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MICROBIOLOGICAL STUDIES ON STREPTOCOCCAL INFECTION IN PIGEONS

By

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ABSTRACT

Streptococcus gallolyticus (*S. gallolyticus*) infection is considered an important septicemic disease in pigeons especially in squabs. In this study, 145 different samples from old pigeons and squabs from internal organs (liver, lung, heart blood and kidney. Samples were collected from pigeon's sailing shops at Cairo and Giza governorates. Samples were cultivated for bacteriological isolation targeting streptococci especially the so called *S. gallolyticus*. Morphological, biochemical and molecular identification of bacterial isolates revealed presence of *S. gallolyticus* with total incidence of 11.72%. The individual incidences were 12% (El Marg), 9% (Ein shams), 8% (Nasr city), 17% (El- Moneeb), (11% (El- Hawemdia) and 12% (El- Badrashin). Isolates were subjected to antibiotic sensitivity test and multiplex PCR targeting two virulence genes namely *Scp*B and *Lmb* as well as 5 antibiotic resistance genes (*Pbp*1A, *erm*B, *tet*O, *aac* (6') and *aph* (2'').

Keywords:

Streptococcus gallolyticus, pigeons, virulence genes, antibiotic resistance genes.

INTRODUCTION

Streptococcosis is an important septicemic disease in pigeons, which is caused by *Streptococcus gallolyticus* (*S. gallolyticus*), formerly identified as *S. bovis* **(Devriese *et al.,* 1990b; De Herdt *et al.,* 1994a and Devriese *et al.,* 1998).**

Madej (1961) described an outbreak of streptococcosis in five small breeding pigeon's lofts in Poland. *S. gallolyticus* has been isolated from different lesions in pigeons and was only infrequently found in the gut and faeces of pigeons without symptoms. It was also isolated from the crop and the pharynx of a minority of healthy pigeons **(Devriese *et al.,* 1990a).**

S. gallolyticus infection in pigeons was reported to be an unusual finding because the microorganism appeared to be rare in other birds where it was not found in the faeces of wild birds **(Mundt, 1963)** or in the caeca of chickens **(Barnes, 1958)** or turkeys **(Harrison and Hansen, 1950).** It has been reported that *S. gallolyticus* represent an important component of the intestinal flora of many mammals, especially farm animals and less often humans. Therefore, *S. gallolyticus* (*S. bovis*) can act as a pathogen and may be involved in septicaemic diseases and endocarditis in man and ruminants **(Parker, 1978).** An outbreak of *S. gallolyticus* infection was detected in a broiler flock, which denoted a clear heterogeneity with pigeon isolates **Chadfield *et al.* (2007).** According what we have in hands of literature, only one paper is found about *S. gallolyticus* infection in pigeons in Assiut governorate of Egypt. Therefore, the current study targeted investigation on *S. gallolyticus* incidence among diseased pigeons sold in live poultry merchandize at different localities of Cairo and Giza governorates.

MATERIAL AND METHODS

Samples:

As shown in (Table 1), 145 samples were aseptically taken from the visceral organs of clinically ill pigeons of various ages raised in different localities and presented for human consumption at Cairo and Giza governorates between the years of 2020 and 2022. These organs included the heart, blood, liver, gall bladder, spleen, lung, and kidney.

Table (1): Number and locations of collected samples from diseased pigeons.

locality	No. of samples
El Marg - Cairo	**25**
Ein Shams- Cairo	**25**
Nasr city- Cairo	**22**
EL Moneeb-Giza	**23**
El Hawemdia-Giza	**26**
El Badrashin-Giza	**24**
Total No.	**145**

Bactrial isolation and identification:

Samples were inoculated onto Edwards medium (Oxoid, UK) and incubated at 37°C for 24 hours, colonies that produced browning and blackening coloration were further characterized.

Those colonies were reinoculated onto sheep blood agar plates and incubated at 37°C for 24 hours. Smears were prepared from non-hemolytic streptococcus-like colonies and stained with the Gram method and examined microscopically. Gram positive cocci were biochemically identified to detect *S. gallolyticus.* The suspected streptococcal isolates were biochemically characterized using catalase, methyl red, Voges - Proskauer and citrate utilization tests **(Chadfield *et al.*, 2007).**

Antimicrobial susceptibility testing (Hemalatha *et al.*, 2006):

The Kirby-Bauer disc diffusion method was employed to investigate the antimicrobial sensitivity patterns of the *S. gallolyticus* isolates. Mueller-Hinton agar was made using a commercially available dehydrated base in accordance with the directions provided by the manufacturer (Oxoid, UK). A uniform depth of about 4 mm was achieved by pouring the freshly prepared medium onto plastic flat-bottomed Petri plates and allowing them to cool.

Discs were applied and plates were incubated and interpreted according to the **CLSI (2021).**

Bacterial DNA extraction and PCR:

DNA was extracted from different *S. gallolyticus* isolates using the QIAamp DNA mini kit following the manufacturer instructions.

Emerald Amp GT PCR mastermix was used to perform the PCR assays (Takara, Japan). The reaction components are illustrated in (Table 2). Also, (Table 3) shows the temperatures of the PCR cycling machine with each targeted gene.

Table (2): The PCR assay reagent components.

Component	Volume/Reaction
Emerald Amp GT PCR mastermix (2x premix)	**12.5µl**
PCR grade water	**5.5 µl**
Forward primer(20 pmol)	**1 µl**
Reverse primer (20 pmol)	**1 µl**
Template DNA	**5 µl**
Total	**25 µl**

Multiplex PCR was carried by using oligonucleotide primers for: 1- conventional identification of streptococcus (*tuf*), 2- detection of virulence genes (*Scpb* & *Lmb*) 3-detection of antibiotic resistance genes (*Pbp1* A, *ermB*, *teto* and Ass *(*6) - *aph* (2).

Table (3): Multiplex PCR cycling temperatures with primers targeting different genes.

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
tuf	**94˚C 5 min.**	**94˚C 30 sec.**	**50˚C 30 sec.**	**72˚C 30 sec.**	**35**	**72˚C 7 min.**
Pbp1A	**94˚C 5 min.**	**94˚C 30 sec.**	**57˚C 40 sec.**	**72˚C 45 sec.**	**35**	**72˚C 10 min.**
ermB	**94˚C 5 min.**	**94˚C 30 sec.**	**51˚C 40 sec.**	**72˚C 45 sec.**	**35**	**72˚C 10 min.**
aac(6')aph(2'')	**94˚C 5 min.**	**94˚C 30 sec.**	**54˚C 40 sec.**	**72˚C 40 sec.**	**35**	**72˚C 10 min.**
tetO	**94˚C 5 min.**	**94˚C 30 sec.**	**56˚C 40 sec.**	**72˚C 45 sec.**	**35**	**72˚C 10 min.**
scpB	**94˚C 5 min.**	**94˚C 30 sec.**	**47˚C 30 sec.**	**72˚C 30 sec.**	**35**	**72˚C 7 min.**
lmb	**94˚C 5 min.**	**94˚C 30 sec.**	**50˚C 40 sec.**	**72˚C 40 sec.**	**35**	**72˚C 10 min.**

Products of PCR amplification were separated by electrophoresis using 1.5% agarose gel in 1x TBE buffer containing ethidium bromide (0.5 µg/ ml) with electric current of 1.5 volts/ 1cm of the agarose length. At the end of the run, the gel was photographed by a gel documentation system and the data was analyzed through computer software **(Sambrook *et al*., 1989).**

RESULTS

Incidences of *S. gallolyticus* in the examined pigeon samples are depicted in (Table 4). It is noticed that, the incidence rates ranged between 8% and 17.39% with the highest incidence in samples collected from El Moneeb of Giza and the lowest incidence in samples collected from pigeons at Nasr City of Cairo. The average incidence rate was 11.72%>.

Table (4): Incidence of streptococci in samples collected from pigeons reared and sold at different localities at Cairo and Giza Governorates.

Region	No. of samples	No. of S. gallolyticus isolates	Incidence %
EL Marg-Cairo	**25**	**3**	**12%**
Ein shams-Cairo	**22**	**2**	**9.09%**
Nasr city-Cairo	**25**	**2**	**8%**
EL Moneeb-Giza	**23**	**4**	**17.39%**
El Hawemdia-Giza	**26**	**3**	**11.53%**
EL Badrashin-Giza	**24**	**3**	**12.5%**
Total	**145**	**17**	**11.72%**

The following table depicts the phenotypic characteristics of the confirmed S. gallolyticus isolates.

Table (5): Morphological and biochemical characteristics of the identified *S. gallolyticus* isolates recovered from pigeons.

Basic Characteristics	Specification
Capsule	**Capsulated**
CAMP	**Negative (-ve)**
Catalase	**Negative (-ve)**
oxidase	**Negative (-ve)**
Gram Staining	**Gram Positive (+ve)**
Hemolysis	**Non Hemolytic**
Motility	**Non-Motile**
OF (Oxidative-Fermentative)	**Facultative Anaerobes**
Shape	**Cocci**
Spore	**Non-Sporing**
Urease	**Negative (-ve)**
Methyl Red (MR)	**Negative (-ve)**
Voges Proskauer (VP)	**Positive (+)**
Citrate	**Negative (-)**

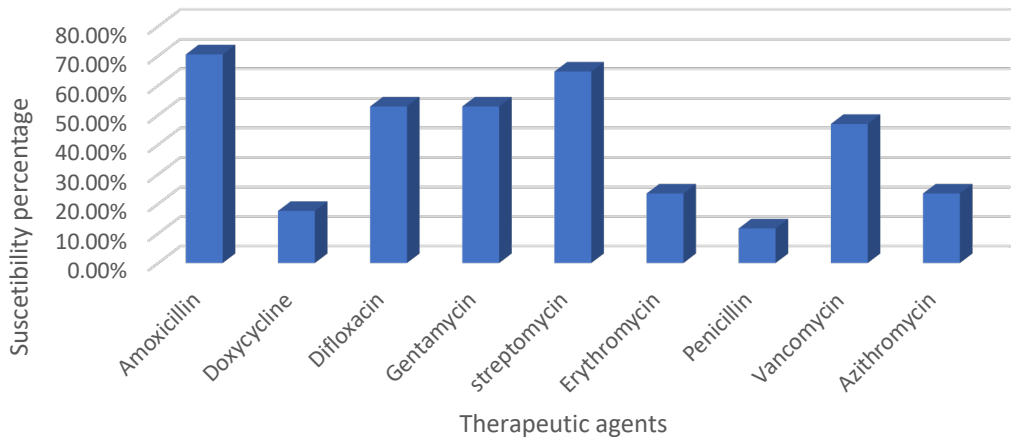
The antimicrobial susceptibility results of 17 *S. gallolyticus* isolates against 9 antibacterial agents.

As shown in (Table 6), Fig. (1), out of 17 tested *S. gallolyticus* isolates, the most effective agent was amoxicillin where 12 isolates were sensitive (70%) followed by streptomycin (64%). Gentamicin and difloxacin were moderately effective (52.9%).The least effect agent was penicillin (11.7%).

Table (6): Antibiotic sensitivity tests of 17 *S. gallolyticus* isolates from pigeons.

Antimicrobial agent	No. of Sensitivity	% of Sensitivity
Amoxicillin	**12/17**	**70.5%**
Doxycycline	**3/17**	**17.6%**
Difloxacin	**9/17**	**52.9%**
Gentamycin	**9/17**	**52.9%**
Streptomycin	**11/17**	**64.7%**
Erythromycin	**4/17**	**23.5%**
Penicillin	**2/17**	**11.7%**
Vancomycin	**8/17**	**47%**
Azithromycin	**4/17**	**23.50%**

Figure 1: *S. gallolyticus susceptibilit to* 9 antibacterial agents



Results of multiplex PCR:

Fig. (2, 3) show the electrophoresis results of multiplex PCR targeting 7 genes in 3 *S. gallolyticus* isolates. Of the 7 genes, 6 were detected in the 3 isolates (*tuf, scp*B, *lmb*, *pbp*1A, *tetO* and *aac (6') aph (2")* genes. On the other hand, *ermB* gene was detected in one isolates only. Table (7) summarizes the results of multiplex PCR assay.

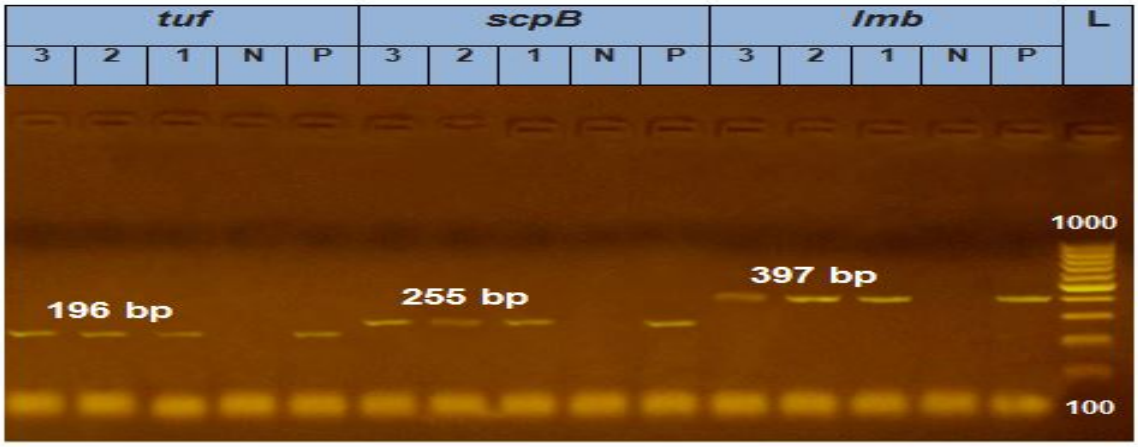


Fig. (2): Agarose gel electrophoresis showing amplification of 196 bp product of *tuf*, 255 bp of the *scp*) gene, 196 bp *Imb* gene. Lane (p): Streptococcus (positive control) and Lane (N): Streptococcus (negative control). Lane (L): DNA Ladder, lanes (1, 2 & 3) streptococcus isolates.

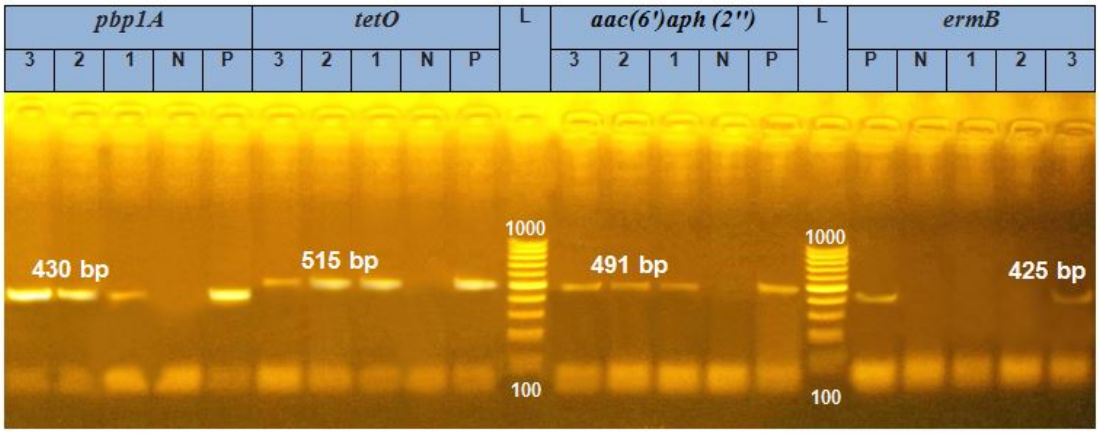


Fig. (3): Agarose gel electrophoresis showing amplification of 430 bp product of *pbp1*A gene, 515 bp of the *tet*O gene, 491 bp of the *aa(6')aph(2")* gene and 425 bp of erm*B* gene. Lane (p): Streptococcus (positive control) and Lane (N): Streptococcus (negative control). Lane (L): DNA Ladder, lanes (1, 2 & 3) streptococcus isolates.

Table (7): Summarized results of multiplex PCR assay targeting 7 genes in 3 *S. gallolyticus* isolates.

Isolate	Targeted genes						
	tuf	***Pbp1A***	***ermB***	***tetO***	***aac(6')aph (2'')***	***scpB***	***lmb***
1	+	+	-	+	+	+	+
2	+	+	-	+	+	+	+
3	+	+	+	+	+	+	+

DISCUSSION

Streptococcosis is an important septicaemic disease in pigeons; it is caused by *Streptococcus gallolyticus* which was formerly identified as *S. bovis* **(Devriese *et al.,* 1990b; De Herdt *et al.,* 1994a and Devriese *et al.,* 1998).**

Devriese *et al.* (1990a) isolated *S. gallolyticus* from different lesions in pigeons. It was announced that *S. gallolyticus* infection in pigeons has been an unusual finding as the bacteria appeared to be rare in other birds and it was not found in the faeces of wild birds **(Mundt, 1963)** or in the caeca of chickens **(Barnes, 1958)** or turkeys **(Harrison and Hansen, 1950).**

However, *S. gallolyticus* is an important component of the intestinal flora of many mammals, especially farm animals and less often in humans **(Parker, 1978).**

In 2007**,** an outbreak of *S. gallolyticus* was reported in a broiler flock and the isolates demonstrated a clear heterogeneity with pigeon isolates (**Chadfield *et al.,* 2007).**

De Herdt *et al.* (1992a) and Vanrobaeys *et al.* (1996) reported that, different types have been recognized within the *S. gallolyticus* species in healthy pigeons and pigeons that died from septicemia in equal frequency. This indicates that *S. gallolyticus* is a facultative pathogen which can belong to the intestinal flora of healthy pigeons.

Devriese *et al.* (1990b) mentioned that, streptococcal septicaemia, is known to exist in pigeons, mainly in squabs, commonly the disease was peracute or acute and rarely chronic. The mortality rate in untreated pigeons reached 78% and was highest in short-beak pigeons of Belgian race. Pure cultures of Streptococci were isolated from the parenchymatous organs and the blood in the majority of the cases. They diagnosed *S. gallolyticus* infection in 20 pigeon lofts submitted for post mortem investigation. Clinical signs were variable and ranged from hyperacute death to chronic lameness with arthritis. Lesions were generally unspecific except for single cases of muscle necrosis with purulent myositis. They found that, St. Gallolyticus infection was an

important etiology ranking next to *Salmonella.* Intravenous inoculations of St. Gallolyticus resulted in prostration, long lasting loss of weight and polyuria. After oral inoculation no clinical signs were seen.

S. gallolyticus has been reported with septicemia in pigeons and is associated with significant lesions including extensive areas of multifocal necrosis in different organs **(Devriese *et al.*, 1990a; De Herdt *et al.*, 1992b; De Herdt *et al.*, 1994a).**

In this study, the overall incidence of *S. gallolyticus* in in pigeons from different places of Cairo and Giza governorates was 11.72%.

In a similar study, **De Herdt *et al.* (1994b)** reported the prevalence of *S. gallolyticus* in 1056 pigeons as 10%. The microbe was isolated from the organs or joints of 106 pigeons.

In another study, **Van der Toorn and Lumeij (2001)** mentioned that *S. gallolyticus* septicaemia in 10% of septicemic necropsied pigeons.

In the current study, we tested *S. gallolyticus* isolates against 9 antibacterial therapeutic agents to detect the most effective ones. The tested isolates showed the following patterns of susceptibility; 70% were highly sensitive to amoxicillin; 64% were highly sensitive to streptomycin, 52% were highly sensitive to gentamicin and difloxacin, 47% were highly sensitive to vancomycin, 23% were highly sensitive to erythromycin and azithromycin, 17% of were highly sensitive to doxycycline and 11% of were highly sensitive to penicillin.

De Herdt *et al.* (1993) *S. gallolyticus* isolates recovered from pigeons were sensitive to penicillins, macrolides, lincomycin, tetracyclines, chloramphenicol and nitrofurans. However, the prevalence of acquired resistance against tetracyclines was approximately 40%. Sulphonamides and trimethoprim had little activity while activity of enrofloxacin, neomycin and gentamicin were in or near to the intermediate range.

Kimpe *et al.* (2002a) studied the susceptibility of 33 *S. gallolyticus* strains isolated from internal organs of homing pigeons to different antimicrobials that is most commonly used to treat pigeons. Aminoglycosides (Gentamicin and kanamycin), trimethoprim and flumequine were relatively inactive. Acquired tetracycline resistance accounted to 85%, and lincomycin and macrolide (erythromycin) resistance to 48% and 45%, respectively. All erythromycin-resistant strains, except one, were also resistance to lincomycin. All strains were susceptible to ampicillin, which agree with the results of this study.

In a similar study, Egypt, **Mohamed and Abd El-Motelib, (2007)** isolated *S. gallolyticus* from 22.5% of healthy and diseased pigeons. The isolates were highly sensitive to ampicillin, enrofloxacin, erythromycin, sensitive to gentamycin, neomycin, penicillin, less sensitve to chloramphenicol, tetracycline and resistant to lincomycin and trimethoprim. **Mohamed *et al.* (2009)** were successful in isolation *S. gallolyticus* with ratio of 21.4% from the examined pigeons. The higher incidence may be referred to inclusion of diseased birds in their studies. However, incidence reported in our study is almost consistent with other reports.

Finally, molecular studies confirmed the conventional identification and indicated that, the tested isolates harbored the targeted resistance and virulence genes.

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