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Preliminary Study on Bacterial Strains Used in the Preparation of Polyvalent Inactivated Vaccine Against Chronic Respiratory Disease in Chickens

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Abstract: The target of this study is to perform the identity tests on certain bacterial strains prior to its usage in the preparation of local polyvalent inactivated vaccine in a trial to protect chickens against the chronic respiratory disease (CRD). These strains include *Mycoplasma gallisepticum* and *Escherichia coli* strains. All of these strains subjected to bacteriological, biochemical and serological identifications in addition to the molecular confirmation. Results of these identity tests ensured that the will be used bacterial strains are *Mycoplasma gallisepticum* S6 strain and the *E. coli* serotypes are *E. coli* O1, *E. coli* O2 and *E. coli* O78. Polymerase chain reaction applied on the same strains for each confirmed these results as the obtained size of PCR product was 185 bp with the *M. gallisepticum* S6 strain when using the specific primers of the 16SrRNA gene of *M. gallisepticum* and were 323 bp with the different *E. coli* serotypes when using the specific primers of STX gene.

Key words: *M. gallisepticum* · *E. coli* · CRD · Vaccine · Identity

INTRODUCTION

Mycoplasmosis is one of the most important poultry diseases and causes significant economic losses either directly or indirectly and it is caused by *Mycoplasma gallisepticum* nfection with or without complicating factors [1]. As poultry industry developed, almost the chickens are grown in crowded manner and with low air condition houses. In such situation many of the flocks which infected by *M. gallisepticum* become predisposed or their disease condition have been aggravated and the chronic respiratory disease (CRD) complex occurs [2].

E. coli infections are responsible for great economic losses in the poultry industry worldwide, bringing serious threat to the poultry industry. Avian pathogenic *E. coli* (APEC) strains are most commonly associated with extra intestinal diseases, mainly respiratory or systemic infections. Among the extra intestinal diseases caused by APEC, colisepticaemia is considered to be one of the most important poultry disease [3].

Colibacillosis in poultry can be either localized or a systemic infection [4] and is characterized by a diverse array of lesions, although airsacculitis, perihepatitis and pericarditis predominate [5]. The causal agents most commonly isolated in such disease syndrome are serovars O1:K1, O2:K1 and O78:K80, which are identified in up to 61 % of tested isolates [6].

This study aimed to ensure, identify and characterize the different bacterial strains that used in the preparation of combined inactivated vaccine for the protection of chicken against chronic respiratory disease (CRD) either Mycoplasma or *E. coli* strains.

MATERIALS AND METHODS

Bacterial Strains: Both Mycoplasma gallisepticum S6 strain and *Escherichia coli* O1, $O_{2,}$ O_{78} strains were supplemented by CLEVB and subjected to bacteriological, biochemical and molecular identification. These strains were used latterly for the preparation of the local polyclonal E. coli and Mycoplasma vaccine.

Identity of *M. gallisepticum* Strain

Purification: The used *M. gallisepticum* S6 strains inoculated onto Mycoplasma agar then incubated at 37°C with humidity and 5% CO_2 and checked for colonies using stereoscopic microscope within 2 weeks post inoculation. If the characteristic Mycoplasma colonies (Fried egg).

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appeared, agar blocks containing Mycoplasma colonies were transferred into broth medium and incubated under the same conditions 2-3 days. When the colour of phenol red changed to orange. The culture was subjected to biochemical confirmation and PCR.

Biochemical Identification: The tested *M. gallisepticum* strain was tested for Digitonin sensitivity [7], Glucose fermentation test: [8], Arginine deamination test [8] and Tetrazolium reduction test [7].

Extraction of Genomic DNA of *M. gallisepticum* strains [9]: A 10 ml of overnight culture from Mycoplasma strain was centrifuged at 14,000 g for 30 minutes. The cell pellets were washed twice in 100 ul of 150 mM PBS (pH 7.2) and suspended in 25 µl PBS. The cell suspension was heated directly at 100°C for 10 minutes and cooled on ice for 10 minutes. Finally, the cell suspension was centrifuged at 14,000 g for 5 minutes chromosomal DNA was collected and stored at 4°C.

PCR

Oligonucleotide Primers for Detection of Mycoplasma [9]: The primers sequences were chosen for the 16srRNA gene:

Forward Primer: 5-GAG CTA ATC TGT AAA GTT GGT C-3

Reverse Primer: 5-GCT TCC TTG CGG TTA GCA AC-3

PCR Amplification of 16srRNA Gene [9]: The PCR reaction mix was prepared from 5ul of DNA template added to 25 ul of master mix, 1 ul of each primer and 18 ul of DDW. The DNA amplification was performed in a DNA thermal cycler under the following condition: 94°C for 3 minutes for one cycle followed by40 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 60 sec.

Agarose Gel Electrophoresis [10]: PCR product was analyzed by an agarose gel electrophoresis. The samples were electrophoresed at a constant 100 volts for 1 hour, visualized under ultraviolet light and photographed.

Identity of *E. coli* Strains Used in Vaccine Preparation

Colonial Morphology of *E. coli* Strains [11]: A loopfull from the selected culture was inoculated into tryptose broth and incubated at 37°C for 24 hours Then streaked onto the surface of MacConkey agar and incubated at 37°C for 24 hours. A separate colony was picked up and stained by Gram's stain then examined microscopically for detection of Gram negative microorganism [12].

Biochemical Identification [13]: Pure culture of each strain was identified biochemically by using API 20E identification system following the kit manual instructions.

Serological Identification of *E. coli*: The selected *E. coli* strains were subjected to serological identification by slide agglutination test according to Edward and Ewing [14] using standard polyvalent and monovalent *E. coli* antisera.

Extraction of DNA from *E. coli* Strains: Following the instruction of the used DNA extraction kit [Isolate Genomic DNA mini Kit (Bioline, Cat. No. BIO-52032)].

Detection of Virulence Genes among *E. coli* Strains Using PCR

Oligonucleotide Primers [15]: The primers sequences were chosen for the STX gene.

Forward Primer: 5- GAG CGA AAT AAT TTA TAT GT-3.

Reverse Primer: 5- CGA AAT CCC CTC TGT ATT TGC C -3.

PCR Amplification of STX Gene: The PCR mix and cyclic conditions applied as described by Read *et al.* [15]. PCR reaction mixture was consisted of 4µl DNA template, 25µl Master mix, 1 µl Forward primer (25pmol), 1µl Reverse primer (25pmol) and 19 µl Double distilled water. The mixture was placed in the thermal cycler and was programmed as follows: 94°C for 3 minutes for one cycle followed by35 cycles of 94°C for1 min., 55°C for 1 min. and 72°C for 1 min.. The amplified and kb DNA marker were run on 1% Agarose gel.

RESULTS AND DISCUSSION

Identity and confirmatory processes should be applied on the master seed of any vaccine prior to the preparation of the target vaccine. A combined vaccine against chronic respiratory disease including *M. gallisepticum* and *E. coli* pathogens are the target of the second part of this study.

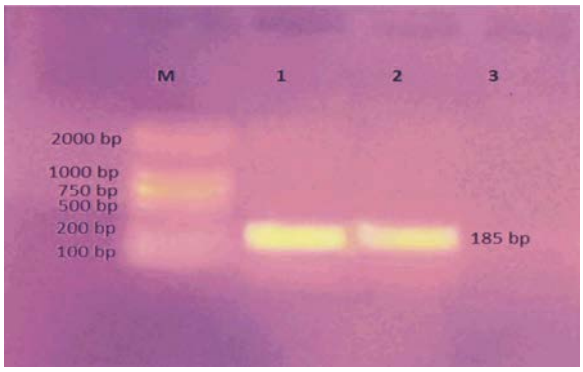

Fig. 1: Agarose gel electrophoresis of 16-S gene PCR product from different strains of *M. gallisepticum*
Lane M:100 bp DNA Marker, Lane 1: *M. gallisepticum* strain Lane 2 Positive *M. gallisepticum* control and Lane 3 Negative control.

Identity of *M. Gallisepticum* strain used in the targeted vaccine preparation was achieved through firstly, the Colonial Morphology where the selected strain of *M. gallisepticum* (S6) showed a characteristic fried egg appearance on the mycoplasma agar and clear growth with change in colour of phenol red indicator to the orange colour on the mycoplasma broth media.

The Biochemical characterization of *M. gallisepticum* culture after filtration through 0.45 um Millipore filters induced glucose fermentation, digitonin sensitivity, argenine deamination and tetrazolium reduction. Enro and Stipkovits [7] reported the capability of *M. gallisepticum* to ferment glucose and based the evidence of investigation on the colour change of phenol red. Also, Woods and Smith [16] recorded the capability of mycoplasma colonies in reducing tetrazolium and development of characteristic colour within one hour. Moreover, Enro and Stipkovits [7] stated that *M. gallisepticum* showed the ability to reduce 2, 3, 5 triphenyl tetrazolium chloride to its brick red form azone in broth culture. In addition, Tully [17] recorded that *M. gallisepticum* showed sensitivity to a paper disc saturated with 1.5% digitonin and the zone of growth inhibition disc indicated the sensitivity to digitonin.

As regards to molecular identification using the PCR *M. gallisepticum* strain were identified and confirmed using a specific primers of the 16 SrRNA gene which was successfully amplified giving rise of a PCR products at the molecular size of 185pb which corresponded to the predicated size as shown in Photo (1). PCR finding was highly confirmatory to that obtained by the biochemical reactions. These results the as described in OIE [9].

E. coli stains strains used in the combined vaccine preparation were adopted through several steps starting with bacyteriological and morphological characterization where the examined *E. coli* strains were Gram negative non-spore forming straight rods and non-motile. It grew onto MacConkey agar media producing rounded, non-mucoid pink colonies (Lactose fermenter) on the surface of the medium.

Concerning the biochemical identification depending on the results of API 20E identification system, the three *E. coli* strains were classified into 2 groups, the first group gave positive reaction with ONPG, LDC, ODC, IND, GLU, MAN, SOR, RHA, SAC, MEL and ARA tests and negative reaction with ADH, CIT, H2S, URE, TDA, VP, GEL, INO, AMY and OX tests as shown in photo (2, A) meanwhile the second group gave positive reaction with ONPG, ADH, LDC, ODC, IND, GLU, MAN, SOR, RHA, SAC, MEL and ARA tests and negative reaction with CIT, H2S, URE, TDA, VP, GEL, INO, AMY and OX tests as shown in photo (2, B). The same finding was mentioned by Kwon *et al.* [18] identified *E. coli* isolates by screening biochemical traits using API 20E identification system.

The Serological identification of *E. coli* strains using polyvalent antisera ensured that, these strains were belonging to belonging to 3 serogroups. These are Polyvalent (1):[O1, O26, O86, O111, O119, O127, O128], Polyvalent (2): [O2, O11, O87, O127, O142] and Polyvalent (3): [O6, O27, O78, O148, O159, O168].The second step of sero-identification was done by using *E. coli* monovalent antisera, it was identified as O1, O2 and O78. These results were confirmed by Salama *et al.* [19] who recovered 5 different *E. coli* serotypes identified as O1, O2, O6, O78 and O126. Similar serotypes (O1, O2 and O78) were obtained by Ibrahim [20] and McPeake *et al.* [21].

Regarding PCR amplification of STX gene, the result of PCR amplification revealed that, the three tested serotypes (O1, O2 and O78) were positive with PCR product of 323 bp as shown in photo (3). This means that, these three serotypes are shiga toxin producing *E. coli* (STEC).

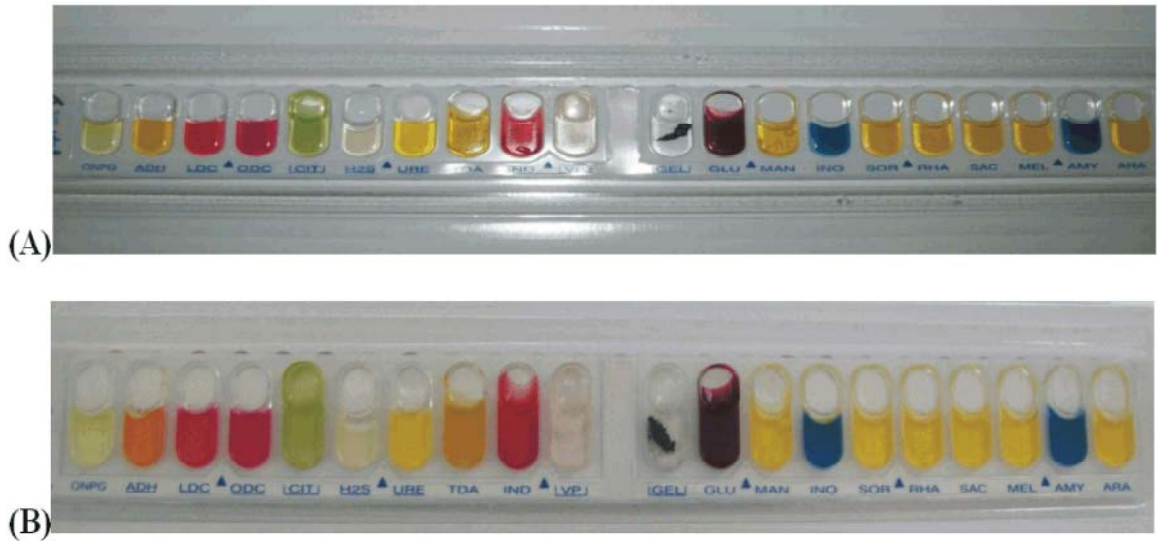

Fig. 2: Showed the API 20E identification system reactions

Fig. 2.A: Showed positive reaction with ONPG, LDC, ODC, IND, GLU, MAN, SOR, RHA, SAC, MEL and ARA tests and negative reaction with ADH, CIT, H2S, URE, TDA, VP, GEL, INO, AMY and OX tests.

Fig. 2.B: Positive reaction with ONPG, ADH, LDC, ODC, IND, GLU, MAN, SOR, RHA, SAC, MEL and ARA tests and negative reaction with CIT, H2S, URE, TDA, VP, GEL, INO, AMY and OX tests

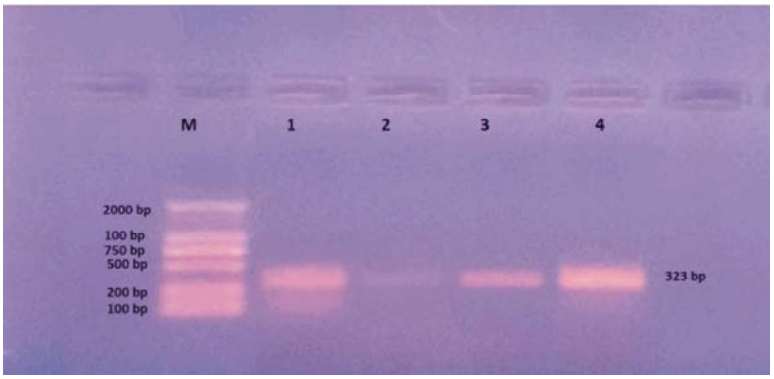

Fig. 3: Shows the PCR amplification of *STX* gene of *E. coli* serogroups:
M: DNA marker; lane 1, *E. coli* serogroup O1, lane2: *E. coli* serogroup O2, lane 3:*E. coli* serogroup O78, lane4: Positive *E. coli* control and lane 5: Negative control

The same results obtained by Parreira and Gyles [22] using the primers of the same gene. Also Tutenel *et al*. [23] examined 3625 meat samples, under the same conditions, only 451 samples were positive for *E. coli*. On the other hand Ghanbarpour *et al*. [13] studied the virulence genotyping of 64 *E. coli* isolates and the results revealed that all the examined isolates were negative for *stx1, stx2* and *eaeA* genes. In contrast to these results Parreira and Gyles [22] examined 34 samples from cellulites cases and the results showed that 5.8% (Two cases) were positive for *stx1* by PCR and 32% (11 cases) in colony hybridization assay.

From the above mentioned data it could be concluded that, the bacterial strains checked in this study are identically approved to be suitable to be used in the preparation of the targeted inactivated polyvalent vaccine to be used in the protection of chickens against chronic respiratory disease under the Egyptian conditions.

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