First record of *Bordetella avium* in Egyptian turkey flocks

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To screen *Bordetella avium* in Egyptian turkey flocks, tracheal swabs and nasal exudates were gathered from 21 turkey farms out of five Egyptian governorates. Bacteriological examination revealed the isolation of 2 strains. Both isolates displayed different resistance rates regarding the ten applied antibiotics. Both strains agglutinated guinea pig RBCs and adhered to the tracheal rings. Confirmatory recA gene PCR was performed. Moreover, blaTEM, tetA(A), aada1, sul1 and dfrA genes were amplified in both *B. avium* strains. Partial sequencing of the amplified 740 bp of recA gene revealed 100% maximum identity with German *B. avium* ATCC 35086 strain and with the American strain 197N. To the best of our knowledge, this is the 1st record of *B. avium* in Egyptian turkey flocks.

**Keywords:** *Bordetella; avium; PCR; sequence; resistance*

**INTRODUCTION**

*Bordetella avium* (BA), that cause turkey bordetellosis, (moreover called turkey coryza) is an exceptionally infectious upper respiratory pathogen that specially infects turkey poult leading to high morbidity and minimal mortality and is frequently followed by serious secondary infections that lead to great and terrible economic impacts for turkey industry. It hasn’t been estimated to be transmitted vertically. But, the survival of BA for at least 6 months in intact damp litter has been reported (Jackwood and Saif, 2008), which contributes to epidemiological situation of *Bordetella avium* infection in turkeys. A review in 2009 ranked *B. avium* as the third infectious pathogen in a list of the most threatening health issues for the American turkey industry as reported by poultry stakeholders (Clark et al., 2011). *B. avium* infection was described as a common infection among turkey flocks that is frequently reported during the whole production cycle and the probability of the past infection increases while birds get older (Śmiałek et al., 2015). The increased incidence of concurrent infections during turkey coryza outbreaks contributes to the poor overall performance of *B. avium*-infected flocks. Antibiotic treatment of turkey coryza usually fails; likely, such antibiotics treat secondary infections instead of treating *B. avium* infection. Four toxins are typically produced by *B. avium*: endotoxin, tracheal cytotoxin, heat-labile dermonecrotic toxin and osteotoxin (Rimler, 1985). Deformation of tracheal rings and damage to articular cartilages that are usually observed in field cases of *B. avium* infections may be related to osteotoxin activity (Yersin et al., 1991). *B. avium* adheres to tracheal epithelium to colonize the turkey’s breathing tract (Arp et al., 1988; Temple et al., 2010). Hemagglutinin produced by *B. avium* in addition to autotransporter proteins and fimbriae play the main role in this process (Moore et al.,...
1994; Loker et al., 2011; Stockwell et al., 2011). Interestingly, the fimbrial locus of \textit{B. avium} is regulated in response to temperature (37°C), and the bacterium is able to adhere to the host’s respiratory epithelium only under such conditions, this was proved by testing the expression of \textit{fimA-D} by real time PCR, when \textit{FimA} gene was only expressed at 37°C, however, expression failed at temperatures below 25°C (Loker et al., 2011). Even though \textit{B. avium} is thought to be strictly an animal pathogen that causes tracheobronchitis in birds, human cases of respiratory disorders related to \textit{B. avium} have been recorded lately in patients with cystic fibrosis (Spiker et al., 2008), therefore demonstrating that \textit{B. avium} and \textit{B. avium}-like organisms are opportunistic human pathogens.

Employing 16S rRNA gene sequencing, two isolates had been identified from human pneumonia that showed 98% and 100% nucleotide similarity to the \textit{B. avium} ATCC 35086 strain (Harrington et al., 2009).

A primary barrier to the improvement of advanced therapy against \textit{B. avium} infections is that very little is known about this bacterium or about the way by which it infects birds to cause disease. However, a major breakthrough in understanding the biology of \textit{B. avium} is rising, because the genome sequence of a representative strain of \textit{B. avium} was successfully generated and analyzed. This has discovered the complete genetic code of the bacterium (Sebaihia et al., 2006).

The target of the present work was the isolation, identification, molecular characterization and pathogenicity testing of \textit{B. avium} from turkey field cases suffering from respiratory troubles.

**MATERIALS AND METHODS**

**Collection of samples.**

Tracheal swabs and nasal exudates have been collected aseptically from 10 pools in each farm out of 21 turkey farms. Sampling was focused on birds showing respiratory manifestations. Sampled farms represented 5 Egyptian governorates that harbor substantial turkey breeding (Giza, Gharbeya, Menofeya, Sharkeya and Faiyum). Samples had been transported in a cooler to the laboratory within 1-3 h. After pooling of the samples (samples from each farm were pooled as one sample), they have been subjected to bacteriological and molecular identification.

**Isolation of \textit{B. avium}.**

Isolation of the microorganism from samples was performed following previously validated protocol (Dufour-zavala et al., 2008). The pooled samples were cultured in blood agar base with 5% of defibrinated sheep blood and in MacConkey agar at 37°C under aerobic conditions for 24-48 h. After the incubation duration, colonies displaying suggestive morphology (pinpoint, convex and glistening with a pearl to gray color) were picked up and subjected to phenotypic characterization.

**Phenotypic characterization.**

The suspected colonies were identified microscopically and biochemically as formerly described (Dufour-zavala et al., 2008; Quinn et al., 1994).

**Antimicrobial susceptibility.**

Antibiogram check was performed following the Kirby Bauer approach, compliant with the standardized protocol established by the documents VET01-A4 and VET01-S2 (CLSI, 2013) using 0.5 McFarland standard adjusted colony at log phase of growth (suspended in Mueller-Hinton broth), zones of inhibition were measured around the antimicrobial disks and then interpreted (CLSI, 2013). The used antimicrobials were ampicillin 10 mcg (AMP), cefotaxime 30 mg (CTX), norfloxacin 10 mg (NOR), ciprofloxacin 10 mg (CIP), erythromycin (E), florfenicol 30 mg (FFC), gentamycin 10 mg, lincomycin (L), sulfamethoxazole/trimethoprim (SXT) and oxytetracycline 30 mg (OT).

**Virulence assay of \textit{B. avium}.**

**Hemagglutination assay.**

A hemagglutination assay using guinea pig erythrocytes was conducted as previously described (Dufour-zavala et al., 2008). Strains were grown on brain heart infusion agar at 37°C for 36-48 h. Bacterial growths were washed from the plates with phosphate buffer saline (PBS) and diluted to a concentration of approximately 5 x 10^9 cell/ml (0.5 optical density at 600 nm). Equal volumes of bacterial suspension and erythrocytes suspension (2% packed-cell volume in PBS) were mixed on a glass slide and observed for agglutination after gentle rocking.

**Tracheal attachment assay.**

A tracheal attachment assay was applied as formerly described (Temple et al., 1998). Transverse 2 mm length tracheal rings were
aseptically obtained from 26 days-old turkey embryos after decapitation. Three tracheal rings were incubated in separate wells containing EBSS culture medium (Sigma Chemical Co., St. Louis, Mo.) and a 0.5 ml of *B. avium* broth culture (of about 2 x 10^7 bacteria/ml) , it was then incubated at 42°C for 3 h, on a shaker incubator until examined by inverted microscope.

**Polymerase chain reaction.**

DNA was extracted from culture samples using QIAamp DNA Mini kit (Qiagen, Germany, GmbH). Unique PCR primers have been supplied from Biobasic (Canada) as shown in Table (1). PCR reactions were carried out in a 25-μl master mix reactions containing 12.5 μl of Emerald Amp Max PCR master Mix(Takara, Japan), 1 μl of each primer (20 pmol conc.), 7.5 μl of PCR grade water and 3 μl of template DNA. PCR was performed in Applied biosystem 2720 thermal cyclers. PCR products were inoculated in agarose gel (Lonza), and Generuler 100 bp ladder (Fermentas, Thermo) was used to check fragments sizes. The gel images had been captured through a gel documentation system (Alpha Innotech, Biometra) and the images were analyzed through the usage of laptop software (Automatic Image Capture, Protein Simple, USA).

Partial sequencing of *recA* gene for 2 strains was done using specific primers. PCR products had been purified by QIAquick PCR Product extraction kit (Qiagen, Valencia). Sequence was performed by Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer), and then it became purified by Centrisep spin column. DNA sequences were performed by using 3130 genetic analyzer (Applied Bio systems, HITACHI, Japan). Sequence homology to GenBank accessions was established through BLAST @analysis (Altschul et al., 1990). The sequence identities were determined by MegAlign module of Lasergene DNAStar (Thompson et al., 1994) and phylogenetic analyses was performed using maximum likelihood, neighbor joining and maximum parsimony in MEGA6 (Tamura et al., 2013).

**RESULTS AND DISCUSSION**

**Clinical signs.**

*B. avium* survives for many months on litter and in drinking water systems, it’s so difficult to eliminate the microorganism from the turkey’s environment (Jackwood and Saif, 2008) and it’s very easy to spread from an infected flock to a healthy one. Infected turkeys suffer from mouth respiration, oculonasal discharge, submaxillary oedema and collapse of the trachea. The same clinical symptoms had been described by previous studies (Jackwood et al., 1995; Stenzel et al., 2017). All of the 21 sampled flocks showed moderate morbidity with variable clinical signs as sinusitis, clear nasal discharge, foamy-watery eyes, coughing, mouth breathing, dyspnea and tracheal rales; some flocks showed marked altered vocalization.

**Table 1: Oligonucleotide primers used in the study**

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Oligonucleotide sequence (5' - 3')</th>
<th>Annealing temperature (°C)</th>
<th>Amplified fragment (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>recA</td>
<td>CCGCTTATGCATGACCCTTT</td>
<td>60</td>
<td>740</td>
<td>Stenzel et al., 2017</td>
</tr>
<tr>
<td></td>
<td>TACAGGGTGGCCGAATCATCA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>blaTEM</td>
<td>ATCAGGAATACCCAGC</td>
<td>54</td>
<td>516</td>
<td>Colom et al., 2003</td>
</tr>
<tr>
<td></td>
<td>CCGGAAGACGTTTTC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aada1</td>
<td>TATCAGAGGTAGTGGCGTCAT</td>
<td>54</td>
<td>484</td>
<td>Randall et al. 2004</td>
</tr>
<tr>
<td></td>
<td>GTTCCATAGGGCTTAAGGTCTTTT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TetA(A)</td>
<td>GTTTCACACGAACGACGTCA</td>
<td>50</td>
<td>576</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CTGTCCGACAAGTTGACATGA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sul1</td>
<td>CGGCGTGTTGCTACTTGAAACG</td>
<td>60</td>
<td>433</td>
<td>Ibekwe et al., 2011</td>
</tr>
<tr>
<td></td>
<td>GCCAGTGCGTGGAAGTGAGGCGGG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TATGTTAGAGGCGAGATCCTGGGA</td>
<td>60</td>
<td>425</td>
<td>Grape et al., 2007</td>
</tr>
</tbody>
</table>
Figure 1. Gel electrophoresis results for confirmatory recA PCR and antibiotic resistance genes for the 2 B. avium strains. L, Generuler 100 bp ladder (Fermentas, Thermo) (100-1000 bp). Lanes 1, 3, 5, 7, 9 and 11 are related to strain Egy 1, while lanes 2, 4, 6, 8, 10 and 12 are related to strain Egy 13.

Figure 2. Phylogenetic tree for recA partial sequences that was generated using maximum likelihood, neighbor joining and maximum parsimony in MEGA6. Phylogenetic tree showed clear clustering of the 2 isolated Egyptian strains with the 2 B. avium strains uploaded from gene bank.
Bacteriological identification.

Bacteriological examination of samples resulted in the isolation of two BA strains (4.76%) out of the 21 tested samples. Colonies were motile gram negative, non-fermentative, strictly aerobic cocobacilli (pinpoint, convex and glistening with a pearl to gray color). Both strains showed positive reactions for oxidase, catalase and citrate; while, they exhibited negative results for urease and nitrate reduction. On triple sugar iron agar (TSI), no acid production in the butt or the slant was determined after overnight incubation, however, H₂S production was observed.

Antibiogram of the isolates towards ten different antibiotics showed that norfloxacin, ciprofloxacin, cefotaxime, florfenicol and to a lesser extent gentamicin (for only one strain) exposed the highest effectiveness towards both Bordetella isolates, respectively. While, ampicillin, erythromycin, oxytetracycline, sulphamethxazole/trimethoprim and lincomycin exhibited the highest resistance rates.

Although, this becomes the 1st record of B. avium isolation in Egyptian turkey flocks, the low isolation prevalence can be related to the existence of different contaminant microorganisms as Proteus spp., Pseudomonas spp. and E. coli which may have overgrown the BA colonies and made it difficult to isolate the microorganism in pure form. Moreover, as the disorder is highly contagious and infection disperse readily through flocks, the damage brought to the airway of turkeys by means of B. avium increases the tendency of those birds to different respiratory infections. Also, the random use of antibiotics might have decreased the isolation rate.

Both isolates showed an antibiotic resistance profile that was so close to that obtained by former studies (Mortensen et al., 1989) when the antimicrobial susceptibility of B. avium and B. bronchiseptica isolates were reported to be resistant to tetracycline, lincomycin and sulfadimethoxine. However, it has been formerly reported that tetracycline resistance has been associated with a limited number of B. avium strains (Cutter and Luginbuhl, 1991). On the other hand, other authors (Beach et al., 2012) tested 17 B. avium strains collected over thirty years and stated that all tested isolates were sensitive to gentamicin, cefoperazone, ceftazidime, piperacillin, and amikacin.

In another study (Malik et al., 2005), four B. avium strains were isolated in Minnesota in 1998–1999 (among others) and were sensitive to both
ampicillin and tetracycline. While, they were resistant to erythromycin. On the other hand, among 50 *B. avium* strains, the range of resistance against cephalosporin, penicillin, erythromycin, and enrofloxacin were >50%, but <25% for all other examined antimicrobials (Nhung et al., 2017). The turkey isolates showed wide spectrum of antimicrobial resistance. This is most probably related to the uncontrolled application of antimicrobials in reared flocks. So, increasing awareness about the use of antimicrobials for treatment of poultry pathogens will decrease treatment failure, and reduce related financial losses.

**Virulence assays of *B. avium*.**

Both harvested strains agglutinated guinea pig RBCs; as *B. avium* Hemagglutinin is a crucial virulence element (Moore et al., 1994), the encountered findings proved the great virulence of the 2 isolated strains. Through a related study (Rumińska and Koncicki, 1999), it was suggested that unique strains of BA are recognized to produce toxins as endotoxin, tracheal cytotoxin, heat-stable dermonecrotic toxin and osteotoxin as well as hemagglutinin that are concerned in pathogenesis of the disease. Also, the findings agreed with the preceding study that stated that *B. avium* agglutinates guinea pig erythrocytes via an unknown mechanism and loss of hemagglutination capability results in attenuation (Temple et al., 2010).

Regarding the tracheal attachment assay, tracheal rings had marked damage, with the disruption of the mucosal architecture. Microscopic observation revealed cilia damage at some areas 4 h. post culturing.

In this study, we utilized embryonic tracheal rings (rather than rings from live poults), as embryonic rings were easily obtained aseptically and show uniform, reproducible results. Moreover, providing an organ that is matching to the in vivo colonization site makes the in vitro and in vivo results more closely comparable. The obtained results for the tracheal attachment assay confirmed that the two isolated strains were highly virulent.

The revealed results in this work are in great concordance with a preceding study (Gentry-Weeks et al., 1988) where *B. avium* was examined for virulence factors together with the filamentous hemagglutinin, and tracheal cytotoxin, and concluded that a dermonecrotic toxin and tracheal cytotoxin are the main putative virulence elements of *B. avium*. Moreover, they detected dermonecrotic toxin (DNT) and tracheal cytotoxin via many Bordetella species and proved that they were concerned in the damage of tracheal tissue and concluded that the capability of a strain to cause an in vivo illness is correlated with its capability to adhere to the ciliated tracheal cells in vitro.

**Polymerase chain reaction.**

As shown in Figure 1, *Bordetella avium* recA gene specific PCR products had been detected in two farms (The 2 cases were confirmed by microbiological isolation). On the other hand, 516 bp, 576 bp, 484 bp, 433 bp and 425 bp for *blaTEM*, *tetA(A)*, *aadA1*, *sul1* and *dfrA* genes, respectively were amplified in both *B. avium* detected strains. The positive results encountered for the antibiotic resistance genes additionally agreed with the revealed profile by disk diffusion method.

**DNA sequence.**

DNA sequencing for 740 bp of *recA* gene from 2 strains have been generated, then submitted (GenBank accession numbers MG821490 and MG821491). As shown in figures 2 and 3, partial sequencing of the amplified 740 bp of *recA* gene revealed a hundred % maximum identity (among each others) and with ATCC 35086 strain (Genbank accession AY124330.1) (Gentry-Weeks et al., 1988), that had been harvested from upper respiratory tract of diseased turkey in Germany. The isolated strains showed additionally one hundred % maximum identity to the American strain 197N (Genbank accession AM167904.1) that was harvested from a diseased turkey in a commercial flock in the early 1980s in Ohio (Sebaihia et al., 2006).

Maximum identity to other Bordetella strains (other than from *B. avium*) ranged from 87.3% (*B. pertussis*) to 89.7% (*B. hinzii*).

**CONCLUSION**

The 1st record of *B. avium* in Egyptian turkeys became encountered that represent a significant trouble to the turkey industry, inflicting poor flock performance and increasing the incidence of other infections. Hence, extensive clean up measures are required to remove the bacterium from contaminated premises, along with complete elimination of all litter, and disinfection of all surfaces, feeders and drinking water systems.

Prevention of infection calls for strict biosecurity measures. Antibiotic treatment of turkey coryza has variable success; it is likely
that antibiotics in fact deal with secondary infections instead of \textit{B. avium} infection.

CONFLICT OF INTEREST
The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS
All authors contributed equally in all parts of this study.

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