Alexandria Journal of Veterinary Sciences 2016, Apr. 49 (2): 163-169
ISSN 1110-2047, www.alexjvs.com
DOI: 10.5455/ajvs.205876

Mycoplasma Synoviae and other Associated Bacteria Causing Arthritis in Chicken

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Key words: Mycoplasma synoviae, other associated bacteria ,Virulance genes , PCR ,Arthritis and Chickens

ABSTRACT

Arthritis in broiler chickens is one of economic problems causing losses in poultry industry**.** Hundred samples (60 samples for Mycoplasma synoviae isolation and 40 samples for isolation other bacteria) from chicken with arthritis symptoms, were collected from different broiler chickens farms. The samples were cultivated on PPIO media for isolation of Mycoplasma synoviae and different media(MaCconky , EMB, X.L.D and salt Mannitol media) for isolation of other bacteria and biochemically identified. The results revealed that out of 40 samples 28(70%) were positive for E. coli isolation,10 (25%) were positive for Staphylococcus aureus isolation,2(5%) were positive for Salmonella Enterica isolation. Out of 6osamples 6(10%) were positive for Mycoplasma synoviae isolation. Amplification of vlhA gene of Mycoplasma synoviae showed that out of 6 isolate 3 (50%) were positive for the gene . Six seogroups of E. coli isolates examined serologically and put into six group, one each group (O128, O125, O146,O27,O114 and O158).The result of multiplex PCR for E.coli virulence genes(ibeA and iss) showed that iss were detected in all serogroups .While ibeA virulence gene was detected in serotypes O125 and O146 only .The result of PCR for amplification of virulance genes of S.aureus(CNA,clfA)reveled that out of 8 isolate of S.aureus,3(37.5%) were positive for clfA. Out of 3 positive S.aureus for clfA,3(100%) were positive for CNA.

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1. INTRODUCTION

Bacterial arthritis in poultry commonly occurred after septicemia or localized infection to the joints is reported to be associated with many bacterial agents including Erysipelothrix, Listeria, Mycoplasma, Staphylococcus, and Escherichia (Mohan et al., 2002).

Arthiritis is most commonly caused by Staphylococcus aureus (McNamee and Smyth, 2000), ,and sometimes involved Escherichia coli (Chansiripornchai, 2009)which is of veterinary importance in broiler breeders.

Mycoplasma synoviae causes a respiratory disorder and infectious synovitis in chicken especially when *Mycoplasma synoviae* combines with other respiratory virus infection, causing significant drop in egg production beside condamination of carcasses due to accumulation of the viscous creamy to grey exudates involving synovial membranes of the tendon sheath, joint,keel bursa and extend even to muscles and air sacs (Kleven, 1997; Ley et al., 2003).

Isolation and identification of *Mycoplasma synoviae* isolates is of critical importance.First isolation of *Ms* was from synovial sheaths and joints from commercial chicken (Morrow et al., 1990) also it was isolated from tracheal swabs as (Bradbury et al., 2001; Poveda et al., 1990; Wissman and Parsons, 1996).

For the first time in Iran, the primers complementary to the single-copy conserved 5' end of *vlhA*gene were used for detection of *MS* by PCR. Results obtained from serology, isolation, and PCR using primers related to 16s rRNA and *vlhA* genes were analyzed and compared. PCR results, in addition to identification of *Mycoplasma*spp, revealed variable sizes of 350-400 bp among standard strain, vaccine strains, and Iranian field isolates. The findings of this study demonstrated that the *vlhA* gene-targeted PCR is a sensitive and specific test for detection of *M. synoviae*, and an efficient tool for primary typing of its different strains(Ghafouri et al., 2011).

E.coli localization in bones and synovial tissue is a common sequeal of colisepticimea affecting birds

likely have in sufficient resistance to compeletely clear bacteria (Huff et al., 2000). E.coli could be colonized in the vesicular sprouts that invade the physis of growing bone provoking an inflammatory condition ,that resulting in osteomylities (Saif et al., 2011).The ibeA gene plays role in the pathogenesis of colibacillosis through invasion assays. After the deletion of the ibeA gene, these authors noted a decrease in virulence(Germon et al., 2005).Increase serum survival gene is the most important and widely distributed virulence marker ofAPEC (Ammar et al., 2011).

Staphylococcus aureus is the most common cause of bacterial arthritis in broiler breeder chickens .They recorded 51.1% mortality in broilers with lesion of swollen joints ,gaseous exudates, cartilage injury, and synovial membrane thickening with infiltration of inflammatory cells(Gu et al., 2013).

The importance of the fibrinogen-bindin adhesin clumping factor A (clfA) in the pathogenesis of Staphylococcus aureus septic arthritis was examined in an animal model(Josefsson et al., 2001).

the role of the collagen-binding function of CNA was examined in a mouse model of septic arthritis by comparing the virulence of isogenic strains of S. aureus expressing (1) wild-type CNA, (2) a truncated form of CNA (CNA35) with a higher affinity for collagen than the wild type, (3) CNA35 containing a single point mutation resulting in loss of collagen binding, (4) CNA lacking the collagen-binding domain, and (5) the collagenbinding domain of ACE (adhesin of collagen from Enterococcus faecalis). Results and conclusions. The results provide, for the first time, direct evidence that the virulence of CAN depends on its collagen-binding ability. Collagen binding facilitated early colonization of the joints of mice(Xu et al., 2004).
The aim of this study was planned to identify Mycoplasma synovia and other associated bacteria causing arthritis in chickens as well as detection of some virulence genes of isolated bacteria.

2. MATERIALS AND METHODS

2.1. Sampling:

Atotal of 100samples (60 samples for Mycoplasma synoviae isolation and 40 samples for isolation other bacteria) from chicken with arthritis symptoms.

2.2. **Isolation and identification of Mycoplasma synoviae:**

A total of 60 collected swabs were inoculated into sealed sterilized tubes containing 2-3 ml of Frey's broth medium then incubated at 37Cfor 3-5 days and examined daily for acidity indicated by change of the color of medium from red to yellowish-orange (Kleven, 1997). The positive inoculated tubes were cultured on Frey's agar medium for 2-3 win sealed container supplied with damp cotton for increasing Humidity and lighted candle for C02 production and O2 exhaustion in the container atmosphere , the incubated plates were examined daily for the growth of colonies by dissecting microscope to demonstrate the characteristic colony.

2.3. Isolation and identification of other bacteria:

Atotal of 40 Bacteriological swabs and synovial fluid were inoculated into tryptic soya broth and incubated at 37C for 18hr then subcultured into 5% blood agar base, nutrient agar , Eosine Methylene agar ,X.L.D , Mannitol salt agar, MacConkey agar and incubated at 37 C for 24-48hr .suspected colony from different media were picked up and subjected for morphological and biochemical identification (Quinn et al., 2011).

Serological identification of E.coli: The serological identification was done at the department of serological unit, Anima health research institute .Cairo. Using available polyvalent and monovalent *E. coli* antisera.

2.4. Molecular identification of isolated bacteria causing arthirtis:

1-DNA extraction:

a.Mycolasma synoviae: DNA was extracted from Mycoplasma Broth using QIAamp DNA Mini Kit.according to the manufacturer instructions.

b.Staphylococcus .aureus and E.coli: Extraction of DNA by boiling method ***(Sambrook and Russell, 2001)***

2- The conventional PCR primers were used in this study showed in this table (1, 2, 3)

Table (1): Oligonucleotide primers encoding for Mycoplasma synoviae :

Primer	Target gene	Primer sequence (5'-3')	Length of amplified product (bp)	Reference
VlhA-F	*vlhA*	TACTATTAGCAGCTAG TGC	350-400 bp	(Jeffery et al., 2007)
VlhA-R		AGTAACCGATCCGCTTAAT		

Table (2): Oligonucleotide primers encoding for *Staphylococcus aureus*

Primer	Target gene	Primer sequence (5'-3')	Length of amplified product (bp)	Reference
clfA-F	*clfA*	GCAAAATCCAGCACAACAGGAAACGA	638 bp	(Mason et al., 2001)
clfA-R		CTTGATCTCCAGCCATAATTGGTGG		
Cna-F	*Can*	AAAGCGTTGCCTAGTGGAGA	192 bp	(Montanaro et al., 1999)
Cna-R		AGTGCCTTCCCAAACCTTTT		

Table (3): Oligonucleotide primers encoding for E.coli:

Primer	Target gene	Primer sequence (5'-3')	Length of amplified product (bp)	Reference
phO	*phO*	CGATTCTGGAAATGGCAAAAG	761	(Hu et al., 2011)
phO		CGTGATCAGCGGTGACTATGAC		
Iss	Iss	ATGTTATTTTCTGCCGCTCTG	266	(JanBen et al,.2001)
Iss		CTATTGTGAGCAATATACCC		
ibeA	*ibeA*	AGG CAG GTG TGC GCC GCG TAC	171	(Johnson and Stell, 2000)
ibeA		TGG TGC TCC GGC AAA CCA TGC		

3-Preparation of PCR Master Mix for

- **a.** Simple PCR:according to Emerald Amp GT PCR mastermix (Takara) Code No. RR310Akit as shown in table (4):
- **b.** b-Multiplex PCR protocol for ibeA and iss genes:
- **c.** According to Emerald Amp GT PCR mastermix (Takara) Code No. RR310Akit as shown in table (5).
- **d.** 4-Cycling conditions of the primers during cPCR :
- **e.** Temperature and time conditions of the primers during PCR are shown in Table (6)

Table (4):component of PCR reaction of simple PCR

Component	Volume/reaction
Emerald Amp GT PCR mastermix (2x premix)	12.5 *µl*
PCR grade water	4.5 *µl*
Forward primer(20 pmol)	1 *µl*
Reverse primer (20 pmol)	1 *µl*
Template DNA	6 *µl*
Total	25 *µl*

Table(5):component of multiplex PCR reaction for gene (ibeA,iss)

Component	Volume/reaction
Emerald Amp GT PCR mastermix (2x premix)	20µl
PCR grade water	2µl
Forward primer (20 pmol) each primer	2µl each
Reverse primer (20 pmol) each primer	2 µl each
Template DNA	14 µl
Total	40µl

Table (6):Different PCR condition of different primers

Gene	Intial denaturation	denaturation	Annealing	Extension	No. of cycles	Final extension
vlhA	94˚C	94˚C	54˚C	72˚C	35	72˚C
	5 min.	30 sec.	45 sec	45 sec		10 min.
clfA	94˚C	94˚C	55˚C	72˚C	35	72˚C
	5 min.	1min.	1min	1min		10 min.
CAN	94˚C	94˚C	55˚C	72˚C	35	72˚C
	5 min	1min	1min	1min		10 min
phO	94˚C	94˚C	58˚C	72˚C	35	72˚C
	5 min.	30 sec.	45 sec	45 sec		10 min
ibeA and ISS	94˚C	94˚C	54˚C	72˚C	35	72˚C
	5 min.	30 sec.	45 sec	45 sec		10 min.

5-DNA Molecular weight marker:

The ladder was mixed gently by pipetting up and down. Six µl of the required ladder were directly loaded.

6-Agarose gel electrophoreses (Sambrook and Russell, 2001)

3. RESULT AND DISCUSSION

In Egypt the swollen arthritic joint in broiler is a matter of economic losses as leads to inability of the chicks to obtain food and water resulting in loss of weight and even mortalities .The annual losses due to skeletal problems in united states were 80$to120$million dollar (Sullivan, 1994) .so the aim of this study to identify bacteria causing arthritis.

In this study *Mycoplasma synoviae* was isolated from arthritic samples at a percentage of 10% .although *Mycoplasma synoviae* could not be isolated by (Bkheet, 2011),who attributed this result to the septic arthritis and pyogenic organisms that present in the joint which may inhibit the Mycoplasma localization within joints and therapeutic may lead to these result ,on the other hand some outhers could detect 17.8% incidence of Mycoplasma as a caustive agent of arthritis in chicken (Abd El-naser et al., 1994).Lower incidence was recorded 4.04% for MG and .8% for Ms (Shaker, 1995).The higher incidence of *Mycoplasma synoviae* in this study was due to the high susceptibility of chicken at young age.

The results of identification of other bacteria isolated from examined arthritic samples cleared that , the most commonly isolated bacteria were E. coli, *Staphylococus aureus, Salmonella enterica* which were isolated at a percentage of 26%, 13%, and 3.3%, respectively .The obtained results were higher than that described by (Mamza et al., 2010), who isolate *E.coli* and *Staphylococcus aureus* from hock joint and digital pad samples of chicken were isolated at a percentage of 4.2% ,8.1% from hock ioint and 14.3%,10.7% from digital pad respectively. These results were lower than that reported by (Rasheed, 2011), who isolate *Staphylococcus aureus*, *E.coli*, at a percentage of 50.98% and 7.8% respectively .This disagreement may be due to joint samples did not have any injuries or wounds , tightly close and opened under sterile condition so that the percentage of E.coli more than *S.aureus* and may be due to breed , age and environment.

Table (7): Results of typing bacteria associated with *Mycoplasma synoviae* isolated from arthritic samples:

Isolated bacteria	No of the samples	Isolated bacteria	
		No	%
Mycoplasma synoviae	60	6	10
E.coli		28	70%
S.aureus	40	10	25%
Salmonella enteriditis		2	5%

Table (8): Results of serotyping of *E. coli* isolate :

Animal species	Organism	Antigenic formula	No of isolate
	E. coli	+ve poly 2 O:125	1
	E. coli	+ ve poly 3 O:158	1
	E. coli	+ ve poly 3 O:114	1
Chickens	*E. coli*	+ ve poly 2 O:146	1
	E. coli	+ve poly 4 O:27	1

in this study serotyping of six E.coli isolated from arthritic samples of chicken indicated that the E.coli isolates were O114,O146,O128,O111,O27,O158 and O125.the serotype O125and O146 nearly similar to the serotype obtained by (Abd El Tawab et al., 20014).

Results of amplification of *Mycoplasma synoviae* (vlhA) coding gene by using PCR: six isolates of Postive Ms broth culture weretested for vlhA using PCR technique. The specifity of the primers was confirmed by positive amplification of fragment with the extracted DNA as shown in figure(1). Out of 6 tested isolates, Three isolates (50%) were positive for the vlhA (figure1).

Results of amplification of *E.coli* (phoA) coding gene by using PCR: **:**Sixteen isolates of biochemically identified *E.coli* were randomly studied for detection of phoA gene using PCR technique. The specifity of the primers was confirmed by positive amplification of fragment with the extracted DNA of the bacterial isolate as shown in (figure 2). Out of 16 tested isolates, Six isolates (37.5%) were positive for the phoA gene (figure 2).. The PCR assay yielded amplified products of 720 bp specific for (phoA) gene;

Results of amplification of E.coli virulence genes by multiplex PCR (iss,,ibeA): Iss and ibeA genes were detected in 6 pathogenic *E. coli* photo(3): Six isolated E.coli used for detection of virulence genes (iss,ibeA)which revealed that All 6(100%) isolate were positive for iss gene while 2 (33,3%) isolate were positive for ibeA

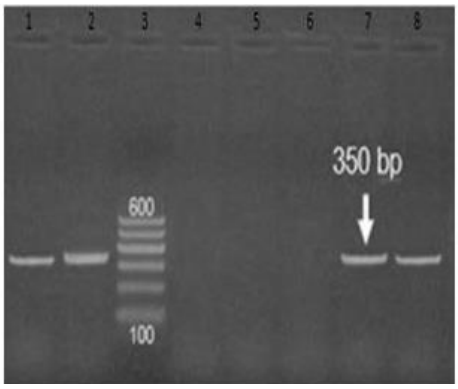

Figure (1): Electrophoretic analysis of PCR amplified DNA of vlhA gene of Mycoplasma synoviae Lane (3):DNA molecular weight ladder 50pb. Lane(2):Control positive**.** Lane (1, 7 and 8): Positive results for vlhA gene. Lane (4,5 and 6): Negative results for vlhA gene

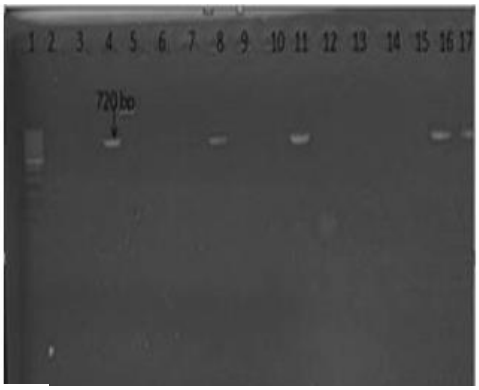

Figure (2): Electrophoretic analysis of PCR amplified DNA of (phoA) gene . Lane (1): DNA molecular weight ladder. Lane (3, 6 ,7,10,15 and 16): Positive results for phOoAgene. The PCR assay yielded amplified products of 720 bp specific for (phoA) gene

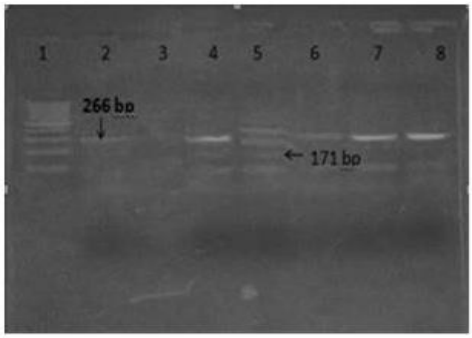

Figure (3): Electrophoretic analysis of PCR amplified DNA of Iss and ibeA genes. Lane (1) : DNA molecular weight ladder. Lane (3) : Control negative.Lane 2 ,4, 5 ,6 ,7 and Lane 8: Positive for Iss gene .

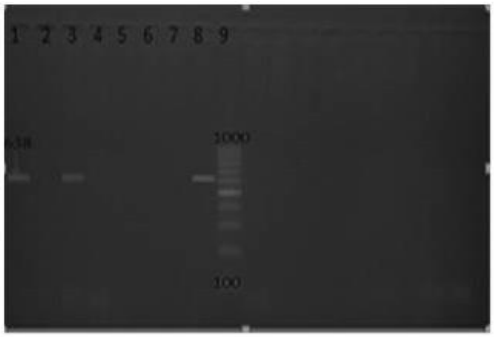

Figure (4): Agarose gel electrophorsesis patterns showing PCR amplification products for the *S. aureus* clfA coding gene . Lane 9, DNA molecular size marker (100 bp ladder) lane 1,3,8 positive for clfA coding gene showing amplification. The PCR assay yielded amplified products of 638 bp specific for (clfA) gene;

The obtained results showed that presence of iss gene in E.coli isolated from arthiritis with apercentage of 100% agree with the result mentioned by(Abd El Tawab et al., 20014),who recorded that PCR for amplification of iss gene of E. coli was 6(100%) of 6 APEC isolatesand higher than the result that obtained by (Jeong et al., 2012)who mentioned that PCR for amplification of iss gene of E. coli was (78.2%) of APEC isolates..This study detected presence of IbeA gene in E.coli isolated from arthritis with a percentage of 33.3%.this result is similar to the result that obtained by (Cunha et al., 2014), who said that percent of PCR for amplification of IbeA gene of E. coli was (31%) of APEC isolates. and higher than the result obtained by (Wang et al., 2010) who identified ibeA gene with a percentage (10.6%)by using PCR.

Results of amplification of *S. aureus* clumping factor A (clfA) coding gene by using PCR which Eight isolates of biochemically identified *S. aureus* were randomly studied for detection of clumping factor gene using PCR technique. Out of 8 tested isolates, three (37.5.%) were positive The specifity of the primers was confirmed by positive amplification of fragment with the extracted DNA of the bacterial isolate as shown in figure (4)

In this study ,the percentage of clfA gene was 37.5% which is lower than the result obtained by (Contreras et al., 2012) who found that clfA gene with percentage of 100%..

Results of amplification of *S. aureus* collagen adhesin (cna) coding gene by using PCR:
Collagen adhesin (cna) genes detected in 6 pathogenic *S.aureus* isolated from Arthiritic samples (figure5).
In this study ,the percentage of cna gene was 100% , this result confirm (Xu et al., 2004)who said that cna is avirulance factor in septic arthritis . this result is higher than obtained from (Arciola et al., 2005),who found that cna gene with percentage of 46% and (Contreras et al., 2012) who found that cna gene with percentage of 78.1%

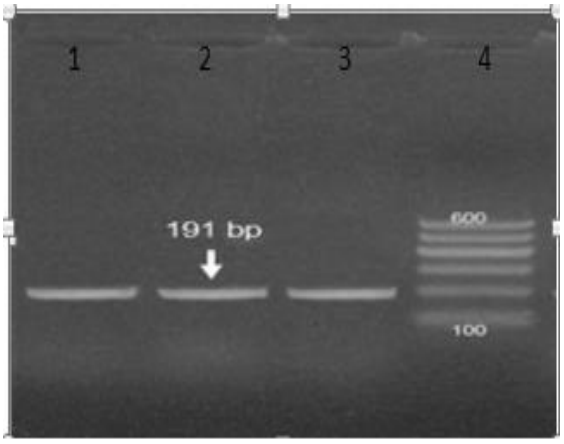

Figure (5): Agarose gel electrophoresis patterns showing PCR amplification products for the *S. aureus* collagen adhesion (cna) coding gene.
Lane 4, DNA molecular size marker (100 bp ladder),
lane 1,2,3 was positive for coding gene showing amplification,lane 5 control positive. The PCR assay yielded amplified products of 191 bp specific for (cna) gene**.**

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