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Comparison of Different Sample Types for *Salmonella* Detection From Chicken Layer Breeder Flocks

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Abstract: The object of this study was to detect *Salmonella* from different chicken samples in same flocks to compare sample types for *Salmonella* detection by both International Organization for Standardization Method 6579:2002/Amd 1:2007 (ISO) and as molecular by a polymerase chain reaction (PCR) method. Salmonellosis is a zoonotic infection and apart from this, infection can be transmitted via vertically to embryo, and this is very important for breeding flocks. A total of 115 samples, comprised of 451 individual samples each pooled into 3, 4, 5 and 6 including 14 drag swabs, 28 pooled wet faeces, 11 pooled embryonated chicken eggs, 62 pooled cloacal swabs, were collected from 14 chicken layer breeding flocks, and tested by culture method (ISO 6579) and conventional PCR. Overall *Salmonella* infection rate in chicken layer breeder flocks by PCR and culture was 18.2% (21/115). According to sample type, *Salmonella* rate in culture positive samples were: 0% (0/14) in drag swabs, 90.9% (10/11) in embryonated chicken eggs, 21.4% (6/28) in wet faeces, 8% (5/62) in cloacal swabs.

PCR results were in 100% agreement (100% sensitivity and specificity) with culture results. We determined *Salmonella* rate in 14 chicken layer breeder flocks by using culture and PCR methods, and the use of embryonated chicken eggs and wet faeces samples, respectively in *Salmonella* detection would yield reliable results. These results indicate that *Salmonella* screening can be done together with different types of sample. And the most reliable and high results were taken from embryonated chicken egg samples for layer breeding poultry. As a conclusion, *Salmonella* infection seems to be the major problem in poultry flocks in Turkey, and both conventional culture method and PCR methods were found sensitive for the detection of *Salmonella* from poultry with different types of sample.

Key Words: *Salmonella*, chicken layer breeder flock, polymerase chain reaction (PCR), culture, zoonosis, different sample types.

Damızlık Yumurtacı Tavuk Sürülerinden *Salmonella* Tespiti için Farklı Örnek Tiplerinin Karşılaştırılması

Özet: Bu çalışmanın amacı, aynı kümeslerden alınan farklı örnek tiplerinin, hem Uluslararası Standardizasyon Teşkilatı 6579:2002/Amd 1:2007 (ISO) kültür metotu hem de moleküler olarak polimeraz zincir reaksiyonu (PCR) ile çalışılarak *Salmonella* teşhisi bakımından karşılaştırılmasıdır. *Salmonella* zoonoz bir enfeksiyondur ve ayrıca vertikal geçiş özelliğinden civcivlere geçebileceğinden dolayı, özellikle damızlık kanatlı yetiştiriciliğinde ayrı bir öneme sahiptir. Çalışmamızda alınan 451 bireysel örnek; 14 adet drag svab, 28 adet pool edilmiş ıslak dışkı, 11 adet pool edilmiş embriyolu yumurta, 62 adet pool edilmiş kloakal svab, 3, 4 ve 5'li bir araya gelecek şekilde gruplandırılarak, toplam 115 adet örnekte, hem ISO 6579 kültür metotu hem de geleneksel PCR metotu ile *Salmonella* aranmıştır. Damızlık yumurtacı tavuklardaki *Salmonella* yaygınlığı, çalışılan iki metotla da %18.2 olarak bulunmuştur. Örnek tiplerine bakılarak değerlendirme yapıldığında ise kültür sonuçları; drag svablarda

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%0, embriyolu yumurtalarda %90.9, ıslak dışkıda %21.4 ve kloakal svablarda %8 olarak bulunmuştur. PCR sonuçları da, kültür sonuçları ile örnek tiplerine göre, yine %100 sensitivite ve spesifite ile aynı bulunmuştur. Türkiye'deki yumurtacı kümeslerde *Salmonella* yaygınlığı PCR ve ISO 6579 kültür metotları ile %18.2 olarak bulunmuş ve damızlık kanatlılarda *Salmonella* taramalarında alınabilecek en güvenli örneklerin embriyolu yumurta ve ıslak dışkı olduğu tespit edilmiştir. Bu sonuçlar aslında, en yüksek oranda *Salmonella* tespiti yapılan örnek tipinin embriyolu yumurtalar olarak bulunmasıyla, özellikle damızlık yetiştiriciliği yapılan kanatlı sürülerinde, *Salmonella* teşhisinde güvenilir sonuçlar alınabilmesi için, klasik olarak kullanılan dışkı kökenli örneklerin dışında, farklı örnek tiplerinin de alınarak hep birlikte değerlendirilmesi gerektiğini de göstermiştir. Sonuç olarak, Türkiye'deki yumurtacı tavuk kümeslerinde *Salmonella* enfeksiyonun önemli problem olarak varlığını sürdürmeyi devam ettiği ve hem geleneksel kültür metotu hem de PCR'ın farklı örneklerden *Salmonella* tespitinde güvenilir yöntemler olarak kullanılabileceği gösterilmiştir.

Anahtar Kelimeler: *Salmonella*, damızlık yumurtacı tavuk, polimeraz zincir reaksiyonu (PCR), kültür, zoonoz, farklı örnek tipleri.

Introduction

Salmonellosis is responsible for about %30 of all food poisoning cases in the United States[28] with an estimated 80.3 million annual foodborne cases[22] in the world, and significant economical losses in poultry sector worldwide. Since poultry is one of the most important reservoirs of *Salmonella* that can be transmitted to humans through the food-chain, public health concerns have increasingly made the prevention of the foodborne transmission of disease to humans an urgent priority for poultry producers. Poultry and eggs remain the major source of infection in developed countries. Traditional microbiological analysis methods are based on bacterial behavior, such as phenotypic or antibody response, which can present problems with cross reaction among related organisms[4]. In addition, *Salmonella* serovars are not detectable in certain clinical samples that contain small number of organisms. The standard cultural method for detecting *Salmonella* require up to 5 days to produce results. To reduce the time required for testing, different methods have been developed[8] including tests based on novel reagents, yet these tests are generally used to supplement rather than replace existing methods. The exception is the methodology based on PCR, which has progressively been replacing with biochemical and agglutination tests[14,30]. With poultry, control of infection depends largely on the identification of the infection in the early stages.

There are lots of works about different sample type for *Salmonella* detection[4,11,14,15,20,22]. Cloacal swabs are individual sample and represent the one chicken. *Salmonella* is disseminated at intervals despite there is an infection, so false negative results can be taken. So, sample type for *Salmonella* must be represent all the flock for herd health. Furthermore, individual samples (cloacal swab, embryonated chicken egg, organ, etc.) can be taken in such cases, because of vertical transmission and septicemic cases of *Salmonella* infection, the latter is generally important for typhoid group *Salmonella* infection[4].

The objective of this study was to compare the different sample types of the detection for *Salmonella* by the cultural with International Organization for Standardization (ISO 6579/A1)[16] and as molecular by PCR methods from chicken layer breeder flocks.

Material and Methods

Salmonella Strains

Salmonella enterica subsp. *Enterica* serovar Enteritidis 64K (M.Y.Popoff, Institut Pasteur, 28 rue du Dr Roux, 75015 Paris Cedex 15, France), *Salmonella enterica* subsp. *Enterica* serovar Typhimurium NCTC 12416 (Refik Saydam National Public Health Agency, Ankara, Turkey), *Salmonella enterica* subsp. *Enterica* serovar Typhimurium ATCC 14028 and *Salmonella enterica* subsp. *Enterica* serovar Enteritidis ATCC 13076 (Uludag University Medical School, Department of Microbiology, Bursa, Turkey) were used as positive controls in PCR and culture.

Clinical Samples

A total of 115 chicken layer breeder samples, comprised of 451 individual samples each pooled into 3, 4 and 5, including; 14 drag swabs, 28 wet faeces, 11 embryonated chicken eggs, 62 cloacal swabs, were collected from 14 layer flocks, each of the samples were submitted to our laboratory in an ice box within 6 hours after sampling in sterile material and screw-cap plastic bottles.

Culture

Bacteriological examination of natural samples was performed as indicated in ISO 6579-A1 (2007)[16]. For cloacal swabs: swabs from 1 and 5 birds, respectively, were pooled and this pooled sample, which weighed approximately 5 g, were transferred into 45 ml Buffered Peptone Water (BPW, Oxoid, CM1049). For samples: wet faeces and embryonated chicken eggs belonging to 3, 4, 5 and 6 samples, each weighing 6-7 g, were pooled, and this pooled 25 g sample was transferred into 225 mL BPW. For drag samples: each swab was cutted and weighed 25 g, were transferred into 225 mL BPW, and incubated at 37°C for 18 hours. After this incubation, semisolid Modified Rappaport Vassiliadis Agar (MSRV, Oxoid, CM1112) plates were inoculated with 3 drops (total 0.1 mL) of BPW culture, and incubated at 41.5°C for 24 hours. Negative plates were reincubated at 41.5°C for 24 hours, and a loopful of growth on MSRV plate was streaked on to both Xylose Lysine Deoxycholate Agar (XLD, Beckton Dickinson, 278850) and Xylose Lysine Tergitol-4 Agar (XLT_4, Beckton Dickinson, 223420). Also, 0,1 ml from BPW culture were inoculated into Tetrathionate Broth (TTB, Oxoid, CM0029B) and incubated at 37°C for 18 hours and was streaked on both XLD and XLT_4 Agar. After selective plating at 37°C for 24 hours, suspect *Salmonella* colonies were subjected to biochemical identification by spesific biochemical agar and broths recommended by ISO 6579:2002/Amd 1:2007 (ISO)[16].

Template Preparation for PCR

Crude DNA was prepared by modifying the method described by Soumet et al.[27]. One milliliter of TTB culture was centrifuged for 4 min at 4,600 *g*. The pellet was suspended in 0.85% saline, was centrifuged, and was resuspended in 20 ml of deionized water. This bacterial suspension was then boiled for 10 min and was centrifuged for 3 min at 18,000 *g*. Five microliters of the supernatant was used as a template in PCR.

PCR primers

We used *Salmonella* genus-specific primers 139 and 141 were described by Rahn et al.[18]. The following nucleotide sequences based on the *invA* gene of *Salmonella*: 5'-GTG AAA TTA TCG CCA CGT TCG GGC AA-3' and 5'-TCA TCG CAC CGT CAA AGG AAC C-3'. Both primers were synthesized in Expedite DNA synthesizer (Perseptive Biosystems, CA, USA) and were purified using reverse phase-High Pressure Liquid Chromatoggraphy (Bio-CAD700E, Perspective Biosystems, USA).

Polymerase Chain Reaction (PCR)

The 25-µl PCR mixture, which contained 0.3 µl of *Taq* DNA polymerase (5 U/µl), 2.5 µl of 10X PCR buffer (3.5 mM $MgCl_2$), 2.5 µl of deoxynucleoside triphosphate (dNTP) mixture (2 mM), 1 µl of each primer (5 pmol/µl), 5 µl of template DNA and 12.2 µl of deionized water, was taken into small microsantrifuge PCR tubes (200µl). The expected product size was reported as 284 bp[25].

PCR reactions were performed using a DNA Air Thermal Cycler, model 1605 (Idaho Technologies). The cycle conditions were as follows: an initial incubation at 94°C for 15 s followed by 30 cycles of denaturation at 94°C for 0 s, primer annealing at 50°C for 0 s, and primer extension at 72°C for 15 s. Following the last cycle, there was a 5-min incubation at 72°C. Amplified products were electrophoresed in 1.5 to 2% agarose gels containing ethidium bromide. A 100 bp DNA ladder was used as molecular size marker.

Results and Discussion

In this study, we compared different sample types for detection of *Salmonella* in chicken layer breeder flock with both ISO 6579 culture and PCR method. The overall incidence of *Salmonella* positivity by ISO culture was found as 18.2% (21/115) regardless of the sample type. Based on sample types, out of 14 drag swab samples, 0/14 (0%) were found positive by ISO culture. Out of 62 pooled cloacal swab samples 5/62 (8.06%), out of 11 pooled embryonated chicken egg samples 10/11 (90.9%), out of 28 pooled wet feces samples 6/28 (21.4%) were found positive, by ISO culture. PCR results were in 100% agreement (100% sensitivity and specificity) with culture results for all sample types. These results showed us that embryonated chicken eggs have the most meaningful sample for *Salmonella* detection from breeder flocks, and the second one was wet feces.

Table 1: Rate of Salmonella isolation from different samples by International Organization for Standardization Method 6579:2002/AMD 1:2007 (ISO) culture and PCR method

Tablo 1: Farklı örneklerden Salmonella izolasyonu için kullanılan Uluslar arası standartlar örgütü 6579:2002/AMD 1:2007 (ISO) kültür metodu ve PCR yöntemleri sonuçlarının oranları

Sample type	Total samples	Number of pooled samples	Number of positive samples by methods	
			Culture %	PCR %
Drag swab	14	14	0 (0)	0 (0)
Embryonated chicken egg	45	11	10 (90.9)	10 (90.9)
Wet faeces	112	28	6 (21.4)	6 (21.4)
Cloacal swab	280	62	5 (8)	5 (8)
Total	451	115	21 (18.2)	21 (18.2)

There are similar previous findings from layer flocks for *Salmonella* detection rates as 0% to 17%[1,10,11,12,18] from our country and as 9.9% to 17.9% from other countries[15,30]. Apart from the effects of '*Salmonella* prevalence within a flock'[2] of the housing system[7,15], and of the flock characteristics[23], the variations in *Salmonella* detection rates have particularly been related to the sample type analyzed and the method used[2,19,26]. In our study for instance, embryonated chicken egg and wet feces samples, non-supported by former study data[1,6,11,1218,24] seemed to work relatively better for *Salmonella* detection both than other sample types especially than drag swabs by ISO culture and PCR,. We found the drag swabs *Salmonella* ratio were 0% with the same methods. In fact, the intestine is the major site of *Salmonella* colonization in the chickens after oral infections[3]. Also, in the feces and intestinal content have present many inhibition factors as bilirubin, enzymes etc.[20]. Our previous incidence study[29] for layer chicken flocks showed that % 61.0 *Salmonella* positive from feces with related samples and these result was as high as 55.6%, 76.9% to 86.5% by Dorn and Schelif[9], Carlı[5] and Li et al.[21] respectively. We speculated that the main reason for this could be due to the low amount of fecal contamination of the cloacal swabs from infected chickens, inhibition from inhibitor factors in the feces and characteristic feature of *Salmonella* dispersed at intervals. To overcome this hindrance, we recommended the poultry companies to take proper drag swabs with sufficient amount of fecal contamination together with other type samples.

Conclusion

As a result, by using PCR and ISO culture, we determined that the *Salmonella* rate in layer flocks in 14 flocks was 18.2%, and that the use of embryonated chicken egg and wet feces samples, respectively in *Salmonella* detection would yield reliable results than drag and cloacal swabs. For the control of *Salmonella* infection, selection of sample type and method for diagnosis are important to routine control programs and embryonated chicken eggs must be included the screening for the *Salmonella* infection, especially for breeding flocks because of vertical transmission of *Salmonella* infection. Throughout the entire study, continuous detection of *Salmonella* regardless of the sample type or the year shows that this pathogen is persistently present in the poultry-related environments in Turkey. This indicates that there is still a failure in the application of general precautions or taking biosecurity actions against *Salmonella* in these areas of concern.

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