolation of A.hemolyticum 1.9%. However, They isolated S.aureus in a rate of 3.8% in mixed infection with Bacillus spp.

Enterobacter aerogenes (E.aerogenes); *Hafnia alvei (H.alvei) S. enterica* subsp. enterica *Typhimurium (S.Typhimurium)* and *Staphylococcus haemolyticus (S. haemolyticus)* were isolated in a lowest rate 0.8% Table (3). On contrary, **da Silva, et al. 2020** isolated E.aerogenes in rate of 5.7%. Hafnia alvei is the only species in the Hafnia genus. The species has been known by the names *Enterobacter hafniae, Bacterium cadaveris, Bacillus asiaticus* and *B. paratyphi alvei* **Janda and Abbott (2006)**.

In mares, H. alvei can produce abortions in different periods of gestation. In 1962, H. alvei was isolated in pure culture from the fetus and placenta. In1983, a case of mare that spontaneously aborted at month 8 of pregnancy was recorded **(Padillaa, et al. 2015)**. This was the first report about the isolation of H. alvei from horses in Egypt. **Abeer (2004)** isolated *S. Typhimurium* in a rate 5% in Egypt. This low rate of isolation may beattributed to the adequate treatment of infection during the previous years.

Conclusion

The present study explains partial situation of different pathogenic microorganism in different samples of Arabian horses (uterine wash, vaginal wash, semen and internal organs of aborted feti. Also new pathogenic isolates may be isolated from aborted Arabian equine for the first time in Egypt *(H.alvei and A.hemolyricum)*

Different types of microorganisms belonging to different bacterial genera isolated from the genital apparatus of Arabian horses, in particular from the uteine wash, vagial wash,aborted feti and semen The vast majority of isolated bacteria are classified as commensal microorganisms that occur in soil, dust, water, skin and mucosal surfaces of domestic and wild animals, on the surface of plants, seeds, fruit and animal or human faeces and are of no clinical importance.

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Table (1): Type, numbers of samples and status of animals

Serial No. of Animals	Age (years)	Type of Samples	Status of Animals
M_1	20	Uterine Wash	5 month abortion following colic
M_2	NA	Uterine Wash	NA
M_3	NA	Uterine Wash	9 month abortion
M_4	NA	Uterine Wash	NA
M_5	NA	Uterine Wash	NA
M_6	NA	Uterine Wash	NA
M_7	NA	Uterine Wash	7 month abortion
M_8	NA	Uterine Wash	NA
M_9	NA	Uterine Wash	8 month abortion
M_{10}	15	Uterine Wash	Previous abortion 1 year ago at 7th month
M_{11}	NA	Uterine Wash	Not getting pregnant after 3 times of mating
M_{12}	NA	Uterine Wash	Sample taken after 2 consecutive times of abortion at the 4th month
M_{13}	NA	Uterine Wash	9 month abortion (stillbirth) with mastitis
M_{14}	6	Uterine Wash	5 month abortion without breeding for 2 years
M_{15}	9	Uterine Wash	9 month abortion
M_{16}	10	Uterine Wash	9 month abortion
M_{17}	8	Uterine Wash	9 month abortion, no breeding since 2016
M_{18}	9	Uterine Wash	9 month abortion, no breeding for 1 year

M_{19}	7	Uterine Wash	5 month abortion
M_{20}	15	Uterine Wash	7 month abortion
M_{21}	4	Uterine Wash	4 month abortion, no breeding for 1 year
M_{22}	10	Uterine Wash	NA
Subtotal of uterine washes		22	
M_{23}	6	Vaginal Wash	5 month abortion, no breeding for 3 years till now
M_{24}	9	Vaginal Wash	9 month abortion
M_{25}	6	Vaginal Wash	Lactating mare
M_{26}	3	Vaginal Wash	NA
M_{27}	7	Vaginal Wash	NA
M_{28}	NA	Vaginal Wash	NA
M_{29}	NA	Vaginal Wash	NA
M_{30}	NA	Vaginal Wash	NA
M_{31}	NA	Vaginal Wash	NA
M_{32}	NA	Vaginal Wash	NA
M_{33}	NA	Vaginal Wash	NA
M_{34}	NA	Vaginal Wash	NA
M_{35}	NA	Vaginal Wash	NA
Subtotal of vaginal washes		13	
F_1	8 months	Internal Organs(6) & placenta	Collected from M_9
F_2	9 months	Internal Organs(6) & placenta	Collected from M_{18}
F_3	5 months	Internal Organs (6)& placenta	Collected from M_1
F_4	9 months	Internal Organs(6) & placenta	Collected from M_3
F_5	7 months	Internal Organs (6)& placenta	Collected from M_{10}
F_6	6 months	Internal Organs (6)& placenta	NA
F_7	7 months	Internal Organs (6)& placenta	NA
F_8	NA	Internal Organs(6) & placenta	NA
F_9	NA	Internal Organs (6)& placenta	NA
F_{10}	NA	Internal Organs(6) &placenta	NA
F_{11}	NA	Internal Organs (6)& placenta	NA
F_{12}	NA	Internal Organs(6) & placenta	NA
F_{13}	NA	Internal organs (6)& placenta	NA
F_{14}	NA	Internal Organs (6)	NA
Subtotal of feti	14	Subtotal of samples	97
Stallion	40	Semen (40)	
Total Number of samples			171

Table (2) Types of different microorganisms isolated from each clinical sample

Serial No. of Animals	Age (years)	Status of Animals	Type of Isolates	No. of Isolates	No. of mixed infection
M_1	20	5 month abortion following colic	*S. enterica* subsp. *arizonae*	1	-
M_2	NA	**NA**	*S. enterica* subsp. *Typhimurium*	1	-
M_3	NA	9 month abortion	*Hafnia alvei* *Streptococcus equi* subsp. *equi*	1 1	1
M_4	NA	**NA**	*Staphylococcus haemolyticus*	1	-
M_5	NA	**NA**	*Streptococcus equi* subsp. *Zooepidemicus*	1	-
M_6	NA	**NA**	*Streptococcus equi* subsp. *Zooepidemicus*	1	-
M_7	NA	7 month abortion	*Listeria monocytogenes*	1	-
M_8	NA	**NA**	*Rhodococcus equi*	1	-
M_9	NA	8 month abortion	*klebsiella pneumoniae* subsp. *Pneumonia*	1	-
M_{10}	15	Previous abortion 1 year ago at 7th month	*Burkholderia cepacia complex Bcc*	1	-
M_{11}	NA	Not getting pregnant after 3 times of mating	*Burkholderia cepacia complex Bcc*	1	-
M_{12}	NA	Sample taken after 2 consecutive times of abortion at the 4^{th} month	*Streptococcus equi* subsp. *Zooepidemicus*	1	-
M_{13}	NA	9 month abortion (stillbirth) with mastitis	*Streptococcus equi* subsp. *equi* *Pseudomonas aeruginosa*	1 1	1
M_{14}	6	5 month abortion without breeding for 2 years	*Listeria monocytogenes*	1	-
M_{15}	9	9 month abortion	*Streptococcus equi* subsp. *Zooepidemicus*	1	-
M_{16}	10	9 month abortion	*Burkholderia cepacia complex Bcc*	1	-
M_{17}	8	9 month abortion, no breeding since 2016	*-ve*	0	-
M_{18}	9	9 month abortion, no breeding for 1 year	*Salmonella enterica* subsp. *Arizonae*	1	-
M_{19}	7	5 month abortion	*Pseudomonas aeruginosa*	1	-
M_{20}	15	7 month abortion	*Rhodococcus equi*	1	-
M_{21}	4	4 month abortion, no breeding for 1 year	*Streptococcus equi* subsp. *Zooepidemicus*	1	-
M_{22}	10	**NA**	*Rhodococcus equi*	1	-

Subtotal No.	Subtotal No. of uterine washes	*Subtotal No. of isolates*	*Subtotal of negative samples*	*Subtotal No. of of mixed infection*	
	22	23	1	2	
M$_{23}$	6	5 month abortion, no breeding for 3 years till now	*Arcanobacterium haemolyticum*	1	-
M$_{24}$	9	9 month abortion	*Arcanobacterium haemolyticum*	1	-
M$_{25}$	6	Lactating mare	*Streptococcus equi* subsp. *equi*	1	-
M$_{26}$	3	**NA**	*-ve*	0	-
M$_{27}$	7	**NA**	*-ve*	0	-
M$_{28}$	NA	**NA**	*Corynebacterium* spp.	1	-
M$_{29}$	NA	**NA**	*Staphylococcus aureus*	1	-
M$_{30}$	NA	**NA**	*Streptococcus equi* subsp. *Zooepidemicus*	1	-
M$_{31}$	NA	**NA**	*Proteus vulgaris*	1	-
M$_{32}$	NA	**NA**	*Enterobacter aerogenes*	1	-
M$_{33}$	NA	**NA**	*Staphylococcus aureus*	1	-
M$_{34}$	NA	**NA**	*-ve*	0	-
M$_{35}$	NA	**NA**	*Streptococcus equi* subsp. *zooepidemicus*	1	-
Subtotal No.	Subtotal No. of vaginal washes	*Subtotal No. of isolates*	Subotal No. of negative samples	*Subtotal No. of mixed infection*	
	13	*10*	*3*	0	
F$_{1}$	8 months	Collected from **M$_{9}$**	*klebsiella pneumoniae* subsp. *Pneumonia*	1	-
F$_{2}$	9 months	Collected from **M$_{18}$**	*S. enterica* subsp. *arizonae* *Pseudomonas aeruginosa* *Streptococcus equi* subsp. *Equi*	1 1 1	1
F$_{3}$	5 months	Collected from **M$_{1}$**	*S. enterica* subsp. *arizonae*	1	-
F$_{4}$	9 months	Collected from **M$_{3}$**	*Streptococcus equi* subsp. *equi*	1	-
F$_{5}$	7 months	Collected from **M$_{10}$**	*Burkholderia cepacia complex Bcc*	1	-
F$_{6}$	6 months	**NA**	*Pseudomonas aeruginosa* *Burkholderia cepacia complex Bcc*	1 1	1

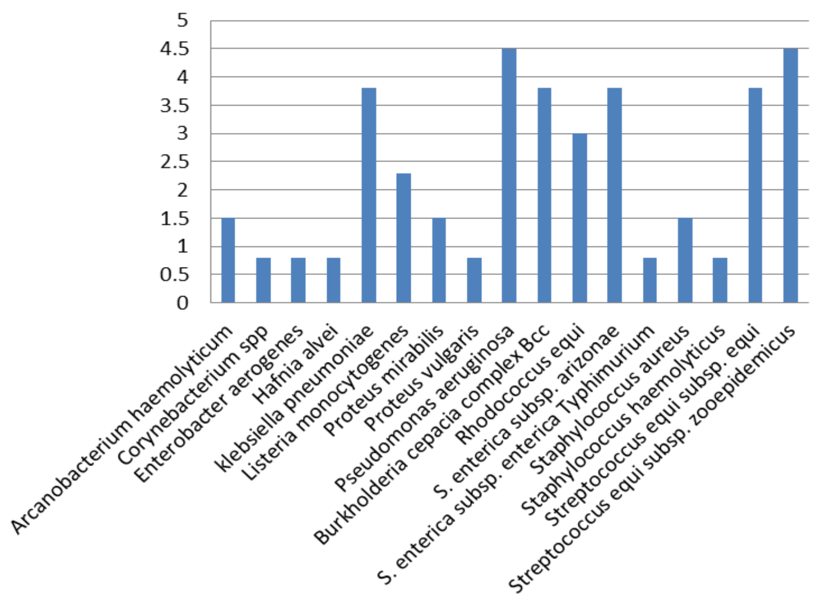
F7	7 months	**NA**	*klebsiella pneumonia subsp. pneumoniae*	1	-
F8	NA	**NA**	*Pseudomonas aeruginosa*	1	-
F9	NA	**NA**	*Listeria monocytogenes* *Proteus mirabilis*	1 1	1
F10	NA	**NA**	*-ve*	0	-
F11	NA	**NA**	*Rhodococcus equi*	1	-
F12	NA	**NA**	*klebsiella pneumoniae*	1	-
F13	NA	**NA**	*Pseudomonas aeruginosa* *Proteus mirabilis*	1 1	1
F14	NA	**NA**	*S. enterica* subsp. *arizonae* *Klebsiella pneumoniae*	1 1	1
Subtotal No.	Subtotal No. of internal organs	*Subtotal No.of isolates*	*Subtotal No of negative samples*	*Subtotal No.of of mixed infection*	
	97	*19*	*1*	5	
Total No. of all examined animals	**Total No. of samples**	**Total No. of Isolates**	**Total No. of negative samples**	**Total No. of mixed infection**	
49	**132**	**53**	**5**	**7**	
Percentage		**40.2%**	**3.8%**	**5.3%**	

Table (3): Rate of isolation of different isolated microorganism

Type of Isolate	No. isolate	Rate of isolation among total No. of samples (132)
Arcanobacterium haemolyticum	2	1.5%
Corynebacterium spp	1	0.8
Enterobacter aerogenes	1	0.8
Hafnia alvei	1	0.8
klebsiella pneumoniae	5	3.8%
Listeria monocytogenes	3	2.3%
Proteus mirabilis	2	1.5%
Proteus vulgaris	1	0.8

Pseudomonas aeruginosa	6	4.5%
Burkholderia cepacia complex Bcc	5	3.8%
Rhodococcus equi	4	3%
S. enterica subsp. *arizonae*	5	3.8%
S. enterica subsp. enterica *Typhimurium*	1	0.8
Staphylococcus aureus	2	1.5%
Staphylococcus haemolyticus	1	0.8
Streptococcus equi subsp. *equi*	5	3.8%
Streptococcus equi subsp. zooepidemicus	6	4.5%

Figure (1): Rate of isolation of different microorganisms (%)



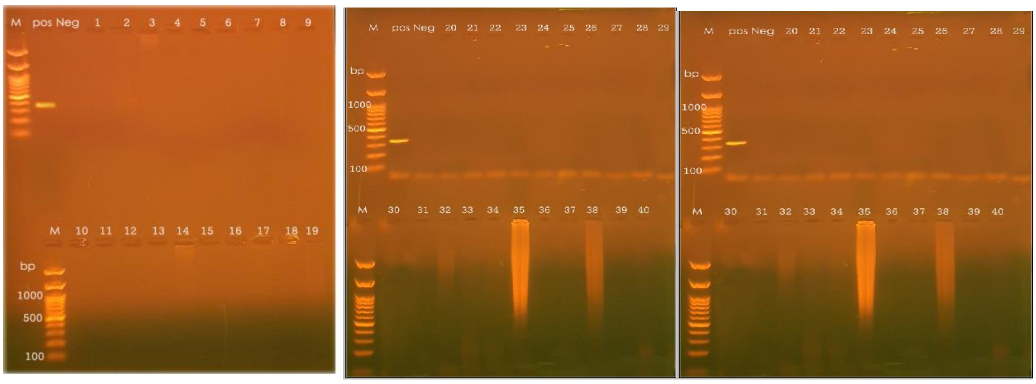


Figure (2):. Representative gel of PCR for detection of Leptospira genus using LA/LB primers.
Lane M: DNA marker (100 bp) Lane; pos: control positive (Leptospira ATCC 43642) Lane; neg: control Negative Lanes1-40: negative semen samples