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Epidemiological Studies on Transmission of some Avian Pathogens from Fish Farms to Water Fowls in Kafr El-sheikh Governorate, Egypt

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ABSTRACT

Integrated Waterfowl is common in Kafr El-Sheikh Governorate. The infection with many bacterial and /or viral diseases due to using poultry litter as a fertilizer in fish farms is studied. Fifty litter samples, sixty nine fish pond water samples, two hundred fecal swabs from integrated waterfowl and sixty samples from liver from these waterfowl were collected for surveying some pathogens which may be present in litter and transported to fish ponds and infect waterfowl. Results of this survey revealed the isolation of 19 *Salmonella* spp. (4 isolates from litter, 2 isolates from fish pond water, 8 isolates from fecal swabs of waterfowl and 5 isolates from waterfowl liver), 17 *Staphylococcus* spp. (7 isolates from litter, 3 isolates from fish pond water, 5 isolates from fecal swabs of waterfowl and 2 isolates from waterfowl liver) and 59 *E. coli* isolates (9 isolates from litter,16 isolates from fish pond water, 22 isolates from fecal swabs of waterfowl and 12 isolates from waterfowl liver) isolates were obtained from different samples.

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Introduction

Kafr El-Sheikh Governorate produce 29% of the total fish production in Egypt (fisheries and fish culture) in a year 2001(GAFRD, 2001). Some of these farms integrated with waterfowl as Duck-fish systems appear to be more favored since ducks fit more easily into aquaculture facilities, performing both vegetation and pest control as well as fertilization roles, with minimum requirement of special facilities and expenditure in warm water systems (Little and Edwards, 2005). Integrated farming is an effective tool for improving rural economy due to its cumulative cost effectiveness, low investment and higher profitability. It optimizes the farm productivity per unit area through incorporation of recycling wastes and residues from one farming system to another due to environmental consideration (Biswas *et al*., 2013). Most of these farms use poultry litter as a fertilizer (El-Dawansy, 2002). Using poultry litter as a fertilizer in fish ponds is important as poultry litter rich in soluble nitrogen and phosphorous, which stimulates algal production that in turn can be consumed by fish directly or after intermediate processing by zooplankton or microbes (Knud-Hansen, *et al*., 1991). Poultry litter is a potential source of protein, calcium, magnesium and other minerals, this encourage fish farmers especially in the integrated farming system to recycle it (SPFG, 1994). Untreated poultry litter contains contaminants (bacteria and viruses) which contaminate the ponds and may infect water fowls (Edwards, 1993). These pathogens as *E. coli*, *Salmonella* spp. *S. aureus*, Newcastle disease virus, avian influenza virus and others. However, the pathogens discharged from the chicken and contaminating the litter, feed, water and thus the nearby birds (Islam *et al*., 2014). The objective of the present study is to survey the predominant pathogens which present in litter and transport to fish ponds during pond fertilization and infect ducks integrated with these fish farms through isolation and identification of some pathogens, which prevailed in each of litter obtained from poultry farms, water of fish ponds and its integrated water fowls.

Materials and methods

Experimental design and sampling

Samples were obtained according to the research design where fifty litter samples, sixty nine fish pond water samples,

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two hundred fecal swabs from integrated water fowls and sixty liver sample were collected from slaughtered ducks from different fish farms along 6 month from April 2015 to October 2015 from different locality in Kafr El-Sheikh Governorate. All samples were labeled and transported to the lab. (Animal Health Research Institute, Kafr El-Sheikh provisional lab., Egypt) and subjected to bacterial and viral isolation and identification.

Bacterial isolation and identification

Bacterial isolation was carried out from litter (Twenty five grams of litter samples through mixed in sterile flask containing 225 ml phosphate buffer saline (PBS, BIO BASIC, Canada), water samples (collected by inverting a 500 ml sterilized flask 30 cm below the pond water surface. 30 ml of water samples were centrifuged by using centrifuge-Universal- Germany at 5000 rpm for 5 minutes), fecal swabs and liver samples (A sterile cotton swabs stabbed into liver parenchyma) by using nutrient broth (Oxoid, UK), where 1 ml of all of these samples inoculated in nutrient broth and incubated at 37ºC for 24 h, then 1 ml of incubated broth were inoculated onto selenite-f-broth (Oxoid, UK) and incubated at 37ºC for 24 h, and a loopful from this broth were streaked onto Salmonella-shigella (SS) agar (Oxoid, UK) and incubated at 37ºC for 24 h, another 1 ml of incubated broth was inoculated onto MacConkey broth (Oxoid, UK), which incubated at 37ºC for 24 h., then a loopful was streaked onto MacConkey agar (Oxoid, UK) and Eosin methylene blue (EMB) agar (Oxoid, UK) and incubated at 37 ºC for 24 h, 1 ml of the previously incubated nutrient broth was inoculated in NaCl broth (nutrient broth containing 6% NACL) and incubated at 37ºC for 24 h, a loopful was streaked onto Mannitol salt agar (Oxoid, UK) and sub cultured on Baired parker (Oxoid, UK). All the suspected pure colonies of Salmonellae, *E. coli* and staphylococci were furtherly subjected to biochemical reactions (Methyl red, Voges-Proskauer, Indole and Urea tests) for Salmonellae and *E. coli* according to Cheesbrough (1985), while Catalase activity, Mannitol test, heamolysis activity test and Coagulase test were achieved for staphylococci isolates according to Finegold and Martin (1982); Bailey and Scott (1998) and Cruickshank *et al*. (1979) respectively. Biochemically positive Salmonellae isolates were finally identified according to Patrick *et al*. (2007) using Salmonella poly "O" antiserum and Salmonella monovalent "O" antiserum (SINIF Co., Germany), and Suspected isolates to be *E. coli* were then subjected to serological identification according to Kok *et al*. (1996) by using Somatic, flagellar and capsular antiserum for *E. coli* (DENKA SEIKEN Co., Japan), while suspected isolates to be staphylococcus were subjected to Molecular (PCR) identification.

Molecular identification (PCR) for Staphylococci spp.

The DNA was prepared from bacterial culture according to Shah *et al*. (2009) where the bacteria streaked on nutrient agar (oxoid UK) plates and after overnight culture, one colony was suspended in 20 ml of sterile distilled water, and the suspension was then heated at 100ºC for 20 minutes, then the bacteria was subjected to DNA extraction by using QIAamp DNA Mini Kit, Catalogue no. 51304 GMBH, Germany, after that the PCR Master Mix was prepared according to Emerald Amp GT PCR mastermix (Takara. China) Code No. RR310A, then DNA amplification was performed in a thermal cycler (Biometra, Germany) using the following conditions:

Initial denaturation (Deionizer Millipore-USA) for 5 min at 94°C followed by 35 cycles of denaturation (94°C for 30 sec.), annealing (55°C for 45 sec.), and extension (72°C for 45 sec.). A final extension step (72 °C for 10 min) was performed after the completion of the cycles. The resultant PCR products were further analyzed by agarose gel electrophoresis (1.5% low-melting temperature agarose Biotechnology grade (Bioshop®, Candainc. lot No: OE16323), stained with ethidium bromide biotechnology grade (Bioshop ® CandaInc, Lot No: 0A14667) and visualized by a UV transilluminator (Spectronics-Corporation-USA).

Viral isolation and identification

The collected samples (poultry litter, fish ponds water, fecal swabs of integrated water fowls and liver of some of these water fowls) were diluted in PBS (BIO BASIC, Canada) at concentration 20% and centrifuged at 3000 rpm for 15 minute, then the supernatant fluid were collected and treated with antibiotic (Penicillin-G-Na 100,000 IU/ml EPICO, Egypt and streptomycin 100 mg/ml CID, Egypt) and antimycotic (Nystatin 100,000IU/ml GSK, Egypt) and left for 2 h at room temperature. These prepared samples were inoculated at 0.2 ml into the allantoic cavity of five embryonated chicken eggs (ECE) at 9 days for each sample. After inoculation, ECE were reincubated for 4-7 days and examined daily by candling up to 7 days and the dead embryos within 24 h. were discarded as nonspecific death, then chilled at 4ºC and the amnio-allantoic fluids were harvested and tested for haemagglutination (HA) activity. Three blind passages were carried out for each sample (Alexander 1989; Beard, 1989).

Results

Results of isolated bacteria are showed in Table 1, from this table, the examination revealed 19 *Salmonella* isolates from poultry litter, fish pond water, fecal swabs of integrated water fowls and liver of integrated water fowls by rate 8, 2.9, 4 and 8.3% respectively while 59 *E. coli* isolates from poultry litter, fish pond water, fecal swabs of integrated water fowls and liver of integrated water fowls by rate 18, 23.2, 11 and 20% respectively. Also 17 *Staphylococcus* isolates were isolated from poultry litter, fish pond water, fecal swabs of integrated water fowls and liver of integrated water fowls by rate 14, 4.4, 2.5 and 3% respectively.

Table 1. Types and numbers of isolated bacteria

Types of samples	Total number of samples	*Salmonella spp.*		*E. Coli*		*Staphylococcus spp.*	
		No. of isolates	%	No. of isolates	%	No. of isolates	%
Litter samples	50	4	8	9	18	7	14
Water samples	69	2	2.9	16	23.2	3	4.4
Fecal* swabs	200 (pooled sample)	8	4	22	11	5	2.5
Liver a	60	5	8.3	12	20	2	3
Total	379	19	5	59	15.6	17	4.3

*: 3 pooled sample (2-3 individual samples) were taken from each farm. a: from scarifying ducks and duckling.

Table 2. Biochemical characterization of isolated Salmonella spp. and *E. coli*

	M.R	V.P	Motility	Indole	TSI				Urea
					Butt	slant	H_2S	Gas	
Salmonella spp.	+ve	-ve	+ve	-ve	Y	R	+ve	+ve	-ve
E. coli	-ve	-ve	+ve	+ve	Y	R	-ve	+ve	-ve

M.R: Methyl red, V.P: Voges-proskauer, TSI: Triple sugar iron agar, Y: Yellow, R: Red

Table 3. Biochemical characterization of isolated *Staphylococcus* spp.

	Coagulase activity		Mannitol test	Detection of Haemolysis		Catalase test
	+ve	-ve		+ve	-ve	
Staphylococcus spp.	7(41%)	10(59%)	+ve	6	11	+ve

Table 4. Serotypes of isolated Salmonellae

Types of samples	Number of samples	No. of isolates	Incidence	Identified serotypes
Litter samples	50	4	8%	*S. kentucky* *S. belgdam* *S. cuckmere* *S. bargny*
Water samples	69	2	2.89%	*S. tshiongwe* *S. gueuletapee*
Fecal swab samples	200 Pooled samples	8	4%	*S. oxford* *S. bargny* *S. atakpame* *S. ferruch* *S. enteritidis* *S. uganda* *S. kentucky* (2 isolates)
Internal organs samples	60	5	8.3%	*S. kentucky* *S. enteritidis* *S. amesterdam* *S. brikama* *S. kulsrivier*

All isolated bacteria (19 *Salmonella* isolates, 17 *Staphylococcus* isolates and 59 *E. coli* isolates) were subjected to biochemical identification. Results of *Salmonella* and *E. coli* were summarized and presented in Table 2. While the biochemical characterization of *Staphylococcus* spp. was mentioned in Table 3.

The biochemically identified *Salmonellae* isolates were typed against known polyvalent and monovalent "O" and "H" *Salmonella* antisera. Results are summarized and presented in Tables 4 and 5. From these Tables, it is evident that out of 19 salmonellae isolates (4) *S. kentucky*; (2) *S. enteritidis*; (2) *S. bargny* and one isolate for each of *S. belgdam*, *S. cuckmere*, *S. tshiongwe*, *S. gueuletapee*, *S. oxford*, *S. atakpame*, *S. ferruch*, *S. uganda*, *S. amesterdam*, *S. brikama* and *S. kulsrivier*.

It is clear that *S. kentucky* is the most frequent isolate with a rate of 21% (4 out of 19 isolate) followed by *S. enteritidis* and *S. bargny* with a rate of 10.5% (2 out of 19 isolate).

Table 5, shows that all Salmonellae isolated were motile containing flagellar antigen "H" with its two phases "H1" and "H2" except *S. belgdam*, *S. gueuletapee*, *S. amesterdam* and *S. enteritidis* contain "H1" only and missing "H2".

The biochemically identified *E. coli* cultures were subjected to serological identification as mentioned in Table (6).

Table 5. Antigenic structure of isolated Salmonellae

Serotype	Antigenic structure profile		
	O antigen	H antigen	
		Phase I	Phase II
S. oxford	3,10,(15), (15,34)	a	1,7
S. bargny	8,20	1	1,5
S. atakpame	8,20	e,h	1,7
S. tshiongwe	6,8	e,h	e,n,z15
S. kulsrivier	1,9,12	g,m,s,t	e,n,x
S. kentucky	8,20	i	Z6
S. belgdam	9,46	g,m,q	-
S. brikama	8,20	r,i	1,w
S. gueuletapee	1,9,12	g,m,s	-
S. ferruch	8	e,h	1,5
S. cuckmere	3,10	i	1,2
S. amesterdam	3,10,(15), (15,34):	g,m,s	-
S. enteritidis	1,9,12	g,m	-
S. uganda	3,10, (15)	1,Z13	15

Table 6. Serological identification of the isolated *E. coli*.

No. of sample	Code	polyvalent	serotype
1	4L	II	O111:K58
2	11L	I	O26:K60
3	7L	III	O25:K11
4	17L	III	O157:K-
5	10L	III	O124:K72
6	50L	II	O55:K59
7	8L	III	O124:K72
8	15L	I	O26:K60
9	33L	I	O114:K90
10	33Liver	III	O78:K80
11	66 liver	-	untypable
12	60 liver	I	O26:K60
13	18 liver	II	O55:K59
14	62 liver	I	O26:K60
15	24 liver	II	O86:K61
16	30 liver	I	O44:K74
17	15 liver	I	O44:K74
18	8 liver	I	O44:K74
19	21 liver	III	O25:K11
20	53 liver	II	O55:K59
21	57 liver	III	O124:K72
22	198 F.S	III	O157:K-
23	10 F.S	III	O157:K-
24	42 F.S	III	O78:K80
25	159 F.S	III	O78:K80
26	174 F.S	I	O125:K70
27	18 F.S	I	O142:K86
28	36 F.S	I	O125:K70
29	149 F.S	I	O142:K86
30	165 F. S	I	O142:K86
31	156 F.S	II	O55:K59
32	109 F.S	II	O55:K59
33	186 F.S	III	O25:K11
34	177 F.S	II	O55:K59
35	103 F.S	III	O103:K-
36	91 F.S	III	O164:K-
37	39 F.S	I	O114:K90
38	81 F.S	I	O114:K90
39	1 F.S	III	O103:K-
40	51 F.S	I	O114:90
41	189 F.S	-	untypable
42	126 F.S	-	untypable
43	15 F.S	-	untypable
44	28 W	II	O128:K67
45	62 W	II	O128:K67
46	15 W	II	O127:K63
47	33 W	II	O91:K-
48	5 W	-	untypable
49	51 W	I	O44:K74
50	67 W	II	O 55:K59
51	18W	II	O55:K59
52	40 W	I	O26:K60
53	50 W	II	O127:K63
54	8 W	II	O55:K59
55	48 W	II	O126:K71
56	30 W	III	O125:K70
57	66 W	II	O126:K71
58	11 W	III	O125:K70
59	69 W	III	O125:K70

The biochemically identified staphylococcus were subjected to PCR, which confirmed all isolates and showed that 4 samples were *S. aureus* (2 from litter, 1 from water and 1 from fecal swab samples) as mentioned in Table (7) and showed in Fig. 1 and Fig. 2. In Fig. 1, detection of 16s rRNA (16S rRNA, 1500 bp) showed that all tested samples were *Staphylococcus* spp, while in Fig. 2, detection of clumping factorA (clfA, 638 bp) showed that 4 tested samples were *S. aureus*.

Viral isolation in embryonated chicken eggs

All collected samples (fecal swabs, water, litter and liver) were negative for viral isolation (NDV and AIV) by using haemagglutination (HA) activity after three blind passages.

Discussion

It is worth stated that *Salmonella* infected-bird are intermittent shedder, In the present work Salmonellae were isolated from litter with a rate of (8%), this rate appears to be higher than that reported in Egypt by Dahshan *et al.* (2015), where they reported a rate of (4%), Al-Nakhli *et al.* (1999) in Saudi Arabia where they reported an incidence of Salmonella (2.3%) and Al-Zenki *et al.* (2007) where they reported Salmonella with a rate of (1.5%) from poultry litter.

Salmonella spp. can reach to aquatic ecosystem through fecal contamination (Lotfy *et al.*, 2011; Musefiu *et al.*, 2011).

In present study, salmonellae were isolated from fish ponds water with a rate of 2.9%. This percent is much lower than that mentioned by Hatha and Lakshmanaperumalsamy (1997), who reported Salmonellae from freshwater lakes with a rate 31%. So we considered that rate of salmonellae isolation from water fish ponds are more or less identical with other worker, and this may be attributed to the original source for pond contamination

In the present study, salmonellae were isolated from fecal swabs of integrated waterfowl (ducks) with a rate of 4% (8 out of 200 pooled samples).This percent is lower than that reported by Orji *et al.* (2005), who reported *Salmonella* from dropping with a rate of 38.3%, Mondal *et al.* (2008), who reported *Salmonella* from fecal swabs from ducks with a rate 13.07 % but it is higher than that reported by Hegazy (1991), who isolated *Salmonella* from fecal swabs of ducks and duckling with incidence 0.98% and 0.72% respectively. This variation in the rate of isolation may be due to the fact that shedding of *Salmonella* in faeces occurs intermittently (Williams and Whitemore, 1976).

Liver showed *Salmonella* isolation with an incidence 8.3%. Many author reported isolation of *Salmonella* from liver with more or less identical incidence as Levine and graham (1942) succeeded to isolate *Salmonella* from the liver but not from the heart blood of 10 weeks old wood ducks, Badr *et al.* (2015) reported four *Salmonella* isolates from internal organs of ducks with percentage 6.45%.

A total of 19 Salmonella isolates were recorded in this study, among the isolated serovars, *S. kentucky*, *S. enteritidis* and *S. uganda*, are zoonotic serovars that were associated with several cases of human food poisoning worldwide Westrell *et al.* (2014).

In this study, *E. coli* was isolated from poultry litter with a rate 18% (9 isolates out of 50 litter samples examined). This rate is matched with that reported by Cookey and Otokunefor (2016), who reported *E. coli* from poultry litter with a rate 20.5%. But it is lower than that reported by Islam *et al.* (2014), where they reported *E. coli* in litter samples with a rate 87.5 % (21 isolates from 24 litter samples).

In present study, *E. coli* was isolated from fish ponds water with a rate 23.2% (16 isolates from 69 water samples). This rate is nearly similar to that reported by Njoku *et al.* (2015), where they reported *E. coli* from fish ponds water with a rate 20.7%. This rate is higher than that reported by Barbosa *et al.* (2013).

Table 7. Identified *Staphylococcus* by PCR

Types of samples	Number of samples	No. of *Staphylococcus spp.* isolates	Incidence	No. of *S. aureus* isolates
Litter samples	50	7	14%	2
Water samples	69	3	4.34%	1
Fecal swab samples	200 Pooled samples	5	2.5%	1
Internal organs samples	60	2	3.33%	0
Total	379	17	4.48%	4

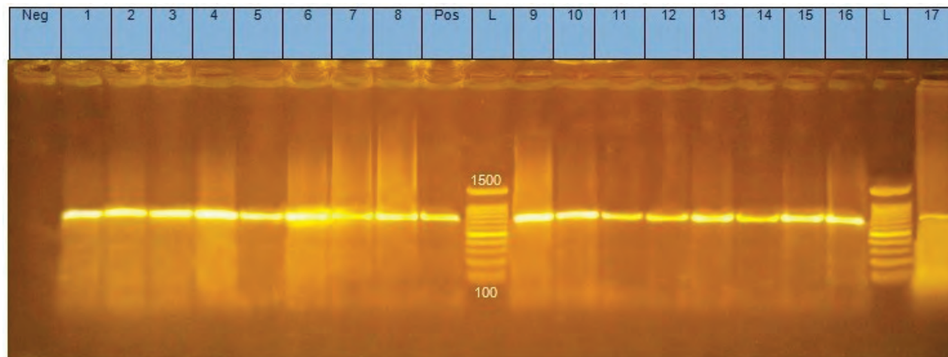

Fig. 1. Detection of 16srRNA for detection of *Staphylococcus* spp.

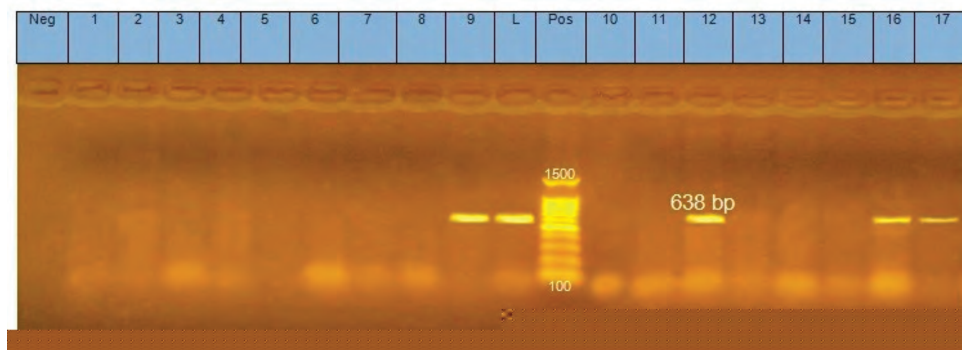

Fig. 2. Detection of clumping factor A for detection of *Staphylococcus* aureus.

They reported *E. coli* with rate 16.5% from fish pond water. The presence of *E. coli* in fish ponds water were an indication of the pond water contamination with fecal materials which may result to the presence of pathogenic organisms in fish and integrated water fowls.

In the present study, *E. coli* was isolated from fecal swabs of integrated ducks with a rate 11% (22 isolates from 200 pooled fecal swab samples). This rate appears to be lower than that reported with Ewers *et al*. (2003), where they reported *E. coli* from fecal swabs in mallard ducks with a rate 82.4% (142 isolates from 175 tested samples), Fallacara *et al*. (2004), where they reported *E. coli* from ducks by a rate 67% (300 isolates out of 450 tested samples), Adzitey *et al*. (2012) where they reported *E. coli* from duck feces with a rate 87.93% (51 out 58), Kim *et al*. (2016), where they reported *E. coli* from ducks by a rate 91% (364 isolates out of 400 fecal samples). However, it is higher than that reported with Sato and Asagi (1979), where they reported a low incidence of Enterobacteriaceae in wild ducks with a rate 6.8% from intestinal contents of ducks.

In this study, *E. coli* was isolated from liver of integrated ducks with a rate 20% (12 isolates out of 60 tested samples). This rate appears to be higher than that reported with Roshdy *et al*. (2012), where they reported *E. coli* from liver of one day old ducks and one week old ducks with a rate 12.4% and 11.4% respectively.

In present study, *Staphylococcus* spp. were isolated from poultry litter with a rate 14% (9 isolates out of 50 tested samples). This study is matched with this reported by Lu *et al*. (2003), where they reported *Staphylococcus* with a rate 13% from poultry litter. This rate is lower than that reported by Dhanarani *et al*. (2009) where they found that *Staphylococcus* made up 29.1% of the total bacteria present in poultry litter, Cookey and Otokunefor (2016), who reported *S. aureus* from poultry litter with a rate 18.6% and Alexander *et al*. (1968) where they reported *Staphylococcus* from poultry litter with a rate 100% (44 isolates from 44 samples).

In this study, *Staphylococcus* spp. were isolated from fish ponds water with a rate 4.4% (3 out of 69 water samples). This rate is lower than that reported by Newaj-Fyzul *et al*. (2008). They reported *Staphylococcus* from fish pond water with a rate 60% (6 isolates from 15 fish water samples). Also it is lower than that reported by Njoku *et al*. (2015), where they reported *Staphylococcus* spp. from fish ponds water with a rate 13.5%.

In our study, staphylococcus was isolated from fecal swabs of integrated ducks with a rate 2.5% (5 staphylococcus isolates from 200 pooled fecal swabs). This rate is lower than that reported with Ali *et al*. (2017). They reported Staphylococcus spp. from cloacal samples with a rate 86.67% (26 out of 30). It is also lower than that reported by Adegunloye and Adejumo (2014) where they reported *Staphylococcus* from fecal swabs of ducks with a rate 11.2%.

Staphylococcus was isolated from liver of integrated water fowls by a rate 3% (2 isolates from 60 liver samples). The literature that discussed staphylococcus isolation from ducks' liver

is little, however it is lower than that reported by Ruzauskas *et al*. (2016), who reported *Staphylococcus* from poultry liver with a rate 96.7 % (116 out of 120 tested samples).

Conclusion

The results of the present work showed that the integration play an important role in transmission of some bacterial pathogens as *Salmonella*, *E. coli* and *S. aureus* and these organisms are potentially to have a socio-economic impact. So, it is recommended to find a suitable way to disinfect the poultry litter before its usage in integration plant. Continuous evaluation of integrated poultry farms for pathogens before it becomes a part in this chain.

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