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Association of Ureaplasma diversum with reproductive diseases in cattle

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In the present study, a total of 120 vaginal swabs were collected from cows suffering from reproductive disorders as well as apparently normal cows. The samples were examined by conventional cultural method for *ureaplasma diversum* isolation as well as by polymerase chain reaction using *U.diversum* primers. The results showed that *U.diversum* was detected from 45 of examined cows in a percent (37.5%) using PCR which is higher than the recovery rate (32.5%) by conventional culture method. The examined cows were divided into two groups, group (A) 75 apparently normal cows, and group (B) 45 cows suffered from reproductive disturbances, vulvar lesions, and abortion. The recovery rate was in a percent (51.1%) of group (B) and in a percent (21.3%) of group (A) by culture method. The recovery rate by PCR was 26 out of 45 group (B) in a percent (57.8%) and 19 out of 75 group (A) in a percent (25.3%).Two isolated strains subjected to sequencing and compared with other strains on GenBank "Asmaaureapl1 and Asmaaureapl2", had very high similarity with each other and with two Canadian strains "ATCC 49783 and PDS-D0801223" (99.79-100%) and had high similarity with the other *Ureaplasma diversum* strains (98.36-99.61%).

Keywords: Ureaplasma diversum, reproductive, cattle, PCR

INTRODUCTION

Reproductive disease related to infection by mollicutes has been described in beef and dairy cattle herds worldwide (Chavien et al. 2013; Doig et al. 1979; Argue et al.2013 and Gaeti et al. 2014).

Despite their ubiquitous character, the importance of these pathogens in the reproductive diseases of cattle is still unclear. This fact may be explained by the challenges experienced during laboratory identification and differentiation of mollicutes by culture and by serology methods that hamper the assessment of their impact in pathogenesis and epidemiology of GVV in cattle worldwide (Buzinhani et al., 2007).

Ureaplasma is the simplest and smallest self-replicating bacteria. They lack a cell wall, which makes them fully resistant to penicillin- and cephalosporin-like drugs (Khan et al. 2010) *Ureaplasma diversum* is one of the most important infectious bacteria causing female reproductive problems Among Ureaplasma species, [Petit et al. 2008]. It has been shown that more than 50% of animals with vaginitis were found to be positive for U. diversum in vaginal swabs than animals without signs of vaginitis (Petit et al. 2008). (Britten et al. 1988) stated that *U. diversum* can adhere to the zona pellucida of the embryo and was not removed by the 10 washes that are recommended by the

International Embryo Transfer Society. Culturing is considered as a gold standard method for its diagnosis, but it requires special media, a culture chamber, and at least 2–3 days (Viscardi, 2014). Molecular diagnosis employing PCR can be an important alternative (Cardoso et al. 2000), The 16S rRNA sequence is the most common genetic marker that is used for various studies such as bacterial phylogeny and taxonomy. The function of 16S rRNA has not been changed over time which makes its gene sequence a valuable tool for the diagnostic purpose (Patel, 2001).

Ureaplasma diversum has been associated with different clinical manifestations including bovine vulvitis, endometritis, salpingitis, spontaneous abortion, and infertility (Cardoso et al. 2000; Miller et al. 1994; Leon et al.1995). Because the isolation of ureaplasma species from specimens (clinical samples) is difficult, so there is a need for improved detection methods as polymerase chain reaction assay (based on the amplification of a region of the gene encoding 16SrRNA.

The traditional means for detection and speciation of mollicutes from clinical samples obtained from diseased animals have been culture and serology (Mcvey et al., 2013). However, as growth in culture requires specialized and enriched media as well as7–10 days of incubation, identification of these microorganisms through culture can present several limitations (Miller et al., 1994; Quinn et al., 2011). Furthermore, contamination of biological samples with other bacteria, inadequate handling and storage conditions, and prolonged intervals between sampling and processing in the laboratory may result in increased false-negative results (Maunsell et al., 2011). Additionally, due to the limited number of biochemical reactions for phenotypic characterization, the differentiation of mollicutes at the species level has been made through serology involving a complete serum panel. This method is labor-intensive, time-consuming, and currently available only in reference laboratories (Pettersson et al. 2000; Volokhov et al.2006). So to overcome difficulties regarding laboratory identification, the work aims to apply the rapid diagnostic technique for detection and identification using PCR assays

So the present study aims to compare the recovery rate of *U.diversum* detection in the genital tract of cows by isolation with the conventional cultural method and using PCR.

Cow's vaginal swabs were collected and examined for the presence of *U. diversum* by broth culture methods as well as PCR assay.

The study aims to verify the presence of *ureaplasma diversum* and elucidate its possible association with reproductive failures after detection *of U. diversum* sequencing and analyze the 16SrRNA partial sequences of different isolates recovered from reproductive tract of cows .

MATERIALS AND METHODS

Samples:

A total of 120 vaginal swabs were collected from 10 farms at Kaliobeya, Dakahlia, and Gharbia Governorates during the period from January 2016 till December 2019. The collected samples were grouped into two groups, group A: 45 cows suffering from reproductive disorders, repeat breading, vaginitis, vaginal discharge, vulvar lesions as hyperemia, ulcers, and abortion.

Group B: 75 apparently normal cows.

All samples were subjected to the conventional culture method for the isolation and also for polymerase chain reaction test (PCR).

Sample collection:

The vulva of each examined cow was previously washed with water and dried with a paper towel and then sampled with a sterile cotton-tipped swab. The swab was directed dorsally then redirected cranially the wiped over the cranial vaginal mucosa. All swabs were introduced directly into sterile screw-capped tubes containing Ureaplasma broth medium (UB medium) and were transferred to the Labe on ice tanks with backs at 4oC for examination.

Cultivation:

According to (Shepard, 1983) Ureaplasma broth cultures were filtered through 0.45 µm Nitrocellulose membrane and diluted in fresh ureaplasma broth then each sample diluted 3 tenfold dilutions and incubated at 37oC /24 hours. The third dilution cultures that have faint pink color at the bottom were streaked on Ureaplasma agar media for Ureaplasma isolation. Ureaplasma agar plates were inoculated on the surface with 200 µL of each sample dilution and incubated at 37oC /24 hours in 5% CO2 tension then examined by adding Urease stain.

DNA extraction:

Genomic DNA was purified from the samples using a QIAamp Kits (DNA Mini Kit Qiagen Germany, Cat. No. 51304) or by boiling method

(Garcia et al. 1997): A 4ml aliquot of a Ureaplasma broth culture was centrifuged in a microcentrifuge (*Hettich centrifuges*, -MIKRO 200*, USA*) at 13,000 rpm for 10 min at 7°C. The cell pellet was washed twice with 200 µL of 150 mM PBS (phosphate buffered saline, pH 7.2) and re-suspended in a final volume of 50 ul of PBS. The cell suspension was heated in a dry-heat block at 95°C for 10 min. and then cooled for 10min at -20 °C, The lysate was centrifuged at 14,000 g for 5 min to remove debris, and the supernatant containing DNA was collected and stored at — 20°C until tested.

Polymerase chain reaction:

The PCR amplification protocol for Ureaplasma diversum (16S rRNA) was reported by (Tramuta et al. 2011). , The following primers were used for PCR, F-5'GTT TGATCC TGG CTC AGG AT 3' and R-5'CTC ATAAGC GAG CC GAC ATT 3', with amplicon 831bp which synthesis PCR reaction was performed in a Gradient Thermal cycler (1000 S Thermal cycler Bio-RAD USA). The reaction mixture (total volume of 50 µl) was 25 µl PCR Master Mix (2X) Thermo Scientific Company, cat., No.K1080, USA.), 5µL DNA template, 20 pmol of each oligonucleotide and ultrapure water. The Ureaplasma diversum strain (GM U132 VUP6, ATCC) was used as a positive control. For the negative control, the DNA template was replaced by water. For electrophoresis, 1.5% agarose gel stained with ethidium bromide (100V/60min), 10 µL PCR reaction mixtures, and 5µL of molecular size marker (GeneRuler 100bp DNA Ladder Thermoscientific Company, Cat.no. SM0323, US) was used.to confirm the species studied, sequencing of PCR positive samples was performed.

PCR program for the amplification of 16S rDNA sequence was carried out in a thermal cycler (S1000-Biorad, USA)

PCR amplification:

The PCR amplification protocol for Ureaplasma diversum reported by (Tramuta et al. 2011).Was as follows: an initial denaturation of 95 oC for 15 min., followed by 40 cycles of denaturation at 94 oC for 60 s, annealing at 52 °C for 60 sec. and extension step at 72 °C for 1min. with a final extension of 72 °C for 10 min.

Amplification products were electrophoresed in agarose gels (1.5% w/v) (Agarose, Sigma, USA) was used for the running of DNA stained with ethidium bromide Using GeneRuler 100bp DNA Ladder: Thermo scientific Company, Cat.No.SM0243, US.

Sequencing and Phylogenetic analysis:

The amplicons (PCR product) were Purified using Thermo Scientific GeneJET PCR Purification kit then sequenced in both directions by a commercial sequencing service (Macrogen Europe). The obtained sequences were edited and assembled using Bioedit software. Nucleotide identity of the U. diversum 16S sequences was determined using the nucleotide BLAST algorithm with GenBank database (http://www.ncbi.nlm.nih.gov) (Johnson et al. 2008). Dendrograms were generated by the neighbor-joining method using the Maximum Likelihood model, by Molecular Evolutionary Genetics Analysis (MEGA 6) (Tamura et al. 2013). Bootstrap values were calculated based on 1,000 replicates and considered significant when >70. In addition to the sequences obtained in this study, published corresponding sequences of reference strains and previously published Egyptian strains were included in the phylogenetic analysis.

RESULTS

In this study the examined cows were 120 cows, 39 of them were positive by culture method (32.5%) as in (Table 1) and 45 by PCR (37.5%) using Ureaplasma diversum primers. The examined cows (Table 2) were divided into two groups, group A:75 apparently normal and group B:45 unhealthy (With reproductive disturbance, vulvar lesions, and abortion). The recovery rate was 23 out of 45 unhealthy cows (51.1%) and 16 out of 75 apparently normal (21.3%) by culture method. The recovery rate by PCR was 26 out of 45 unhealthy (57.8%) and 19 out of 75 apparently normal (25.3%). these results indicated a high prevalence of *U. diversum* in examined cows and the higher sensitivity of PCR assay as compared to culture

Table 1: Comparison between the recovery rate of Ureaplasma species using the conventional culture method and PCR

Total examined cow's vaginal swabs.	Recovery rate of *U.divesum* by Conventional culture method and percent.	Recovery rate by PCR for *U. diversum* detection and percent
120	39 (32.5%)	45 (37.5%)

Table 2: Recovery rate of Ureaplasma from diseased and apparently normal cows

Examined cows		Conventional Culturemethod	using PCR
group	No.		
group A	75	16 (21.3%)	19(25.3%)
group B	45	23 (51.1%)	26(57.8%)

Nucleotide differences:

· U. urealyticum has different sequences at nucleotides 31,171,186,287, 329-331,351, 361, 368, 435, 497, 514, 552-554, 557, 569 and 572 and it is differing from Asmaaureapl1 and Asmaaureapl2 and other U. diversum strains.

· Isolate GI has a different sequence at nucleotide 250(C) while other (T).

· Asmaaureapl1 strain sequence starts from nucleotide 95.

· Asmaaureapl2 strain has different sequences at nucleotide 582 (G) while others are (A) and nucleotide 584 (A) while others don't have this nucleotide.

· PDS-D0801223 strain starts from nucleotide 140 and has different sequences at 612-613(AG) which are not present in others.

Pyrogenicity and similarity:

U urealyticum had Percent Identity from 94.42 to 95.97% with other Ureaplasma diversum strains.

· Asmaaureapl1 and Asmaaureapl2 strains had very high similarity with each other and with ATCC 49783 and PDS-D0801223 strains (99.79-100%).

· Asmaaureapl1 and Asmaaureapl2 strains also had high similarity with the other U. diversum strains (98.36-99.61%).

· Other U. diversum strains had high similarity with each other (98.36-100%).

Table 3: Names and Accession numbers of isolated and compared strains

Accession number	Strain
MW090794	Asmaaureapl1
MW090795	Asmaaureapl2
NR_025878	Strain A417
KU886091	isolate GI
GU227397	ATCC 49782 Partial seq
CP009770	ATCC 49782 genome
JN935894	strain T95
GU227398	ATCC 49783
EU714185	PDS-D0801223
M23935	U. urealyticum

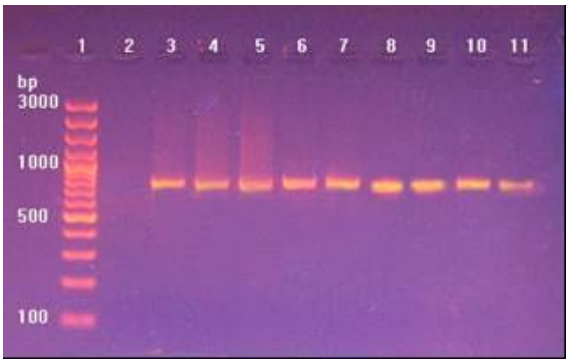

Figure1: Agarose (2%) gel electrophoresis of 16srRNA gene amplified products.
Lane 1: 100 bp DNA ladder.
Lane 2: control positive
Lane 3 :control Negative
Lane 4-12 Ureaplasma diversum positive isolates

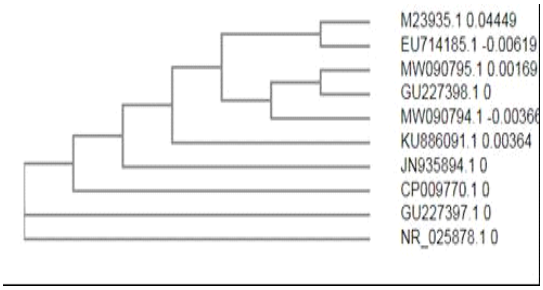

Figure2: phylogentic tree of nucleotide sequence

DISCUSSION

Ureaplasma has previously been implicated in various bovine reproductive disorders that have economic effects

In the present study, the results showed that: from 120 examined cows the recovery rate of *U.diversum* primers by PCR was 45 (37.5%) which is higher than the isolation rate by conventional culture method 39(32.5%).

These examined cows were divided into 75 apparently normal and 45 unhealthy (With reproductive disturbance, vulvar lesions, and abortion) and the recovery rate was 23 out 45 unhealthy cows (51.1%) and 16 out 75 apparently normal (21.3%) by culture method while recovery rate by PCR was 26 out of 45 unhealthy (57.8%) and 19 out of 75 apparently healthy (25.3%).

Results showed that PCR was more sensitive than culture method similar results were shown by (Cardoso et al. 1999)

Table 4: CLUSTAL O (1.2.4) multiple sequence alignment

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EU714185.1 ------------------------------------------------------------ 0
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MW090794.1
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    CP009770.1     AAAATGCGTAG--  609
    GU227397.1     AAAATGCGTAG--  609
    NR_025878.1     AAAATGCGTAG--609
    JN935894.1     AAAATGCGTAG--  609
    GU227398.1     AAAATGCGTAG--  609
    MW090794.1      AAAATGCGTAG--516
    EU714185.1     AAAATGCGTAGAG         473
            ***********
```

Table 5: Percent Identity Matrix - created by Clustal2.1

95.97	95.16	94.58	94.75	94.75	94.75	94.75	94.42	94.42	100	M23935.1	**1**
99.79	99.81	99.84	98.36	98.52	98.52	98.52	98.36	100	94.42	MW090795.1	**2**
99.79	99.42	98.52	99.34	99.51	99.51	99.51	100	98.36	94.42	KU886091.1	**3**
100	99.61	98.69	99.84	100	100	100	99.51	98.52	94.75	CP009770.1	**4**
100	99.61	98.69	99.84	100	100	100	99.51	98.52	94.75	GU227397.1	**5**
100	99.61	98.69	99.84	100	100	100	99.51	98.52	94.75	NR_025878.1	**6**
100	99.42	98.52	100	99.84	99.84	99.84	99.34	98.36	94.75	JN935894.1	**7**
100	100	100	98.52	98.69	98.69	98.69	98.52	99.84	94.58	GU227398.1	**8**
100	100	100	99.42	99.61	99.61	99.61	99.42	99.81	95.16	MW090794.1	**9**
100	100	100	100	100	100	100	99.79	99.79	95.97	EU714185.1	**10**

who stated that culture isolation was (35.7%), while PCR-specific amplification was (52.9%) also, (Silva et al. 2020) who found that positive PCR and sequencing samples were cultured in UB medium and monitored daily for 21 days. However, there was no growth of characteristic colonies.

The high recovery rate of U. diversum from both apparently normal cows was also reported by (Azevedo et al. 2017) as a total of 25% of dairy cows with vulvovaginitis were positive for U. diversum. (Macêdo et al. 2018) who had a recovery rate of 21.69% and (Diaz et al. 2019) who recovered U. diversum at a rate of 64% from granular vulvovaginitis lesions and 36% in cows without lesions.

Recovering from diseased cows was at a higher rate than from apparently normal this was in agreement with (Ruhnke et al. 1978 and Diaz et al. 2019).

The high similarity with other strains of our isolates (Asmaaureapl1 and Asmaaureapl2) with the Ureaplasma strains (over 98.3%) indicates these isolates were Ureaplasma diversum strains.

From the epidemiological view, our isolates (Asmaaureapl1 and Asmaaureapl2) had very high similarity with each other and with ATCC 49783 and PDS-D0801223 strains which are of Canadian origin.

CONCLUSION

The present study indicates the presence of Ureaplasma (which was similar to the Canadian strains) as a cause of reproductive disorders in Egypt and in asymptomatic carrier cows which act as silent transmitters, We recommend using PCR which is more accurate than culture isolation and includes contact cows in treatment and control programs.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

AE collected the data, participated in the design of the work protocol, performed the laboratory work, and was involved in the preparation of the manuscript. AE and NH found the research idea, shared in the performed data and designed the work protocol. FE par-ticipated in the design of the work protocol, contributed to the manuscript review and interpreted the results. YMH performed the PCR and sequence. All authors were involved in the preparation of the manu-script and had read and approved the final manuscript.

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