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Michael Lierz, Nils Hagen, Nigel Harcourt-Brown, Stephen J Hernandez-Divers, Dörte Lüscho, Hafez Mohamed Hafez

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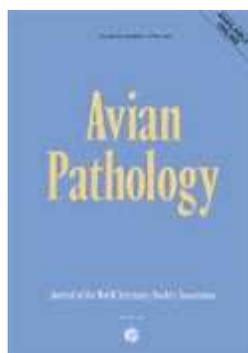
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Lierz, M.^{1*}, N. Hagen¹, N. Harcourt-Brown², S.J. Hernandez-Divers³, D. Lueschow¹ and H.M. Hafez¹

Institute for Poultry Diseases, Free University of Berlin¹ Veterinary Practice, Crab Lane, Harrogate, UK² Department of Small Animal Medicine & Surgery, College of Veterinary Medicine, University of Georgia, USA³

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* Corresponding author:

Institute for Poultry Diseases, Free University of Berlin, Koenigsweg 63, 14163 Berlin, Germany; e-mail: lierz.michael@vetmed.fu-berlin.de; Tel.: +49-30-83862699; Fax: +49-30-83862690

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Summary

Mycoplasmas are commensals and pathogens of different avian species, especially poultry and passeriforms. The role of mycoplasmas in raptors has not yet been completely determined, and especially not the possibility of vertical transmission. Therefore 424 raptor eggs were examined for the occurrence of mycoplasmas using culture and 155 of these eggs with a *Mycoplasma* genus-specific polymerase chain reaction (PCR) assay. This PCR was tested for its sensitivity and specificity, especially for use in a bird population of unknown mycoplasma status (prevalence and species). The size of the amplified PCR product was large (1013 bp) to enable use of the product for species differentiation by sequencing. Culture and PCR yielded only one positive result, in an egg of a Northern goshawk (*Accipiter gentilis*). The isolate was identified as *M. lipofaciens* using an immunobinding assay, as well as by sequencing part of its 16S rRNA gene.

Introduction

Mycoplasma species are well-known pathogens of domestic poultry, causing significant economic losses. In contrast the role of mycoplasmas in birds of prey is poorly understood. Respiratory dysfunction, air sacculitis, pneumonia and tracheitis have been reported as clinical signs of mycoplasma infection in raptors but the specific pathogen involved was not identified (Furr *et al.*, 1977; Gylstorff & Grimm, 1987; Gerlach, 1994; Heidenreich, 1997). *M. gallisepticum*, *M. gallinaceum*, *M. gallinarum*, *M. iners*, *M. anatis* and *M. columborale* have been isolated from raptors with respiratory signs (Poveda *et al.*, 1990, Morishita *et al.*, 1997) and *M. corogypsi* has been repeatedly isolated from raptors with pododermatitis (Panangala *et al.*, 1993; Morishita *et al.*, 1997). In contrast, *M. buteonis*, *M. falconis* and *M. gypis* (Poveda *et al.*, 1994), as well as *Mycoplasma vulturii* sp. nov." (Oaks *et al.*, 2004), have been isolated from healthy raptors. Mycoplasmas were isolated from 82% of nestlings, 26% of juveniles and 50% of adult raptors found injured or debilitated, and these isolates were found to be *M. gypis*, *M. meleagridis*, *M. falconis* or *M. buteonis* using an immunobinding assay (IBA) (Lierz *et al.*, 2000). None of the birds had any signs of any respiratory disease or conjunctivitis, but the authors suggested that *M. falconis*, *M. buteonis* and *M. gypis* may have pathogenic potential. Interestingly a prevalence of mycoplasma infection of 100% was found in healthy captive falcons in a private collection, with the isolates characterized as *M. meleagridis*, *M. corogypsi*, *M. falconis* or *M. buteonis* (Lierz *et al.*, 2002).

The significance of vertical transmission of mycoplasmas is well established in poultry and parent flocks are tested to ensure they remain free of pathogenic *Mycoplasma* species (*M. gallisepticum*, *M. synoviae*, *M. meleagridis* and *M. iowae*). They are able to cause egg infertility, embryonic death (reduced hatchability), poorly developed embryos and weak poults, sometimes even without any clinical signs in the parent flock (Carnaghan, 1961; Yamamoto & Orthmeyer, 1966; Matzer & Yamamoto, 1970; Vardaman, 1976, Carpenter *et al.*, 1981; Lin & Kleven, 1982;

Glisson & Kleven, 1984; Glisson & Kleven, 1985).

However, there are no published reports assessing the significance of vertical transmission of *Mycoplasma* spp. in raptors. Birds of prey are commonly bred by commercial breeders and species conservation projects. Because of the high prevalence of mycoplasmas in captive falcons (Lierz *et al.*, 2002), vertical transmission of mycoplasmas might affect the breeding success of birds of prey. Thus, this investigation was carried out to determine the prevalence of mycoplasmas in eggs obtained from several raptor species using culture as well as polymerase chain reaction (PCR) assays.

To investigate the prevalence of *Mycoplasma* spp. in a bird population using PCR, the species expected must be known to enable use of a specific PCR assay. This is not known in most bird populations. In addition unknown *Mycoplasma* species may be present (Poveda *et al.*, 1990, Lierz *et al.*, 2000, Lierz *et al.*, 2002, Oaks *et al.*, 2004). Therefore a generic *Mycoplasma* PCR is needed to investigate the prevalence of *Mycoplasma* spp. in raptor samples. A genus-specific PCR for the detection of mycoplasmal 16S rRNA gene has been described by Kuppleveld *et al.* (1992), and amplifies a product of 715 base pairs (bp). However sequence analysis of this fragment does not allow the differentiation of all avian *Mycoplasma* species, so a larger fragment would be useful. In addition, although the specificity of this PCR was described for several *Mycoplasma* species, each representing a subgroup according to its phylogenetic classification, the reference avian *Mycoplasma* strains were not tested. Therefore a mycoplasma-genus-specific PCR was designed and its sensitivity and specificity evaluated for use on samples from raptors.

Material and Methods

Collection of eggs from birds of prey To investigate the potential vertical transmission of *Mycoplasma* spp in birds of prey, eggs of different raptor species were examined. The eggs

originated from the collections of five different raptor breeders (4 in Germany, 1 in the UK). The eggs were collected from birds ranging from 2 to 18 years of age that were fed with a mixture of day old chicks, mice, rats, freshly slaughtered quail, pigeons and chicks that were a few weeks old. The birds were maintained using the standard breeding practices described by Heidenreich (1997).

In total 424 eggs (not incubated and known to be infertile (n=8); incubated and infertile (n= 255; embryonic death (n=161)) were sampled. Table 1 shows the species distribution of the eggs. The incubation period prior sampling ranged from 4 days to the estimated hatching time (32-55 days, depending upon the species). Eggs were examined within 24 hours after removal from the incubator. After disinfection, the egg shell was opened and two samples of vitelline membrane and, if an embryo was present, the trachea and oesophagus were collected using aseptic technique. One sample was placed into mycoplasma broth for culture, while the other was stored at -80°C for investigation by PCR.

Of the 424 eggs sampled, 155 (infertile (n = 68); embryonic death (n = 87)) were selected for PCR analysis. The number of eggs tested from each species is shown in Table 1. The eggs tested were selected randomly, except for the eagle and vulture eggs because of the small numbers of these available.

Additionally 3 Peregrine falcon and 2 Northern goshawk chicks, which died one day after hatching, were sampled (vitelline membrane, oesophagus and trachea) and examined as described above.

Culture, Differentiation of *Mycoplasma* spp and PCR. The samples were cultured for *Mycoplasma* spp. immediately after sampling in broth and on agar as described by Bradbury (1998), except that thallos acetate was not added. Briefly, the mycoplasma broth containing the sample was incubated at 37°C in an atmosphere containing 5% CO₂ for 5 days, followed by

subculture onto agar. The agar was observed using a light microscope for the occurrence of mycoplasma colonies for up to ten days.

Isolated mycoplasma colonies were subjected to an immunobinding assay (IBA) as described by Kotani & McGarrity (1985) using antiserum against the reference strains of *M. anseris*, *M. buteonis*, *M. falconis*, *M. gallisepticum*, *M. gypis*, *M. iowae*, *M. lipofaciens*, *M. meleagridis* and *M. synoviae*.

A PCR specific for the genus *Mycoplasma* was developed using the specific reverse primer MGSO described by Kuppleveld *et al.* (1992), in combination with a new forward primer, chosen to amplify a larger PCR product.

The primer was designed based on comparison and alignment of 16S rRNA genes of several known mycoplasma species using Clustal W (European Molecular Biology Laboratory, Heidelberg, Germany) (Higgins *et al.*, 1996). The specificity of the PCR was confirmed using the reference strains of avian *Mycoplasma* species, other *Mycoplasma* species and walled bacteria (Table 2).

The sensitivity of the PCR was evaluated using 10 fold serial dilutions of *M. synoviae* and *M. gypis* DNA, as well as cultures (CFU) of the *M. synoviae* and *M. gypis* reference strains (Albers & Fletcher, 1982).

For DNA extraction from the samples, 25 mg tissue (vitelline membrane, organs) was cut into small pieces and incubated with 180 µl lysis buffer (ATL) and 20 µl proteinase K overnight at 56 °C. After addition of 200 µl lysis buffer (AL), DNA was extracted from the samples using the QIAmp®DNA Mini Kit (Qiagen, Hilden, Germany). Extraction procedures were performed according to the manufacturer's instructions, as modified by Lüscho *et al.* (2004). The concentration of DNA was measured photometrically at a wave length of 260 nm (Bio Photometer 6131, Eppendorf AG, Hamburg, Germany). DNA extraction from mycoplasma cultures was also carried out using the QIAmp®DNA Mini Kit.

The PCRs were carried out using Taq PCR Master Mix (Qiagen) according to the manufacturer's instructions, but with only half of the suggested amount of sample. Briefly, a 50 µl reaction contained 25 µl Taq PCR Master Mix, 25 pmol of the primers, 50 ng of DNA and nuclease free water up to a volume of 50 µl. Amplification was conducted using an initial incubation for 4 min at 94 °C, followed by 35 cycles of denaturation for 30 sec at 94°C, annealing for 30 sec at 55.9°C and synthesis for 30 sec at 72°C, and then a final extension for 10 min at 72°C. The amplified PCR product (10 µl) was then separated in a 0.5% agarose gel and visualized by ethidium bromide staining and ultraviolet transillumination.

DNA sequencing Each mycoplasma isolate recovered was also differentiated by DNA sequence analysis. For sequencing, the primer set described above (GPF/MGSO) was used. The PCR was performed with DNA extracted from the fifth single colony subculture of the isolate.

The PCR product was gel purified using the Invisorb Spin DNA Extraction Kit (Invitex, Berlin, Germany) and sequenced by using the same forward and reverse primers by a commercial DNA sequencing service (Agowa GmbH, Berlin, Germany). The sequence of the product was compared to the 16S rRNA gene sequences of *Mycoplasma* spp. in GenBank using BlastN (NCBI, USA) (Altschul *et al.*, 1990) and Clustal W (Higgins *et al.*, 1996).

Results

Isolation and identification of mycoplasmas from eggs. *Mycoplasma* spp. were isolated from only one egg (0.2%) of a Northern goshawk (*Accipiter gentilis*). The cultures from 52 (12.3%) eggs were heavily contaminated by bacteria or fungi (distributed across all species) and therefore a result was not obtained. Culture for mycoplasmas were negative in 371 (87.5%) of the egg samples. Interestingly, the positive egg originated from an imprinted 4 year old female that was not used for breeding the year of this study. As the egg was known to be infertile it was used for

this study one day after being laid. The previous year the female was artificially inseminated but all subsequent eggs were infertile.

The immunobinding assay differentiated the isolate (strain ML 64) as *M. lipofaciens*. As all colonies on the mycoplasma agar plate bound only the antiserum against *M. lipofaciens*, it was presumed to be a single-species infection. Mycoplasmas were not isolated from the three Peregrine falcons or the two Northern goshawk chicks.

PCR The *Mycoplasma* genus-specific PCR amplified a fragment of the expected size from the DNA of all mycoplasma isolates tested as well as from *Acholeplasma laidlawii*, but not from walled bacteria (Figure 1). The sensitivity of the PCR was 1 pg (1 CFU). The primers, their binding sites within the 16S rRNA gene, the size of the amplicon and the sensitivity of the assay are shown in Table 3.

Using the *Mycoplasma* genus-specific PCR, a product was only amplified from the same Northern goshawk egg that had been positive on culture. Sequence analysis of the PCR product (928 bp obtained with both primers) from the 16S rRNA gene of this isolate found a 100% match with the 16S rRNA gene sequence of *M. lipofaciens* strain R 171 (T) (GenBank Accession No: AF 221115). The sequence of the product was deposited in GenBank and has the accession number DQ653410.

None of the other 154 egg samples nor the samples from the three Peregrine Falcons or the two Northern goshawk chicks were positive using the *Mycoplasma* genus-specific PCR.

Discussion

In spite of the high prevalence of mycoplasma infections in birds of prey (Morishity *et al.*, 1997, Lierz *et al.*, 2000, 2002), their pathogenic significance in raptors remains unclear,

suggesting that the species found regularly are probably commensals rather than primary pathogens. However it is possible that mycoplasmas may harm the embryo even though adults remain clinically normal (Yamamoto & Orthmeyer, 1966; Carpenter *et al.*, 1981). Our study appears to be the first to investigate the potential for vertical transmission of *Mycoplasma* spp. in raptors. Cultures revealed only 1 of 424 raptor eggs to be positive for *Mycoplasma* spp. This low prevalence was confirmed by PCR, as no additional eggs were found to be positive using a *Mycoplasma* genus-specific PCR. As only 155 eggs were selected for the PCR, it is possible that the examination of the remaining eggs may have yielded a few more positive results. Thus, even though there is a high prevalence of mycoplasma infection in raptors (Lierz *et al.*, 2000, 2002), it appears that vertical transmission is rare. This may be because the *Mycoplasma* spp. infecting raptors are commensals of the tracheal mucosa of raptors (Lierz *et al.*, 2002), and the prevalence of infection in other tissues, particularly the genital tract, is low. However, the prevalence of vertical transmission of mycoplasmas in poultry is also low, with as few as 1 of 50 eggs from an infected breeder flock carrying the organism (Seneviratna, 1969). This low rate of transmission is still important because mycoplasmas quickly spread among chicks. It also needs to be borne in mind that the eggs sampled in our study had already been incubated and that the sample quality was sometimes poor. This is especially true for dead-in-shell embryos, as breeders tend to leave an egg that is suspected dead in the incubator longer to ensure that the embryo has died. During this process mycoplasmas are highly likely to die and the DNA may also be degraded, resulting in false negative results. This possibility needs to be further investigated. It is unlikely that there are novel *Mycoplasma* spp. in raptors that were unable to be detected by the genus-specific *Mycoplasma* PCR, but to rule out this possibility culture should always be performed in addition to PCR (Bradbury, 2005). The use of a *Mycoplasma* genus-specific PCR is preferable when investigating the prevalence of mycoplasmas in diverse bird populations as the species present are often unknown. Major benefits of the PCR assay include its ability to identify mycoplasmas even when they are no

longer viable because of transport. In addition the PCR assay is not affected by contaminating bacteria or fungi, a regular problem in culture, as shown by their presence in 12.3% of samples in this study. The use of thallos acetate within the mycoplasma medium would be likely reduce this contamination rate, but poses a health risk for laboratory staff. Unlike the assay described by Kuppelveld *et al.* (1992), the PCR assay used in this study was evaluated for all known avian *Mycoplasma* species, including the species described in raptors. The PCR was able to amplify a product from all avian mycoplasma reference strains, but not from walled bacteria. Therefore the specificity of this PCR is adequate for its use in assessing the prevalence of mycoplasmas in an avian population. The modification of the primer did not reduce the sensitivity. The product amplified by this modified PCR of this study was larger, allowing the differentiation of mycoplasma species by sequencing the PCR product.

It is unlikely that the mycoplasma detected was a contaminant from the female's feet, as the other three eggs from the same female were negative and the mycoplasma was isolated from the vitelline membrane, which had not come into contact with the shell.

In conclusion, vertical transmission of a mycoplasma was demonstrated in raptors and should be included as a differential diagnosis in the examination of raptor eggs and dead-in-shell embryos.

The isolate recovered (strain ML 64) was *M. lipofaciens*, a species previously isolated from a chicken (Bradbury *et al.*, 1983) as well as from turkeys and ducks (Bencina *et al.*, 1987). This species does not seem to be specific to a single order of birds. Previous studies have not detected *M. lipofaciens* in raptors (Lierz *et al.*, 2000; 2002), but undifferentiated *Mycoplasma spp.* were isolated in these earlier studies and antiserum against *M. lipofaciens* was not available, suggesting the presence of this species may not have been detected.

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Table 1: Species distribution of the eggs sampled for mycoplasma culture and PCR

Species	Total number	Mycoplasma culture	PCR
Gyr-Saker- Hybrid falcon (<i>Falco rusticolus</i> * <i>Falco cherrug</i>)	145	145	59
Peregrine Falcon (<i>Falco peregrinus</i>)	120	120	34
Saker Falcon (<i>Falco cherrug</i>)	64	64	14
Barbery Falcon (<i>Falco pelegrinoides</i>)	34	34	12
Gyrfalcon (<i>Falco rusticolus</i>)	20	20	12
Gyr-Hybrid of unknown species (Originated from the UK)	13	13	8
Northern Goshawk (<i>Accipiter gentiles</i>)	19	19	7
Steller's Sea-eagle (<i>Haliaeetus pelagicus</i>)	3	3	3
Bald eagle (<i>Haliaeetus leucocephalus</i>)	2	2	2
White-tailed Sea- eagle (<i>Haliaeetus albicilla</i>)	1	1	1
Golden Eagle (<i>Aquila chrysaetos</i>)	2	2	2
Eurasian Griffon vulture (<i>Gyps fulvus</i>)	1	1	1
Total	424	424	155

Table 2: *Mycoplasma* reference strains and bacteria used for establishment of the PCR's

Reference strain	Provided by	Reference strain	Provided by
<i>M. anatis</i> 1340	IOM	<i>M. gallisepticum</i> – FI	IPD
<i>M. anseris</i> 1219	IOM	<i>M. synoviae</i> – FI	IPD
<i>M. buteonis</i> Bb/T2g	IOM	<i>M. iowae</i> – FI	MG-BUT
<i>M. corogpsi</i> BV1	IOM	<i>M. arginini</i> NC 10129	IMT
<i>M. cloacale</i> 383	IOM	<i>M. arthrididis</i> NC 10162	IMT
<i>M. columborale</i> MMP-4	IOM	<i>M. canis</i> NC 10146	IMT
<i>M. columbinasale</i> 694	IOM	<i>M. equigenitalium</i> T 37	IMT
<i>M. columbinum</i> MMP-1	IOM	<i>M. felis</i> C0	IMT
<i>M. falconis</i> H/T1	IOM	<i>M. fermentans</i> NC 10117	IMT
<i>M. gallopavonis</i> 1197	IOM	<i>M. hyorhinis</i> ATC 23839	IMT
<i>M. gallinarum</i> 1504	IOM	<i>M. salivarium</i> NC 10113	IMT
<i>M. gallisepticum</i> 75969	IOM	<i>M. spumans</i> PG 13	IMT
<i>M. gallinaceum</i> 887	IOM	<i>Acholeplasma laidlawii</i> NC 10116	IMT
<i>M. glycyphilum</i> 486	IOM	<i>Pasteurella avium</i> 1158	IMT
<i>M. gypis</i> B1/T1	IOM	<i>Pasteurella gallinarum</i> 1586	IMT
<i>M. iowae</i> DK - CPA	IOM	<i>Pasteurella multocida</i> 338	IMT
<i>M. imitans</i> 4229	IOM	<i>Pseudomonas aeruginosa</i> 9380	IMT
<i>M. iners</i> D1-E3	IOM	<i>Ornithobacterium rhinotracheale</i> – FI	IPD
<i>M. lipofaciens</i> R 171	IOM	<i>Salmonella typhimurium</i> – FI	IPD
<i>M. meleagridis</i> 17529	IOM	<i>Escherichia coli</i> - FI	IPD
<i>M. pullorum</i> 111R	IOM	<i>Streptococcus mutans</i> ATCC 35668	IPD
<i>M. sturni</i> ATCC 51945	IOM	<i>Clostridium perfringens</i> ATCC 13124	IPD
<i>M. synoviae</i> NVU 1853	IOM		

FI= Field isolate; IMT= Institute of Microbiology, Free University Berlin, Germany; IOM= International Organization for Mycoplasma, Department of Veterinary Pathobiology, Purdue University, IN, USA; IPD= Institute of Poultry Diseases, Free University of Berlin, Germany; MG-BUT = Mark Grant, British United Turkey, UK.

Table 3: *Mycoplasma* genus-specific PCR amplifying a product within the mycoplasmal 16S rRNA gene.

Primersequence (5'-3')	Polarity	Position	Size of amplicon (bp)	Sensitivity	Source
GPF					
GCT GGC TGT GTG CCT AAT ACA	Forward	1-20*			This study
MGSO					
TGC ACC ATC TGT CAC TCT GTT AAC CTC	Reverse	986-1013*	1013*	1 pg 1 CFU	Kuppleveld et al. (1992)

*Varies between *Mycoplasma* species

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Fig. 1a-d: Electrophoresis of *Mycoplasma* genus-specific PCR products of the specificity test using the following reference strains: Lane 1, 15, 28, 40: 1 kbp-ladder; lane 14, 27, 39, 49: negative control; lane 2: *M. gallisepticum*, 3: *M. synoviae*, 4: *M. iowae*, 5: *M. meleagridis*, 6: *M. buteonis*, 7: *M. corogypsi*, 8: *M. falconis*, 9: *M. gypis*, 10: *M. anatis*, 11: *M. anseris*, 12: *M. cloacale*, 13: *M. columborale*, 16: *M. columbinasale*, 17: *M. columbinum*, 18: *M. gallopavonis*, 19: *M. gallinarum*, 20: *M. gallinaceum*, 21: *M. glycyphilum*, 22: *M. iners*; 23: *M. lipofaciens*, 24: *M. pullorum*, 25: *M. sturni*, 26: *M. imitans*, 29: *M. arginini*, 30: *M. arthrididis*, 31: *M. canis*, 32: *M. equigenitalium*, 33: *M. felis*, 34: *M. fermentans*, 35: *M. hyorhinis*, 36: *M. salivarum*, 37: *M. spumans*, 38: *A. laidlawii*, 41: Positive control- *M. gallisepticum*, 42: *P. avium*, 43: *P. gallinarum*, 44: *P. multocida*, 45: *P. aeruginosa*, 46: *O. rhinotracheale*, 47: *S. typhimurium*, 48: *E. coli*

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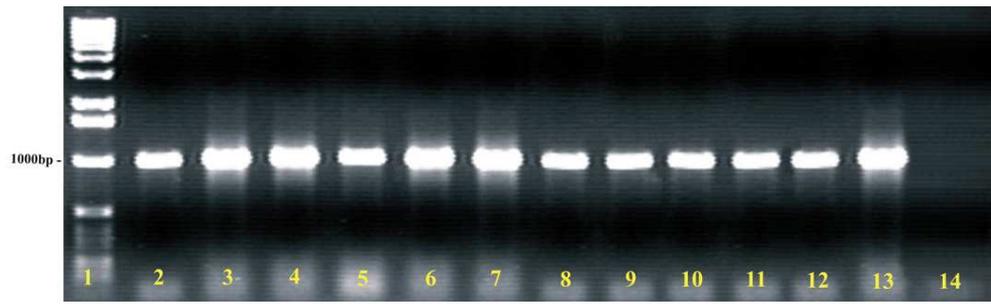
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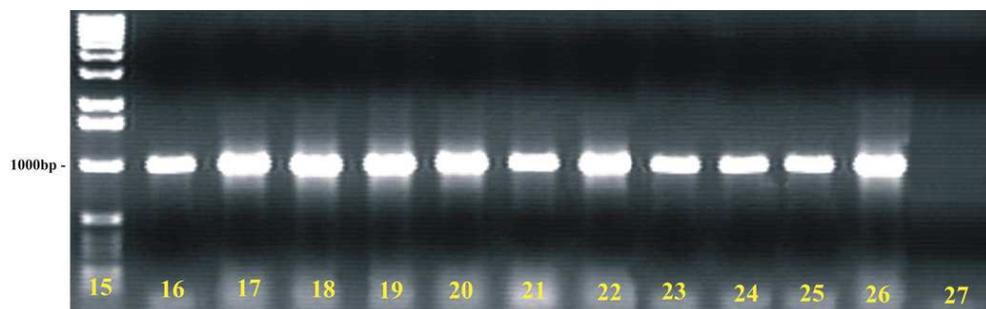
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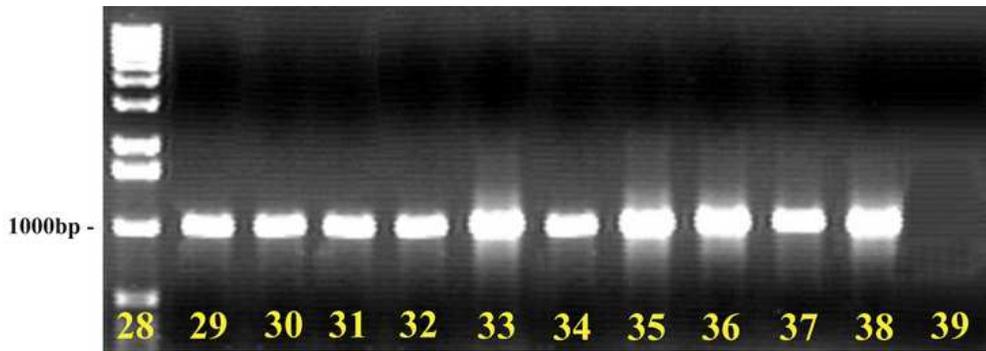
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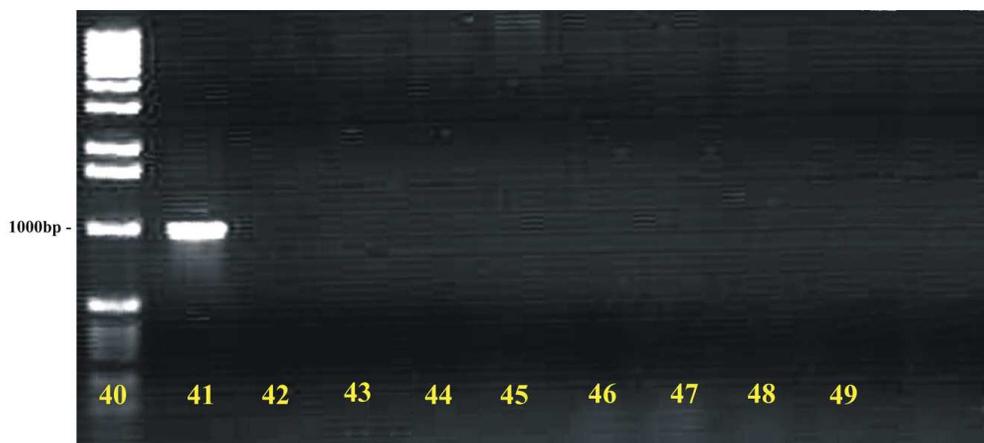
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