

## Use of a multiplex-polymerase chain reaction for detection of *Salmonella* and *Chlamydomphila psittaci* from caged birds

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**Summary:** In this study, development of a multiplex-PCR (m-PCR) assay for identification of Salmonellosis and Psittacosis in caged birds was aimed. DNAs extracted from *Salmonella* spp. strains from the collection of our department and *Salmonella* spp. isolates from caged birds in addition to the extracted DNA samples belong to two different *Chlamydomphila psittaci* clinical strains (strain 6BC and a strain isolated from a parakeet), constituted the material of the study. A m-PCR assay was developed which use iroBF and iroBR primers to specifically amplify 606 bp sequence of *iroB* gene of *Salmonella* spp. DNA in the same reaction mix and with the same conditions, CpsiA and CpsiB primers were used to amplify 300 bp sequence of *pmp* gene of *C. psittaci*. The specificity and the sensitivity of the assay were determined by using positive controls (*Salmonella* and *Chlamydia* DNA samples). We believe that the newly developed m-PCR assay is a fast, reliable and an economic assay, which has the potential for direct identification of *Salmonella* spp. and *C. psittaci* from the clinical samples.

Key words: Caged birds, *Chlamydomphila psittaci*, identification, multiplex-PCR, *Salmonella* spp.

### Kafes kuşlarında *Salmonella* ve *Chlamydomphila psittaci*'nin saptanmasında multiplex-polimeraz zincir reaksiyonu tekniğinin kullanılması

**Özet:** Bu çalışmada, Salmonellosis ve Psittacosis'in tanısında kullanılmak üzere bir multipleks-polimeraz zincir reaksiyonu (m-PZR) tekniği geliştirilmesi amaçlandı. Ankara Üniversitesi Veteriner Fakültesi Mikrobiyoloji Anabilim Dalı suş koleksiyonunda yer alan *Salmonella* suşları, kafes kuşu orijinli *Salmonella* izolatları ve bunlardan izole edilen DNA örnekleri dışında yurt dışından sağlanan ve iki farklı *Chlamydomphila psittaci* klinik suşundan izole edilmiş DNA örnekleri çalışma materyalini oluşturdu. Çalışmada, *Salmonella* türlerinde *iroB* genini kodlayan sekansın 606 bp'lik porsiyonunu spesifik olarak amplifiye eden iroBF ve iroBR primer çifti ile *Chlamydomphila psittaci*'nin *pmp* genlerinin 300 bp'lik sekansını amplifiye eden CpsiA ve CpsiB primer çiftinin aynı reaksiyon karışımı ve şartlarında çalışacağı bir m-PZR tekniği geliştirildi. Pozitif kontrol *Chlamydomphila psittaci* DNA'larının dışında, kendi laboratuvar izolatları ve suşlarından izole edilen DNA örnekleri kullanılarak tekniğin spesifikite ve sensitivitesi de belirlendi. Sonuç olarak, klinik materyallerden direkt tanı potansiyeli de bulunan tekniğin kafes kuşlarında *Salmonella* spp. ve *C. psittaci*'nin tanısında hızlı, güvenilir ve ekonomik bir teknik olduğu düşünülmektedir.

Anahtar sözcükler: *Chlamydomphila psittaci*, kafes kuşları, multiplex-PCR, *Salmonella* spp., tanı.

### Introduction

Salmonellosis and Psittacosis are two of the most important infections of caged birds caused by *Salmonella* spp. and *Chlamydomphila psittaci* (*C. psittaci*), respectively. Both of the agents are significant, in that, they cause zoonoses (8, 20).

Laboratory diagnosis of *Salmonella* and *C. psittaci* requires time and labor intensive procedures. Diagnosis is by culture of feces, blood, spleen, liver and intestinal contents (20, 23). Since the culture of clinical material and final identification of the causing agent lasts for at least a week, alternative identification methods such as molecular detection methods like polymerase chain reaction (PCR) have been developed (4, 7, 10, 13, 22).

A number of reports on the use of PCR assays to detect *C. psittaci* have appeared in the literature (6, 11,

12, 16, 17). These sensitive assays were able to rapidly detect *C. psittaci* DNA in samples of tissues, feces and choanal cleft and cloacal swabs and performed better than traditional tissue staining methods and culture especially with mishandled and improperly sampled specimens (18, 19).

Multiplex-PCR (m-PCR) is a type of PCR in which two or more target sequences are simultaneously amplified in the same reaction (9). Since more than one target sequence can be amplified with this assay, it has the potential to produce considerable savings of time and effort within the laboratory without compromising test utility (5).

We aimed to develop and use a m-PCR assay which simultaneously detects two important pathogens which cause significant infections in caged birds.

## Materials and Methods

### *DNA of laboratory strains and clinical isolates:*

Laboratory strains of *Salmonella enterica* subsp. *enterica* serovar Pullorum (AUM21), *S. Gallinarum* (AUM27), *S. Enteritidis* (AUM32), *S. Typhimurium* (AUM41) from the archive of Department of Microbiology, Faculty of Veterinary Medicine, Ankara University, as well as, *Salmonella* spp. isolates from clinical isolates originated from caged birds were used as positive control *Salmonella* DNA. *C. psittaci* (strain 6BC and a strain isolated from a parakeet) and *Chlamydia trachomatis* DNAs were kindly provided by Prof. Bernhard Kaltenboeck (Department of Pathobiology, College of Veterinary Medicine, Auburn University), were used as positive and negative controls, respectively.

*DNA extraction from Salmonella serovars:* For extraction of *Salmonella* DNA from plate cultures incubated overnight, one or two colonies were suspended in 200 µl of DEPC treated water (Fermentas, Vilnius, Lithuania). The suspension is added to spin columns of QiaAmp DNA mini kits (Qiagen, Hilden, Germany) and extraction protocol was followed according to the instructions reported in the kit manual.

*DNA extraction from necropsy materials obtained from a caged bird:* DNAs were extracted from the swab samples (ocular and cloacal), internal organs (liver, spleen, lungs), intestines with Qiagen DNA tissue kit and feces with Qiagen DNA stool kits (Qiagen, Hilden, Germany) according to the kit instructions.

### *PCR detection of iroB and pmp genes:*

Oligonucleotides were *iroBF*, 5'-TGCGTATTCTGTTT GTCGGTCC-3' and *iroBR*, 5'-TACGTTCCCACCATT CTTCCC-3' which specifically amplify 606 bp portion of *iroB* (iron regulated locus B, glucosyl-transferase-like protein) gene in *Salmonella* spp. (3), and *CpsiA*, 5'-ATG AAACATCCAGTCTACTGG-3' and *CpsiB*, 5'-TTGTG TAGTAATATTATCAA-3' those specifically amplify approximately 300 bp sequence of *pmp* (polymorphic membrane protein) genes of *C. psittaci* (14). These primer pairs were analyzed in BLAST (<http://www.ncbi.nlm.nih.gov/BLAST>) searches for determination of probable interactions. PCR assays described in the study of Baumler et al. (3) and Laroucau et al. (14) were used for the detection of *iroB* and *pmp* genes.

*M-PCR:* A m-PCR was developed following a series of standardization studies aiming to find the optimal primer, dNTP, MgCl<sub>2</sub> concentrations, as well as the optimization trials for the establishment of the appropriate amplification conditions such as the determination of the ideal annealing and elongation temperatures and durations. The need for the addition of PCR enhancers were also considered and two of the

mostly used PCR enhancers (bovine serum albumin, BSA and dimethyl sulphoxide, DMSO solutions) were comparatively evaluated in the PCR assays. Following the optimization studies m-PCR mix and amplification conditions was determined. M-PCR mix contained 200 µM of each dNTP (Fermentas, Vilnius, Lithuania), 3 mM MgCl<sub>2</sub>, 5 µl 10xPCR buffer, 10 µM primers, 0.2% DMSO, 2.5 U Taq polymerase (Fermentas, Vilnius, Lithuania). Two µl of template DNA was included into final volume of 50 µl m-PCR reaction mix. Amplification consisted of 94°C 2 min, pre-denaturation and 30 cycles of PCR steps of 94°C 30 sec, denaturation; 50°C 30 sec, annealing; 65°C 2 min extension, and a final extension of 72°C 10 min.

*Determination of the specificity of the assay:* For the determination of the specificity, the m-PCR assay was tested against the DNAs obtained from bacteria such as *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus* spp., *Pasteurella multocida*, *Chlamydia trachomatis* (Table 1).

*Determination of the sensitivity of the assay:* For the determination of the sensitivity, concentration of the DNA samples (*C. psittaci* DNAs and DNAs from the selected *Salmonella* from our laboratory) was calculated with a UV spectrometer. DNAs were 10-fold diluted serially beginning from 20 ng to 2 pg dilutions and equal volumes from these dilutions were used as template DNAs in the multiplex reactions.

## Results

*PCR detection of iroB and pmp genes:* Six hundred and six base pair portion of the *iroB* gene was specifically detected in all of the extracted *Salmonella* DNAs (Figure 1). Specific bands were observed of a size of approximately 300 bp in *Chlamydia* DNAs, but not in *C. trachomatis* (Figure 2).

*M-PCR:* Both of the amplicons with specific bands sizes of 606 and 300 bps were detected with different *Salmonella* serovars and *C. psittaci* strains, respectively (Figure 3). *Salmonella* and *C. psittaci* specific bands were detected with m-PCR tests performed with DNA extracted from the necropsy material and feces of a canary.

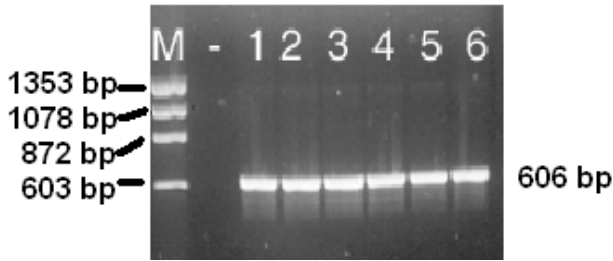
*Specificity of the assay:* Neither amplification nor non-specific bands was observed with assay performed with bacteria such as *Staphylococcus aureus*, *Bacillus* sp., *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus* sp., *Pasteurella multocida* (Table 1).

*Sensitivity of the assay:* M-PCR assay was able to detect as low as 20 pg of both DNA (Figure 4).

Table 1. Bacterial strains tested in PCR assays.

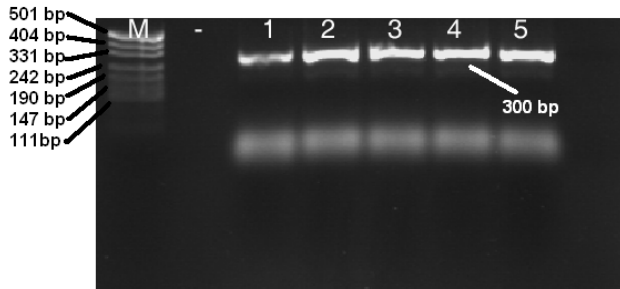
Tablo 1. PCR testlerinde kullanılan bakteri suşları.

Strain	<i>iroB</i> PCR	<i>pmp</i> PCR	m- PCR
<i>Staphylococcus aureus</i>	-	-	-
<i>Bacillus</i> sp.	-	-	-
<i>Klebsiella pneumoniae</i>	-	-	-
<i>Escherichia coli</i>	-	-	-
<i>Pseudomonas aeruginosa</i>	-	-	-
<i>Proteus</i> sp.	-	-	-
<i>Pasteurella multocida</i>	-	-	-
<i>Salmonella</i> Enteritidis	+	-	+
<i>Salmonella</i> Typhimurium	+	-	+
<i>Salmonella</i> Gallinarum	+	-	+
<i>Salmonella</i> Pullorum	+	-	+
<i>Salmonella</i> spp. isolate from a caged bird	+	-	+
<i>Chlamydomphila psittaci</i> strain 6BC	-	+	+
<i>C. psittaci</i> strain isolated from a parakeet	-	+	+
<i>Chlamydia trachomatis</i>	-	-	-

Fig. 1. Detection of *iroB* gene by PCR.Şekil 1. *iroB* geninin PCR ile saptanması.

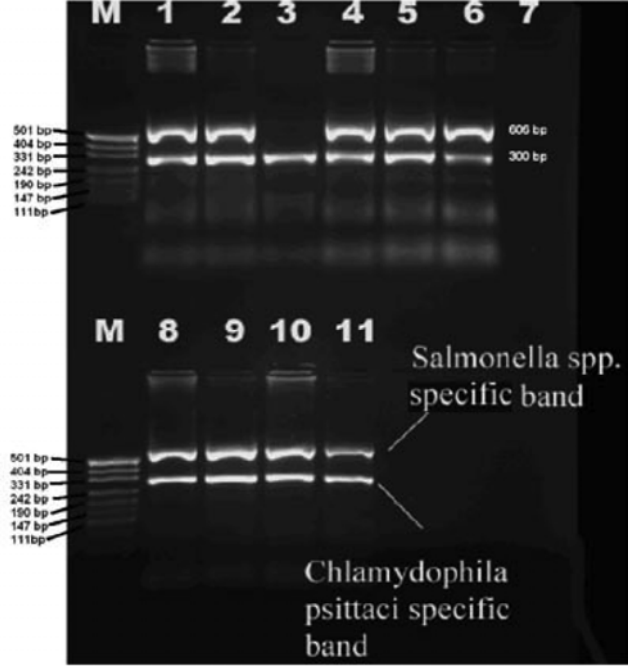
M. 100-bp DNA ladder (Fermentas, Vilnius, Lithuania). -: *Escherichia coli* as negative control. Lane 1: positive control. Lanes 2-6: PCR amplicons of *Salmonella* strains. Lane 2: *S. Enteritidis*. Lane 3: *S. Typhimurium*. Lane 4: *S. Gallinarum*. Lane 5: *S. Pullorum*. Lane 6: *Salmonella* spp. isolate from a caged bird.

M. 100-bp'lik DNA marker (Fermentas, Vilnius, Litvanya). -: Negatif kontrol, *Escherichia coli*. Hat 1: pozitif kontrol. Hatlar 2-6: *Salmonella* suşlarından elde edilen PCR ürünleri. Hat 2: *S. Enteritidis*. Hat 3: *S. Typhimurium*. Hat 4: *S. Gallinarum*. Hat 5: *S. Pullorum*. Hat 6: *Salmonella* spp. isolate from a caged bird.

Fig. 2. Detection of *pmp* genes by PCR.Şekil 2. *Pmp* genlerinin PCR ile saptanması.

M. pUC19 DNA/*MspI* (25-bp) Marker, 23 (Fermentas, Vilnius, Lithuania). -: *C. trachomatis* as negative control. Lane 1: *C. psittaci* strain 6BC. Lane 2: *C. psittaci* strain isolated from a parakeet. Lanes 3-5: DNAs extracted from ocular swab, intestine, feces, respectively, of a death caged bird.

M. pUC19 DNA/*MspI* Marker, 23 (Fermentas, Vilnius, Litvanya). -: Negatif kontrol, *C. trachomatis*. Hat 1: *C. psittaci* 6BC suşu. Hat 2: Bir papağandan izole edilmiş *C. psittaci* suşu. Hatlar 3-5: Ölü bir kafes kuşundan örneklenen göz, bağırsak ve dışkı svablarından ekstrakte edilmiş DNA'lar.

Fig. 3. Multiplex-PCR detection of *iroB* and *pmp* genes.Şekil 3. *IroB* ve *pmp* genlerinin m-PZR ile saptanması.

M. pUC19 DNA/*MspI* (25-bp) Marker, 23 (Fermentas, Vilnius, Lithuania). Lanes 1 and 2: positive controls. Lane 1: *S. Enteritidis* with *C. psittaci* strain 6BC. Lane 2: *S. Typhimurium* with *C. psittaci* strain isolated from a parakeet. Lane 3: DNA extracted from the ocular swab from the caged bird. Lanes 4-6: DNA extracted from internal organs (liver, spleen, lungs). Lane 7: DNA extracted from *E. coli* strain as negative control. Lanes 8 and 9: DNA extracted from intestines. Lanes 10 and 11: DNA from cloacal swab and feces, respectively.

M. pUC19 DNA/*MspI* Marker, 23 (Fermentas, Vilnius, Lithuania). Hat 1 ve 2: pozitif kontrollörler. Hat 1: *S. Enteritidis* ve *C. psittaci* 6BC suşu. Hat 2: *S. Typhimurium* ve bir papağandan izole edilmiş *C. psittaci* suşu. Hat 3: Kafes kuşundan alınan göz svabından ekstrakte edilmiş DNA. Hatlar 4-6: İç organlardan (karaciğer, dalak, akciğerler) ekstrakte edilmiş DNA'lar. Hat 7: *E. coli* suşundan ekstrakte edilmiş DNA, negatif kontrol. Hat 8 ve 9: Bağırsaklardan ekstrakte edilen DNA'lar. Hat 10 ve 11: Sırasıyla, kloakal svab ve dışkıdan elde edilmiş DNA'lar.

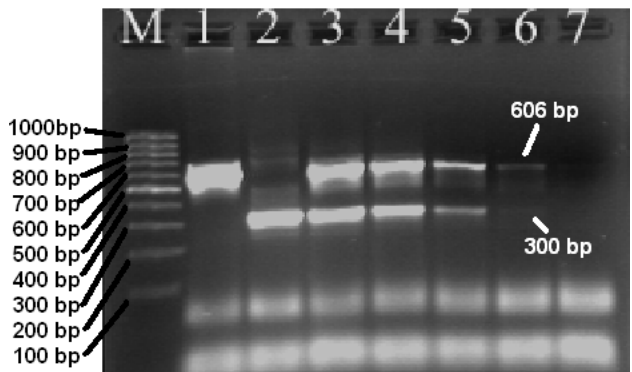


Fig. 4. Determination of the sensitivity of m-PCR assay.

Şekil 4. M-PZR tekniğinin sensitivitesinin belirlenmesi.

M. GeneRuler™ 100-bp DNA ladder (Fermentas, Vilnius, Lithuania). Lane 1: M-PCR with *Salmonella* DNA only. Lane 2: M-PCR with *C. psittaci* DNA only. Lanes 3-7: M-PCR tests with *Salmonella* and *C. psittaci* DNAs of respectively 20 ng, 2ng, 200pg, 20 pg and 2 pg.

M. 100-bp DNA marker (Fermentas, Vilnius, Litvanya). Hat 1: Sadece *Salmonella* DNA ile yapılan m-PZR. Hat 2: Sadece *C. psittaci* DNA ile yapılan m-PZR. Hatlar 3-7: Sırasıyla 20 ng, 2ng, 200pg, 20 pg ve 2 pg'lik *Salmonella* ve *C. psittaci* DNA'sı içeren örneklerle yapılan m-PZR testleri.

### Discussion and Conclusion

We established and used a m-PCR assay for the detection of *Salmonella* and *C. psittaci* in caged birds. Isolation of *C. psittaci* from clinical samples is very difficult and laborious and requires inoculations to cell cultures and embryonated eggs. Additionally, it is important to apply appropriate biosafety precautions while working with *C. psittaci*. Therefore, we included *C. psittaci* primers to *Salmonella* PCR mix to establish a primer cocktail that could detect any or both of the agents, which will increase the practicability of detection. M-PCR assays are becoming prevalent for the simultaneous detection of toxins, virulence factors and pathogens in clinical and environmental specimens (24). Although there are studies of m-PCR for the investigation of avian agents in avian medicine, particularly, in chickens (1, 21), we encountered a few in the case of caged birds (17).

It is likely that the assay is specific for the detection of *Salmonella* and *C. psittaci*, since we detected only *Salmonella* spp. and *C. psittaci* DNA in the test, and no non-specific amplification of DNA from different bacteria was observed.

The m-PCR assay was found sensitive, since it could detect both DNAs at dilutions of 20 pg. Laroucau et al. (14) detected up to 5 pg of *C. psittaci* DNA dilution with the same primers. This slight difference could be originated from the multiplexing of PCR, interactions between the primer pairs and the amplification mixes, and also from the different DNA extraction methods used in the studies.

The test lasted for 4 h overall, from extraction to electrophoresis. The assay is economic, because it employs the same reaction reagents and consumables for the investigation and detection of two different bacteria simultaneously from the common DNA preparation in the same tube.

In this study, we managed to detect both *Salmonella* and *C. psittaci* DNA directly from internal organs, intestines and feces obtained from a dead canary. Molecular detection of *Salmonella* in clinical materials may require a pre-enrichment procedure. PCR with pre-incubation in an enrichment broth has been found to be a useful and more rapid method because it increases the number of viable *Salmonella* spp. in the sample and, therefore, increases the sensitivity of the assay (4). We believe implication of a pre-enrichment procedure to the method should increase the sensitivity of the assay. The efficacy of the assay has to be determined with sufficient number and diversity of both artificially spiked tissue samples and clinical materials such as feces and other tissue samples. Feces or blood contain PCR inhibitors that hinder the detection of specific DNA and result with

false negative results (15). Either the dilution of the extracted DNA and repeat of PCR or addition of PCR enhancers (2) or implying nested primers to PCR (nested PCR) to improve test sensitivity is suggested (25). Another choice is addition of an internal control into PCR reactions which we believe is another strategy to determine the possible participation of inhibitors. We compared BSA and DMSO as the probable enhancer to be used in the assay and gathered better results with the addition of DMSO at a concentration of 0.2 % (v / v) (data not shown).

As a conclusion, we believe this is the first report of a m-PCR assay for the detection of *Salmonella* spp. and *C. psittaci* DNA. Also reported for the first time was the co-existence of both agents in avian clinical samples (feces, intestines and internal organs) obtained following the necropsy of a dead caged bird.

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