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ORIGINAL ARTICLE

Public Health Hazards Related to *Streptococci Species* Isolated from Milk, Chickens and Fish

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Abstract
Streptococcus is a genus of spherical Gram-positive bacteria belonging to the phylum firmicutes and the lactic acid bacteria group. Members of the genus Streptococcus cause mild to severe bacterial illnesses in humans and animals. This work was designed to determine the biochemical, serological, and molecular characterization of *Streptococci species isolated* from cow`s milk, broilers chickens, and Nile tilapia. Bacteriological examination was carried out on 255 Samples (120 cow' milk, 65 broiler chickens, and 70 Nile tilapia) collected during the period from July 2010 to June 2013 from various localities in Beni-Suef Governorate, Egypt. Identification of the isolated strain revealed *S. agalactiae*, *S. equi* subsp. *zooepidemicus*, *E. fecalis*, *S. iniae*, *E. avium* and *L. lactis* subsp. *Lactis* were found in the isolated strains. Furthermore *S. agalactiae* (Lancefield group B), *S. equi* subsp. *zooepidemicus* (Lancefield group C) and *E. faecalis* (Lancefield group D) were found in the agglutination test for Lancefield groups. A multiplex PCR assay involves amplifying the multiple gene products in a single reaction using primers derived from *16S rRNA* genes of *S. agalacitae*, *S. equi* subsp. *zooepidemicus* and *lct*O gene of *S. iniae*. The amplified products showed amplification of 220bp, 679bp and 870bp of amplification respectively. The sensitivity of *streptococci species* to antimicrobials exhibited that *S. agalactiae*, *S equi* subsp. *zooepidemicus* and *S. iniae* showed high sensitivity to both enrofloxacin, erythromycin followed by cephalexin.

Keywords
Antimicrobial Assay, Chickens, Fish, Milk, Multiplex PCR, *Streptococci*

1. Introduction
Streptococcus is a genus Gram-positive bacteria with a spherical shape. Bacterium is divided along a single axis which causes them proliferate into pairs or chains. They are classified on the basis of colony morphology, hemolysis, and serologic specificity into the Lancefield group taxonomic system. Many of them are facultative anaerobe, non-pathogenic, some *streptococci* can cause severe diseases and health issues, such as bovine mastitis. Here, the most relevant species are *S. agalactiae*, *S. dysgalactiae subsp. dysgalactiae* (hereinafter referred to as *S. dysgalactiae)* and *S. uberis*. Streptococcal pathogens rarely associated with bovine mastitis are *S. canis*, *S. lutetiensis* and *S. equinus* **(Ruegg, 2017).**

Recently, many members of *Streptococci* were separated into the genera *Enterococcus* and *Lactococcus* **(Facklam, 2002).** The principle streptococcal disease in cattle is mastitis and the species involved include *S. uberis*, *S. agalactiae*, *S. dysgalactiae* and *S. zooepidemicus* **(Bramley and Dodd, 1984).** *Streptococcus equi zooepidemicus* is a zoonotic pathogen with adhesive and invasive properties. It can be responsible for septicemia, meningitis, arthritis and several other serious diseases. It affects most commonly people who have been consuming unpasteurized cow and goat milk products, or having close contact with horses or pigs **(Aida et al., 2020).** Bovine mastitis represents a disease of high incidence in dairy cattle herds worldwide is regarded as one of the most economically damaging diseases in the dairy industry globally due to decrease in milk yield, poorer quality milk, increased culling rate among dairy cattle as well as increased in the cost of veterinary services and medications globally **(Rahman et al., 2009; Schlesser, 2017).**

Poultry are regarded as the most appropriate source of animal protein supply of high nutritive value for man. One of the major problems in the poultry industry is the control of infectious diseases that cause significant economic losses. Problems due to streptococcus species in poultry result from infections causing growth depression and increased mortality without obvious clinical signs **(Chadfield et al., 2004).**

Streptococcosis in chickens is a group of pathogens that cause a range of syndromes, most commonly septicemia, peritonitis, salpingitis **(Edwards and Hull, 1937)** and endocarditis **(Jortner and Helmboldt, 1971)**. Infections are often thought to occur secondarily to other diseases. It has been reported in numerous bird species throughout the world. There are two forms of the disease, an acute septicemic form and a chronic form. Flock mortality can be as high as 50% **(Morishita, 2020).**

The demand for fish is expanding rapidly throughout the world representing an important component of human food and animal feed too. However, fish may harbor several pathogens that may threaten the public health, beside their impact on the fish industry. Streptococcus species represents one of the most important fish-borne pathogens, particularly *S. iniae* that causes recurrent outbreaks of disease in many species of cultured fish **(Woo and Bruno, 1999)**. *Streptococcus iniae* has been directly linked to massive economic losses in both marine and freshwater aquaculture environments, with mortality rates reaching 75% in tilapia farms for example **(Francis et al., 2014)**. Most streptococci outbreaks rather than *S. iniae* were associated with aqua-cultured ponds fertilized with caw dung and/or poultry droplets. As a result, *S. uberis*, *S. agalactiae*, *S. dysgalactiae* were found to be associated with fish streptococcosis outbreaks **(Mata et al., 2004; Nomoto et al., 2004).** Some aquatic *Streptococcus* species may cause disease in human in unusual circumstances. In addition to bacteria in the genus *Streptococcus*, there are several other closely related groups of bacteria that can cause similar disease in fish, including *Lactococcus*, *Enterococcus*, and *Vagococcus*. All of these bacteria and the disease itself were referred to as streptococcosis **(Yanong and Francis-Floyd, 2002).**

In Egypt, streptococcus and related genera are expected to cause detectable impact on cattle, poultry and fish industry, beside their potential hazards to the public health. Therefore, the present study was undertaken in Beni-Suef governorate, Egypt for isolation and identification of streptococci from different sources including cow's milk, broiler chickens and tilapia fish. Special consideration was applied to organisms that have public health hazards using molecular and antimicrobial assays as tools for concurrent diagnosis and treatment, respectively.

2. Materials and Methods

This study performed at various localities in Beni-Suef governorate, Egypt during the period from July 2010-June 2013. A total of 255 samples were collected; included 120 cow' milk, 65 broiler chickens and 70 Nile tilapia fish samples. These samples were exposed to bacteriological examination for the presence of *Streptococcus* species.

2.1. Milk Samples

The age, number of parity and current or previous illness problems of 100 individual cow were all recorded. The udders of each cow were examined for signs of mastitis in each quarter. Samples were collected according to **Blood and Handerson (1968).** In addition; 20 raw cows' milk samples (30ml) from dairy shops in Beni-Suef governorate were collected and transferred directly from the vendors' containers into sterile glass bottles. All samples were directly transported in ice box to the microbiology laboratory at Department of Bacteriology, Mycology and Immunology, Faculty of Veterinary Medicine, Beni-Suef University, Egypt, for microbiological investigations. The collected milk samples were subjected to bacteriological tests in accordance with **Mahy et al., (2010)**. Briefly, the samples were centrifuged for 20 minutes at 10°C and 3.000 *rpm*. Then the cream and supernatant fluids were removed and a loopfull from the sediment of each sample was directly inoculated into sterile tryptone soya broth.

2.2. Chicken Samples

A total of 65 broiler chickens of varying ages (5-8 weeks) were obtained from poultry farms through in Beni-Suef Governorate. Age, clinical symptom and postmortem lesions were noted. All chickens had respiratory symptom (coughing, sneezing, nasal discharges and sometimes swollen infraorbital sinuses) that were either unilateral or bilateral sinuses. The chickens were subjected to clinical, postmortem and bacteriological examination besides samples were collected from air sacs, lungs, trachea, pericardial sac, heart blood and liver. All samples were quickly transported to the laboratory in an ice box of where they immediately, analysis was directly operated. Air sacs, lungs, trachea, pericardial sac, liver and heart blood swabs were taken aseptically from butchered diseased and freshly dead chickens. Samples were directly inoculated into sterile tryptone soya broth.

2.3. Nile Tilapia Samples

At fish market in Beni-Suef governorate, Seventy Nile tilapia *(Oreochromis niloticus)* individuals with an average weight of 40-60 gm were collected from. Special considerations were taken for fish with Streptococcosis-like symptom, such as eye hemorrhages and opacity, exophthalmia and skin hemorrhages. Bacteriological examination of the examined fish was carried out according to **(Austin and Austin, 2007).** Briefly, the outer surfaces of the examined fish were disinfected by ethyl alcohol 70%. Then, using sterile sharp scissors and forceps, one cut was made in the dorsal surface at the middle of the fish spine for taking a kidney swab. An additional swab was taken from the eyes, if there was an ocular lesion. Samples were directly inoculated onto sterile Todd Hewitt broth (Sigma).

2.4. Bacteriological Examination

2.4.1. Isolation of Bacterial Agents

Loopfulls from the incubated broth were streaked onto tryptone soya agar (TSA), 5% Sheep blood (SBA) and /or Todd Hewitt agar (THA) and then incubated aerobically at the appreciate temperatures ranging from 25-37°C for 24 to 48 h. morphological and culture characteristics of The colonies obtained were studied. *Streptococcus* spp. Colonies with characteristics morphology (smooth round translucent glistening colonies, 0.5-1.5 mm in diameter) were chosen for further examination. Smear from colonies were stained with Gram's staining technique and examined microscopically. For each plate, one single pure colony representing typical colonial morphology was picked up, cultivated on TSA slopes and then kept at 4°C for further investigation **(Cruickshank et al, 1975).**

2.4.2. Morphological Examination of the Isolated Bacteria

Small tiny, creamy translucent rounded and slightly raised colonies suspected to be *Streptococcus* or *Enterococcus* species were picked up and examined microscopically by Gram's Method to observe the morphology, arrangement and staining reaction. Those revealed Gram positive, spherical or ovoid bacteria, non-sporulating arranged in pairs or in short or long chains were selected for further identification.

2.4.3. Biochemical Identification *Streptococci Spp.*

API 20 Strept, **(Quinn et al., 1999; Mahy et al., 2010)** was used to identify *streptococci* and associated species according to the manufacture instructions.

2.4.4. Serological Identification

Serological agglutination latex agglutination kits (Welcome Diagnostic; Difco Laboratories; Scott Laboratories and Diagnostic Corporation®) were used to perform serological agglutination test for identifying Lancefield groups A, B, C, D, F and G according to the manufacture recommendations.

2.4.5. Antibiogram Assay

The disc diffusion method was conducted according to **Finegold and Martin (1982).** The isolated strains were tested against 10 antibacterial agents, namely, Ampicillin (10µg), Ciprofloxacin (5µg), Neomycin (30µg), Amoxycillin (10µg), Erythromycin (15µg), Enrofloxacin (10ug), Cephalexin (30µg), Streptomycin (10µg), Penicillin-g (10 IU) and Tetracycline (30µg) were obtained from Oxoid (Oxoid, UK). The microorganisms were sub-cultured into TSB, then incubated at 37 ºC for 24 hours and adjusted to McFarland's opacity tube No. 0.5 (corresponding to 1.5×108 CFU/ml) with sterile saline. The standardized culture was swabbed thoroughly on Mueller Hinton agar medium (Oxoid) then dried for 5-10 min before placing the antibiotic discs. The zones of inhibition were measured after test plates were then incubated at 37ºC for 24h. The interpretation of zones of inhibition to detect whether the bacteria were sensitive or resistant to the used agent was estimated manually according to the limits given by Oxoid.

2.4.6. Multiplex PCR

2.4.6.1. Extraction of DNA

The DNAs extraction from the selected streptococcus isolates was performed according to **(Ausubel et al., 2003).** Briefly 1ml of overnight BHI of each selected streptococcus isolate was placed in a 1.5ml tube then centrifuged for10min at 12000 rpm to achieve the bacterial pellets. The obtained bacterial pellets were washed with PBS buffer for 3 times. Then, 20µl of 20mg/ml Proteinase K was added to each subjected isolate and incubated at 56°C for 30 min (mix occasionally to aid in digesting). The cellular debris were separated by centrifugation at 12000 rpm at 4°C and the supernatants were collected separately for further DNAs extraction. The DNAs were extracted using a mixture of phenol/chloroform/isoamyl alcohol mixture (25: 24: 1). Then the isolated DNAs were then precipitated with 100% ethanol, dried and re-suspended in 100 µl of purified molecular grade DW (Thermo Scientific, USA) using spectrophotometer. The concentration and purity of the isolated DNAs were assessed by measuring the optical density at a wave length of 260 and 280nm using the

2.4.6.2. Primers

Three sets of primers belonging to genus streptococci were selected for the study based on the earlier reports. The primer set of *S. agalactiae* amplifies a 220 bp fragment of *16S rRNA* gene and primers of *S. iniae* amplify a 870bp fragment of the Lactate oxidase (*lct*O) gene **(Mata et al., 2004)** and the prims set for *S. equi* subsp. *zooepidemicus* amplifies a 679bp fragment of *16S rRNA* gene **(Timoney and Artiushin, 1997).** All the primer sequences were synthesized by Thermo Scientific, USA. All Oligonucleotide primer sequences and size of the PCR-targeted products of the selected *Streptococcus* isolates are given in **Table (1).**

2.4.6.3. Multiplex PCR Assay

The amplified reactions were performed in 50 µl volumes in micro-amplification tubes (PCR tubes). The reaction mixture consisted of 10 µl (200 ng) of extracted DNA template from streptococcus isolates, 5µl 10X PCR buffer, 0.375µl $MgCl_2$ (1.5 mm), 1.25 µl dNTPs (250 µM), 0.25 µl (1.25 Unit) Ampli Taq DNA polymerase, 0.25 µl (0.5 µM) from each primer pairs and the volume of the reaction mixture was completed to 50 µl using DDW. Then all tubes were overlaid with 20 µl mineral oil. The multiplex PCR conditions was consists of initial denaturation at 94°C for 4 min, amplification for 30 cycles as denaturation at 94°C for 1 min, annealing for 55ºC for 1 min, final extension at 72°C for 1 min and final extension at 72°C for 7 min. All multiplex PCR assays were performed using FPTC-100 Programmable Thermal Controller (Peltier – Effect Cycling, MJ Research Inc.).

2.4.6.4. Screening of PCR Products by Agarose Gel Electrophoresis

After amplification, 3µl of each reaction mixture was separated by electrophoresis on a 1.5% agarose gel containing 0.2 µg of ethidium bromide per milliliter. The gel was run in 1X Tris– Acetic acid–EDTA (TAE) buffer at 100 V with standard DNA size markers for 20 min and visualized under short wave UV trans-illuminator, then photographed in order to obtain a permanent record using digital camera (Acer CR-5130, China).

Table (1): Oligonucleotides primers sets used for PCR assays of Streptococci.

Primer name		Primer sequence	Amplification product
16S rRNA *(S. agalactiae)*	F	5'-GAG TTT GAT CAT GGC TCA G-3'	220 bp
	R	5'-ACC AAC ATG TGT TAA TTA CTC-3'	
Lactate oxidase (*lct*O) *(S. iniae)*	F	5'-AAG GGG AAA TCG CAA GTG CC-3'	870 bp
	R	5'-ATA TCT GAT TGG GCC GTC TAA -3'	
16S rRNA *(S. equi* sub spp. *zooepidemicus*	F	5'- TGCATAAAGAAGTTCCTGTC-3'	679 bp
	R	5'-GATTCGGTAAGAGCTTGACG-3'	

3. Results

3.1. Frequent Distribution of *Streptococcus* and Related Genera in Examined Milk Samples

Out of 80 milk samples from apparently healthy cows 7(8.75%) isolates were recovered; 3 isolates (3.75%) were identified as *E. faecalis* and 4(5%) as *Lactococcus lactis subsp. lactis.* On the other hand, out of 20 milk samples of clinically mastitic cow's milk, 3(15%) streptococcus isolates were recovered; 2 isolates (10%) were identified as *S. agalactiae* and 1(5%) as *S. equi subsp. zooepidemicus*. Additionally, out of 20 milk samples collected from dairy shops 7(35%) isolates were recovered; 5(25%) were identified as *E. faecalis* and 2(10%) as *Lactococcus lactis subsp. lactis* **(Table, 2).**

Table (2): frequent distribution of streptococcus and related species in milk samples.

Examined Sample	Apparently Healthy cow's milk No = 80		Mastitis cow's milk No = 20		Dairy shops cow's milk No = 20	
Bacterial Isolates	No	%	No	%	No	%
S. agalactiae	2	10	-	-	-	-
S. zooepidemicus	1	5	-	-	-	-
E. faecalis	-	-	3	3.75	5	25
L. lactis	-	-	4	5	2	10
Total	3	15	7	8.75	7	35

No: Number of positive cases, %: was calculated according to the number of examined cases.

3.2. Occurrence of Streptococcus and Related Species in Chicken Samples

Denoted that out of 65 chickens suffering from respiratory disorders 6 (9.23%) streptococcus isolates were recovered. Four isolates (6.15%) were identified as *S. equi subsp. zooepidemicus* and 2 isolates (3.08%) as *E. avium* as shown in **Table (3).**

3.3. Occurrence of Streptococcus and Related Species in Apparently Healthy and Clinically Diseased Nile tilapia Samples

Revealed that out of apparently healthy fish (60) only 2 (3.33%) *E. faecalis* isolates were obtained in contrast to diseased fish that revealed 5 Streptococcus isolates out of 10 samples examined; 2 isolates (20%) were identified as *S. iniae*, 2 isolates (20%), as *S. agalactiae* and 1 isolate (10%) as *E. faecalis* **(Table, 4).**

3.4. Lancefield Gouping of Streptococcus and Related Genera

The obtained results in **Table (5)** revealed that isolates of *S. agalactiae*, *S. zooepidemicus* and *E. faecalis* were found to be belonged to Lancefield group B, C and D, respectively. On contrary, *S. iniae* couldn't be serologically identified. Concerning the hemolytic activities of the serologically identified stains, *S. agalactiae*, *S. zooepidemicus* and *S. iniae* showed β hemolysis while *E. faecalis* showed α hemolysis. On the other hand no hemolytic activities were expressed with *L lactis* and *E. avium.*

Table (3): Occurrence of streptococcus and related species in broiler chickens.

Bacterial isolates	No.	%
S. zooepidemicus	4	6.15
E. avium	2	3.08
Total	6	9.23

No: Number of positive cases, %: was calculated according to the number of examined cases (65).

Table (4): Frequent distribution of streptococcus and related species in apparently healthy and clinically diseased fish.

Nile tilapia	Total examined (No)	*S. iniae*		*S. agalactiae*		*E. faecalis*		Total	
		No	%	(No)	(%)	(No)	(%)	(No)	(%)
Apparently healthy	60	0	0.0	0	0.0	2	3.33	2	3.33
Diseased	10	2	20	2	20	1	10	5	50
Total	70	2	2.86	2	2.86	3	4.29	7	10

Table (5): Lancefield grouping and hemolytic activity of streptococcus and related genera.

Bacterial species	Serological identification	Hemolytic activity
S. agalactiae	Lancefield group B	β hemolysis
S. zooepidemicus	Lancefield group C	β hemolysis
L lactis	No typing	No hemolysis
E. faecalis	Lancefield group D	α hemolysis
S. iniae	No typing	β hemolysis
E. avium	No typing	No hemolysis

3.5. Efficiency Evaluation of Streptococcus and Related Species against Antimicrobials

S. agalactiae exhibited high sensitive to penicillin-G, enrofloxacin and streptomycin, followed by intermediate resistance to ampicillin, amoxycillin, ciprofloxacin, erythromycin cephalexin and tetracycline but resistant to neomycin. *S. equi subsp. zooepidemicus* are sensitive to erythromycin cephalexin, penicillin-G, tetracycline and intermediate interaction to ampicillin, ciprofloxacin, amoxicillin, enrofloxacin, and streptomycin but resistant to neomycin. On the other hand, *S. iniae* isolates were sensitive to ampicillin, enrofloxacin and intermediate action to ciprofloxacin neomycin, cephalexin, and penicillin-G but resistant to amoxicillin, erythromycin and streptomycin. Additionally, *L. lactis* was sensitive to penicillin-G and intermediate reaction was shown to ampicillin, amoxycillin enrofloxacin cephalexin while it was resistant to the other antibiotic used **(Table, 6).**

Table (6): Antimicrobials assay of Streptococcus and related species.

Isolates / Chemo-therapeutic agents	*S. agalactiae*	*S. zooepidemicus*	*E. faecalis*	*E. avium*	*S. iniae*	*L. lactis*
Ampicillin (10 µg)	+	+	++	-	++	+
Ciprofloxacin (5 µg)	+	+	++	-	+	-
Neomycin (30 µg)	-	-	-	+	+	-
Amoxycillin (10 µg)	+	+	+	+	-	+
Erythromycin (10 µg)	+	++	-	-	-	-
Enrofloxacin (10 ug)	++	+	+	+	++	+
Cephalexin (30 µg)	+	++	+	-	+	+
Streptomycin (10 µg)	++	+	-	-	-	-
Penicillin-G (10 IU)	++	++	+	+	+	++
Tetracycline (30 µg)	+	++	+	++	-	-

Interpretation: (++): Sensitive (+): Intermediate (-): Resistant

3.6. Multiplex PCR (M-PCR)

Agarose gel electrophoresis showing amplification product of 220 bp, 679 bp, fragment of 16srRNAgene of *S. agalactaie* and *S. equi subsp. zooepidemicus* respectively and 870 bp fragment of lctO of *S. iniae* performed with specific primers **(Fig. 1)**

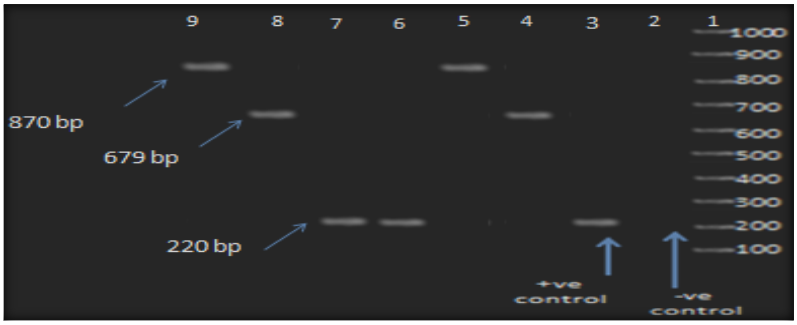

Fig. (1). Agarose gel electrophoresis performed with specific primers. L1: 100-1000bp DNA ladder, L2: negative control (*E. coli*), L3: positive control (*S. agalactiae*), L4: positive control (*S. equi. subsp. zooepidemicus*), L5: positive control (*S. iniae*), L6:*S.agalactiae* isolated from cow milk, L7:*S.agalactiae* isolated from cow milk, L8: *S. equi* subsp. *zooepidemicus* isolated from cow milk, L9: *S. iniae isolated* from fish.

4. Discussion

S. agalactiae is a highly infectious pathogen that can rapidly spread among a herd from a single infected animal. The main source of the infection is the udder of infected cows. Although, when hygiene is poor, contamination of the environment may also provide an additional source **(Radostits et al., 2000; Meiri–Bendek et al., 2002)**. It is clear that the *Streptococcus* species, inhabiting the udder and causing mastitis. The transmission of mastitis from infected udder to healthy udder is through hands during milking processes and possibly flies **(Argaw and Tolosa., 2008)**. *S. agalactiae* is a leading cause of both subclinical and clinical mastitis in dairy cattle worldwide. Good farm management and a high level of veterinary monitoring and treatment, may allow control of these pathogens in a herd. However, diagnosis is difficult, because of normally subclinical expression of these pathogens **(Keefe, 1997)**. Therefore, early diagnosis of the presence of new infection in a herd is important for an effective control.

Streptococci are non-motile, Gram-positive, non-spore forming bacteria, established or suspected pathogens in most groups of vertebrates. These include various mammals, birds and fish. A wide range of disease signs were recorded with different affected hosts. Infection can be mild to severe and sometimes with fatal termination depending the disease signs, host immunity and seasonal incidence as well. In cattle streptococcal mastitis is considered as the most common affection in Egypt and worldwide, in particular in dry areas, causing economic losses in dairy farm. Members of streptococci, including *S. agalactiae*, are responsible for persistent disease in adult and neonate human; however, the same pathogen causes serious infection among dairy cattle, poultry and fish. Additionally, the disease signs associated with such affections are characterized by occurrence of inflammation, cell necrosis and the formation of granuloma in the infected host(s) leading to high mortality rates.

The results illustrated in **Table (2)** revealed 3(15%) *streptococcus* isolates recovered from 20 milk samples of clinically mastitic cows; 2(10%) of them were identified as *S. agalactiae* and the third one (5%) as *S. equi* subspecies *zooepidemicus*. The total recovery rate of *Streptococcus* spp. is lower than **Dego and Tareke (2003)** (23.6%**)** and so lower than **Adesola (2012)** (55.38%**)** and **El-Jakee et al., (2013)** **(**55%**)**. Nevertheless, the isolation rate of *S. agalactiae* is relatively in agreement with those obtained by **Almaw et al., (2008)** 8%, but it is lower than **Adesola (2012)** (12.31%) and **Kivaria et al., (2007)** (15.4%), and very lower than **El-Jakee et al., (2013)** (19.3%)**, Amosun et al., (2010)** (24.6%) and **Tenhagen et al., (2006)** who recorded that *S. agalactiae* was isolated from 29% of the herds.

For human, *S. agalactiae* cause severe invasive illness in adults and neonates, it has thought to have caused 16,880 human cases, including 1,650 deaths, in the United States alone in 1998 **(Schrag et al., 2000).** Human infection with *S. equi* subspecies *zooepidemicus* whereas most cases occurring in people consuming unpasteurized milk or dairy products or who are in close contact proximity with horses **(Barnham et al., 1989)**.

The obtained *S. equi subsp zooepidemicus* (5%) are in harmony with that isolated by **Amosun et al., (2010)** (3.9%) and **Adesola (2012)** (2.31%). The isolation of zoonotic *streptococcus* spp. from the examined milk draws the attention to the importance of adequate boiling or pasteurization of raw milk to prevent the spread of infection via milk sold in Beni-Suef Governorate. On the other hand, the obtained results showed that seven isolates were obtained from 80 milk samples from apparently healthy cows (8.75%) 3 isolates (3.75%) were identified as *E. faecalis* and 4(5%) as *L. lactis* as shown in table (2) The *E. faecalis* results obtained are lower than **Seputiene et al., (2012)** that recovered 42 strain *E. fecalis* from 80 enterococcal isolates while, **Moschetti et al., (2001)** recovered 25 strains of *E. fecalis* from 80 *enterococcal* isolates. Other strains were identified as and *E. durans* and *E. gallinarum* **(Moschetti et al., 2001)**.

Investigation have shown that commensal enterococci play an essential role in animal infections **(Seputiene et al., 2012)**. *Enterococci* have been shown to increase virulence potential during a poly-microbial infection in animal models suggesting a synergistic effect between *enterococci* and virulent bacteria **(Montravers et al., 1997; Lavigne et al., 2008).** The given results are nearly agree with **Abou-Younes et al., (2007)**; who isolated *E. fecalis* with percentage of 21%; but are higher than isolation percentage of **Ryšánek et al., (2009)** (16.1%). On contrary, the results of the present study are lower than those obtained by **Citak et al., (2005)** (54.2%).

Kagkli et al., (2007) recorded that bovine feces were not an important source of contamination of raw milk with enterococci or coliforms. The major source of these bacteria in the milk was the milking equipment.
This suggests that the healthy mammary gland is of equal importance as suitable environmental hygienic conditions for high quality of raw milk intended for dairy processing **(Ryšánek et al., 2009)**.

The results showed in **Table (3)** denoted that out of 65 chickens suffering from respiratory disorders 6 (9.23%) streptococcus isolates were recovered; 4 isolates (6.15%) were identified as *S. equi* subsp *zooepidemicus* and 2 isolates (3.08%) as *E. avium*. These results are lower than those recorded by **Al-Barrodi et al., (2011)** who recovered *S. equi* subsp *zooepidemicus* with a percentage of (10%) and (20%) in 2 different flocks

S. equi subsp *zooepidemicus*' bacteria are active when a decline in the immune system of poultry took place due to any disease as influenza virus **(Hofstad et al., 1978)**. *E. avium* was recovered with low rate by **Champagne et al., (2011)** who recovered 2 isolates from 9 commercial and experimental broiler farms, and **Hedegaard et al., (2008)**

who also recovered 3 isolates from two consecutive broiler parent flocks on a farm.

Public health may be threatened by VRE release, particularly if the organisms reach ground water, which may be consumed without treatment, or if they impact recreational waters. Evidence exists that *Enterococcus* spp. can proliferate in subtropical and tropical soils and waters **(Fujioka et al., 1998; Roll and Fujioka, 1997);** therefore, introduction of VRE into such environments may be especially problematic.

Tilapias have become a perfect host for streptococcus infection. **Labrie et al., (2009)** mentioned that two species of streptococci; *S. agalactiae* and *S. iniae* are generally considered the most important agents of streptococcal disease in tilapia. *S. iniae*, known to infect certain fish species, has caused disease in human, including one group of people in Canada who handled live tilapia. However, these people were considered much more susceptible to disease than the general population because of their advanced age and underlying health problems. Additionally, puncture wounds or cuts were required to initiate infection in this group. Normal, healthy persons are at minimal risk of acquiring this disease from sick fish **(Yanong and Francis-Floyd, 2002).**

Results illustrated in **Table (4)** showed that out of 70 Nile tilpia samples; 7(10%) *streptococcus* isolates were recovered; 3 isolates (4.29%) were identified as *E. faecalis*, 2 isolates (2.85%) as *S. iniae* and 2 isolates (2.85%) as *S. aglactiae*.

This result agrees with that obtained by **Shoemaker et al., (2001)** who recorded 1.67% prevalence of *S. iniae* in market-size tilapia and **Chen et al., (2012)** who recovered *S. iniae* with 2.3% prevalence. On the other hand; the obtained result is lower than that recorded by **Abuseliana et al., (2010)** who recovered streptococcal isolates (5%) from healthy fish. Additionally, our result is very low when compared with that obtained by **Torky et al., (2005)** who recovered *E. fecalis* with a percentage of 29.48%.

The present study the isolation rate of *S. iniae* and *S. agalactiae* are equal while **Chen et al., (2012)** isolated 105 streptococci from tilapia in 2006-2007 where 94.7% and 5.3% were *S. iniae* and *S. agalactiae*, respectively, while during the period of 2009-2011 the percentages were 2.3 and 97%, respectively.

Antibiotic resistance is an increasing concern worldwide, and there is an agreement that improved surveillance is needed **(Anon, 1998; Livermore and Chen, 1999).** The uncontrolled usage of antimicrobial agents is recognized as the most important factor that favours the development and spread of resistant microorganisms **(Burch, 2005)**.

Results shown in **Table (6)** revealed that *S. agalactiae* is highly sensitive to penicillin-G, enrofloxacin and streptomycin, followed by intermediate resistance to ampicillin, amoxicillin , ciprofloxacin, erythromycin cephalexin and tetracycline but resistant to neomycin **.**

The obtained results are in agreement with **Schrag et al., (2000)** who declared that penicillin is the drug of choice for treatment of both human and bovine *S. agalactiae* infections, **Al-Marzouk et al., (2005)** reported that *S. agalactiae* isolates were sensitive to amoxicillin and showed resistance to neomycin and **Figueiredo et al., (2006)** found that *S. agalactiae* samples were resistant to neomycin, nalidixic acid and gentamicin similarly **Abuseliana et al., (2010)** concluded that *S. agalactiae* was resistant to neomycin, however**,** **El-Jakee et al., (2013)** mentioned that *S. agalactiae* were sensitive to ampicillin and penicillin.

The obtained results in the present study disagree with **Uh et al., (2001)** who reported that the prevalence of resistance to erythromycin has been increasing in *S. agalactiae*. In addition **Guérin-Faublée et al., (2002)** mentioned that *S. agalactiae* strains isolated from mastitic cows were resistance to tetracycline and **Acikgoz et al., (2004)** proved that high rate of tetracycline resistance in Turkey Group B hemolytic streptococci especially *S. agalactiae*. Moreover, our results disagree with **Ebrahimi et al., (2008)** who mentioned that *S. agalactiae* demonstrated high level of resistance against streptomycin and penicillin besides low level of sensitivity to other tested antimicrobials; cephalexine, amoxicillin, tetracycline ampicillin, chloramphenicol and erythromycin. **Abuseliana et al., (2010)** concluded that *S. agalactiae* were resistant to streptomycin. **Jain et al., (2012)** declared that the highest resistance was found by disc diffusion method for *S. agalactiae*, was the streptomycin.

Table (6) revealed that *S. equi* subsp. *zooepidemicus* are sensitive to erythromycin cephalexin, penicillin-G, tetracycline and intermediate interaction to ampicillin, ciprofloxacin, amoxicillin, enrofloxacin, and streptomycin but resistant to neomycin. These results on contrary to those obtained by **Adesola (2012)** who reported 100%, resistance, to ampicillin, tetracycline, but coincided in the results of neomycin and streptomycin (intermediate).

In this study *S. iniae* isolates were sensitive to ampicillin, enrofloxacin and intermediate action to ciprofloxacin neomycin, cephalexin, and penicillin-G but resistant to amoxicillin, erythromycin and streptomycin.

This result nearly agree with **Facklam et al., (2005)** who found that penicillin appears to be the drug of choice for managing *S. iniae* infections and **Suanyuk et al., (2010)** who reported that *S. iniae* was sensitive to ampicillin and penicillin. On the other hand revealed that *S. iniae* was sensitive to erythromycin. Generally**;** streptococci are naturally resistant to aminoglycosides because they lack the

active transport mechanism that is required for these drugs to be taken up into the bacterial cell **(Teale et al., 2004)**.

Iversen et al., (2002) reported that during the last decades, *Enterococci* have emerged as important nosocomial pathogens. Their role in such infections has increased due to their ability to acquire resistance to various antimicrobial agents, which renders them difficult to treat. Two strains related to *Enterococcus* group (*E. faecalis* and *E. avium*) were recovered. *E. fecalis* was sensitive to ampicillin and ciprofloxacin with intermediate reaction to enrofloxacin, cephalexin, penicillin-G, tetracycline and amoxycillin and resistant to the other antibiotics used. *E. avium* was sensitive to tetracycline and showed intermediate reaction to penicillin-G, enrofloxacin, amoxycillin and neomycin while the organism was resistant to the other antibiotics tested.

These results nearly agree with **Del Campo et al., (2000)** who recorded that the combination of a cell wall-active agent ampicillin, penicillin has been used to effectively treat entercoccal infection. Also the results agree with those of **Gianneechini et al., (2002)** who found that most *Enterococcus* strains were susceptible to penicillin and **Arvanitidou et al., (2001)** who recorded the resistance patterns of *Enterococcus* to streptomycin and ciprofloxacin while show difference with *E. fecalis* which exhibited sensitivity to these antibiotics. Also **Kolar et al., (2002)** recorded that *Enterococcus* strains isolated from poultry were resistant to tetracycline, erythromycin. Moreover, **Sood et al., (2008)** reported high resistance level of most significant *Enterococci* to amino-glycosides, ampicillin (caused by beta-lactamase production), and glycopeptides including vancomycin resistance.

Identification based on biochemical and antigenic characteristics can be barely differentiating between streptococci, however, they are limited by the length of time required to complete the assays **(Zlotkin et al., 1998).** Additionally, isolation of those pathogens, particularly in a case of mixed infection, is considered as time and effort consuming and sometimes lead to misdiagnosis **(Hussein and Hatai, 2006).**

Molecular diagnosis protocols have been the most effective methods for diagnosis of bacterial agents involved in streptococcal outbreaks because they permit more specific and sensitive detection than do serological assays **(González et al., 2004).** Therefore, PCR can target unique genetic sequences of microorganisms and has previously been developed for the identification of pathogenic bacteria using a primer specific to target a gene segment of a given bacterium. Alternatively, bacterium-specific gene can be used as targets for PCR amplification to permit more specific detection as well as subspecies and strain differentiation.

Previous conventional PCR studies demonstrated that the primers *LOX-1/LOX-2* could be used successfully to aid in the identification of *S. iniae* via the generation of a specific 870-bp product **(Mata et al., 2004; Hussein and Hatai, 2006).** On the other hand, **Priestnall et al., (2010)** confirmed that the 2 primers pairs targeting *sze*F gene have the capability to identify *S. equi* subsp. *zooepidemicus* isolated from dogs suffered from acute fatal pneumonia giving a specific PCR amplicon of 679-bp band. Moreover, **Martinez et al., (2001)** described a primer set sensitive enough to detect specific sequences of the 16S *rRNA* gene of *S. agalactiae* in caw milk by amplification of the specific 220-bp band.

In the presented study, we designated a multiplex PCR assay involves amplifying the multiple gene products in a single reaction based on primers deduced from the regions carrying the *16S rRNA* genes of *S. agalactae*, of *S. equi* subsp. *zooepidemicus* and *lct*O gene of *S. iniae*, respectively **(Martinez et al., 2001; Mata et al., 2004; Hussein and Hatai, 2006; Priestnall et al., 2010).**

The specificity of the developed PCR assay using the above-mentioned primer sets was confirmed by the fact that only specific bands were amplified equivalent to 220, 679 and 870 bp, which are characteristics for *S. agalactae*, *S. equi* subsp. *zooepidemicus* and *S. iniae*, respectively.

These results are in agreement with those obtained by **Mata et al., (2004)** for *S. iniae*, **Priestnall et al., (2010)** for *S. equi* subsp. *zooepidemicus* and **Martinez et al., (2001)** for *S. agalactae* when they used single PCR. In addition, same results were reported by **Itsaro et al., (2012)** for *S. iniae* and *S. agalactae* but not for *S. equi* subsp. *zooepidemicus* because the later was not included in their m-PCR. Moreover, our m-PCR could detect the three pathogens at low concentrations of (3×10^2) indicating its specificity as well as sensitivity.

From these data, it can be concluded that the proposed m-PCR assay can be useful not only as a diagnostic tool but also for epidemiological surveys and could be efficient to establish preventive measures. Furthermore, the m-PCR offers a rapid and reliable procedure for detection of infection and for implementing prompt measures to prevent the spread of disease from locality to another.

5. Conclusion

Streptococci were isolated (15%) from the examined milk samples (20) of mastitic cows, and identified as *S. agalactiae* and *S. equi-zooepidemics*. While, streptococci were isolated (8.75%) from apparently healthy cow's milk samples (80) and identified as *E. faecalis* and *L. lactis*. Out of 20 milk samples were collected from dairy stores (35%), isolated and classified into *E. faecalis* and *L. lactis*. For broiler chickens (65) suffering from respiratory disorders (9.23%), streptococci were isolated, recovered, and identified as *S. equi* subsp. *zooepidemicus* and *E. avium*. Out of apparently healthy fish (60), only *E. faecalis* were isolated and obtained in contrast to diseased fish that revealed *Streptococci* isolates out of 10 examined samples and identified as *S. iniae*, *S. agalactiae* and as *E. faecalis.* The results of isolates were tested for sensitivity against 10 types of antibiotics as

follows: *S. agalactiae* was highly sensitive to Penicillin-G, Enrofloxacin, and Streptomycin, *S. equi-zooepidemics* was sensitive to Cephalexin, Erythromycin, Penicillin-G, Tetracycline and *S. ineae was* sensitive to Ampicillin, and Enrofloxacin. PCR for regions carrying the gene (16srRNA) of *S. agalactiae* and *S. equi-Zoepidmics* gave a product at molecular weight 220 and 679 bps, respectively. While, *S. aniai* gene (lctO) gave a product at a molecular weight of 870 bps

6. Authors Contributions

All authors contributed equally to study design methodology, interpretation of results and preparing of the manuscript.

7. Conflict of Interest

The authors declare no conflict of interest.

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