Original paper

Isolation and phenotypic characterization of Gallibacterium anatis biovar haemolytica from a hen with hemorrhagic ooforitis

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Abstract

Objective: the isolation and phenotypically identification of a Gallibacterium anatis biovar haemolytica strain from a hen with hemorrhagic ooforitis; the antimicrobial susceptibility testing of this isolate.

Methods and results: a strain of G. anatis biovar haemolytica, was isolated and phenotypic identified by morphological, cultural and biochemical characters examination, with API 20 E, API 20 NE, API STAPH, API ZYM tests and ABIS online software. The antimicrobial susceptibility of the isolate was performed using the standard disk diffusion method.

Conclusions: a strain of G. anatis biovar haemolytica was isolated and phenotypically identified from a hen. From our knowledge, this is the first reporting in Romania of isolation and identification of G. anatis biovar haemolytica. The Gah IDSA 161 strain could be phenotypic identified only by ABIS online software, Pasteurellaceae Database version, unifying the results of four API kits and other biochemical tests. The isolate showed a multi-drug resistant profile to tetracyclines (tetracycline, oxitetracycline, doxycyclin), floroquinolones (enrofloxacin, ciprofloxacin), ampicillin, trimethoprim, nalidixic acid, clindamycin, and it was susceptible to sulfonamide, sulfmethoxazole/trimethoprim, gentamicin, cephalothin, streptomycin, amoxicilin/clavulanic acid.

Keywords Gallibacterium anatis biovar haemolytica, hen, phenotypic identification, antimicrobial susceptibility testing
Introduction

Gallibacterium anatis is part of the normal chicken microbiota in the upper respiratory, lower genital and terminal digestive tracts (Christensen et al., 2003; Bojesen et al., 2003a). However, G. anatis is defined as an opportunistic pathogen associated with pathological changes in the reproductive organs (oophoritis, follicular hemorrhage, salpingitis), gastrointestinal (inflammation of intestine and peritoneum, hepatic necrosis) and respiratory systems (necrosis, inflammation), septicemia and pericarditis (Paudel et al., 2013; Persson and Bojesen, 2015; Krishnegowda et al., 2020). The pathogenicity of G. anatis is determined by virulence determinants of bacterial strain (toxin A, outer membrane vesicles, F 17-like fimbriae, capsule, metalloproteases, biofilm formation, hemagglutinin) and route of infection (Persson and Bojesen, 2015; Krishnegowda et al., 2020). The physiological status of host (stress, immune status, age, hormones), co-infection with other pathogens (Escherichia coli, Avibacterium paragallinarum, Mycoplasma gallisepticum, infectious bronchitis virus) and abrupt changes in environment (cold stress, deficient nutrition, poor ventilation etc.) exacerbate the disease (Krishnegowda et al., 2020). Diagnosis of the Gallibacterium anatis infection is based on isolation and identification by phenotypic characterization (Christensen et al., 2003). Other tools have been used, also, for the bacterial identification: matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, gyrB gene-based quantitative PCR (qPCR) assay, PCR restriction fragment length polymorphism (PCR-RFLP) assay, fluorescence in situ hybridization (FISH) (Bojesen et al., 2003b; Alispahic et al., 2012; Wang et al., 2016; El-Adawy et al., 2018).

The genus Gallibacterium has four named species (G. anatis, G. mellospitsitai, G. trehalosiifermentans and G. salpingitidis), three Gallibacterium genomospecies (1, 2 and 3) and an unnamed group V (Christensen et al., 2003; Bisgaard et al., 2009). Genus Gallibacterium belongs Pasteurellaceae family (Christensen et al., 2003; Bisgaard et al., 2009). G. anatis is divided in two biovars: haemolytica and anatis. G. anatis bv. haemolytica comprises β-haemolytic, D-arabinose and L-fucose positive isolates (includes strains from former biovars 1, 3, 4, 11, 12, 15, 17-20, 22, 24 of the avian [Pasteurella haemolytica]-‘Actinobacillus salpingitidis’ complex). G. anatis bv. comprises non-haemolytic, trehalose-positive and D-arabinose-, L-fucose-, maltose- and dextrin-negative isolates (includes strains from former P. anatis) (Christensen et al., 2003). G. anatis has been isolated from domestic and non-domestic birds (chicken, turkey, goose, duck, budgerigar, parrot, peacock, partridge, pheasant, guinea fowls), other domestic animals (cattle, pigs, sheep, horse and rabbits) (Christensen et al., 2003; Rzewuska et al., 2007; Krishnegowda et al., 2020) and human (an immunocompromised women with bacteremia and diarrhea) (Aubin et al., 2013). G. anatis is distributed in Europe (Poland, Germany, Denmark, Switzerland, Norway, England, Sweden, Austria, Czech Republic, Africa (Egypt), Asia (Iran), Australia, North (Mexico) and South America (Rzewuska et al., 2007; Persson and Bojesen, 2015; Krishnegowda et al., 2020).

The aim of our work was to communicate the isolation and phenotypically identification of a G. anatis biovar haemolytica strain from a hen with hemorrhagic ooforitis and antimicrobial susceptibility testing of this isolate. From our knowledge, this is the first reporting of G. anatis biovar haemolytica isolation and identification in Romania.

Materials and Methods

Pathological material

Heart, lung, spleen, liver, kidney, ileum and genital tract samples from a hen were collected for bacteriological examination. Hemorrhagic ooforitis was observed. The hen came from a 30 birds flock, Iliov county, Romania.

Isolation of bacterial strains

The specimens were streaked on nutrient agar (Oxoid) supplemented with 5% sheep blood and inoculated media were incubated at 37°C for 24-48 hours in aerobic conditions. The colonies with large β-haemolysis zone from blood agar plates were investigated for cell morphology in Gram-staining slides and inoculated in nutrient broth (Oxoid) supplemented with 5% horse serum and 3% glucose for isolation of bacterial strain.

Identification of bacterial strains

Phenotypic identification of isolated bacterial strain was performed by morphological, cultural and biochemical characters examination, according with Christensen et al. 2003, Christensen et al. 2007, Rzewuska et al., 2007, Bisgaard et al., 2009 and ABIS on line software, Stoica and Sorescu, 2020. Cell morphology was observed in Gram-staining slides, mobility was appreciated in semisolid medium (Mobility-Indol-Urea medium) and cultural characters were investigated with blood nutrient agar and nutrient broth with serum and glucose. Biochemical characters of the isolated strain were determined using MIU, TSI (Triple-Sugar-Iron) and Simon’s citrate media, API 20 E, API 20 NE, API STAPH and API ZYM tests (bioMerieux, France), according to the manufacturers instructions. The catalase (3% H2O2), oxidase (Sigma) and ONPG (Oxoid) tests were performed, also.

Antimicrobial susceptibility

The antimicrobial susceptibility of the isolated strain was performed using the standard disk diffusion method according to the Clinical Laboratory Standards Institute (CLSI) 2002 (M31-A2), CLSI VET08-ED4:2018 (zone diameter for Actinobacillus pleuropneumoniae) and CLSI 2020 (M100, ed. 30, zone diameter for Haemophilus influenzae and H. parainfluenzae). Mueller Hinton Agar with 5% sheep blood (BioMaxima, Poland) and disks containing specified amounts of antibiotics (Oxoid) were used. The plates were incubated at 37°C, 24 hours, in aerobic conditions.

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Results and discussion

A strong β-haemolytic strain was isolated from heart, ovaries, lung and spleen of a hen with hemorrhagic ooforitis. This strain wasn’t isolated from liver, kidney or ileum. A non-haemolytic Escherichia coli strain was isolated, from ovaries, also.

The taxonomic classification of isolated bacterial strain in Gallibacterium anatis biovar haemolytica was performed by morphologically, culturally and biochemically characteristics. It is Gram-negative, polymorph, non-motile rod, shorter or cocobacillar on blood agar media and longer rod in serum glucose broth. The bacteria occurs singly, in pairs or, in liquid media, in short chains. Colonies are strong β-haemolytic, circular with regulated edges, transparent/semi-transparent, smooth, non-pigmented, up to 1 mm in diameter after 24 hours or 1.5-2.0 mm after 48 hours of aerobic incubation at 37 °C on agar media (nutrient agar, Columbia agar, Tryptone Soya agar) supplemented with 5% sheep blood (Fig. 1). It determines small, pinpoint, opaque, pink or red colonies on MacConkey agar after 24-48 hours of incubation. No V-factor requirement. The strain grow moderate, with small, granular deposit and weak turbidity in nutrient broth supplemented with 5% horse serum and 3% glucose after 24-48 hours of incubation. It grows better on Brain Heart Infusion (Oxoid) broth supplemented with 5% horse serum. Positive reactions are obtained for catalase, oxidase and ONPG tests. Nitrate is reduced. α-glucosidase (PNPG), alkaline phosphatase, acid phosphatase, esterase (C4), esterase-lipase (C8), leucine arylamidase, trypsine, β-galactosidase, α-chymotrypsine, α-fucosidase, arginin dihydrolase, lysine decarboxilase, ornithine decarboxilase, troposphane deaminase, Voges-Proskauer (acetoin production) and gelatinase. Acid is formed without gas from D-glucose (could be weak), D-mannitol, inositol, D-fructose, D-mannose, D-trehalose, D-xilose, N-acetylglycosamine. Negative in Simmon’s citrate, TSI, triple sugar iron, indole and urease tests in MIU. Negative tests are further observed with lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-hemoglobinidase, α-galactosidase, β-glucuronidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, arginin dihydrolase, lysine decarboxilase, ornithine decarboxilase, troposphane deaminase, Voges-Proskauer (acetoin production) and gelatinase. Acid is not produced from D-melibiose, amygdalin, L-arabinose, D-maltose, lactose, xylitol, D-rafinose and metil melibiose, amygdalin, L-arabinose, D-maltose, lactose, D-sorbitol, L-rhamnose (29 hours of incubation), D-sucrose, gas from D-glucose (could be weak), D-mannitol, inositol, phosphohidrolase tests are positive. Acid is formed without gas from 1% C4, esterase-lipase (C8), leucine arylamidase and naphtol-β-naphthofenhidrolase (PNPG), alkaline phosphatase, acid phosphatase, esterase oxidase and ONPG tests. Nitrate is reduced.

The Gah IDSA 161 strain  was susceptible to sulfonamide, sulfomethoxazole/trimethoprim, gentamicin, cephalothin, streptomycin, amoxicilin/clavulanic acid, moderate susceptible to erythromycin and was resistant to tetracycline, oxitetracycline, doxicyclin, trimethoprim, nalidixic acid, clindamycin, enrofloxacin, ciproflaxacin, ampicillin. In Table 1 are presented the diameter of inhibition zone for every tested antibiotic.

<table>
<thead>
<tr>
<th>No. crt.</th>
<th>Antibiotic</th>
<th>Diameter of inhibition zone (mm)</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Amoxicilin/acid clavulanic</td>
<td>23</td>
<td>S</td>
</tr>
<tr>
<td>2.</td>
<td>Ampicillin</td>
<td>18</td>
<td>R</td>
</tr>
<tr>
<td>3.</td>
<td>Cephalothin</td>
<td>23</td>
<td>S</td>
</tr>
<tr>
<td>4.</td>
<td>Ciproflaxacin</td>
<td>14</td>
<td>R</td>
</tr>
<tr>
<td>5.</td>
<td>Clindamycin</td>
<td>6</td>
<td>R</td>
</tr>
<tr>
<td>6.</td>
<td>Doxicyclin</td>
<td>9</td>
<td>R</td>
</tr>
<tr>
<td>7.</td>
<td>Enrofloxacin</td>
<td>6</td>
<td>R</td>
</tr>
<tr>
<td>8.</td>
<td>Erythromycin</td>
<td>15</td>
<td>MS</td>
</tr>
<tr>
<td>9.</td>
<td>Gentamicin</td>
<td>25</td>
<td>S</td>
</tr>
<tr>
<td>10.</td>
<td>Nalidixic acid</td>
<td>6</td>
<td>R</td>
</tr>
<tr>
<td>11.</td>
<td>Oxicylcycline</td>
<td>6</td>
<td>R</td>
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<tr>
<td>12.</td>
<td>Streptomycin</td>
<td>19</td>
<td>S</td>
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<tr>
<td>13.</td>
<td>Sulfomethoxazo le/trimethoprim</td>
<td>23</td>
<td>S</td>
</tr>
<tr>
<td>14.</td>
<td>Sulfonamide</td>
<td>25</td>
<td>S</td>
</tr>
<tr>
<td>15.</td>
<td>Tetracycline</td>
<td>6</td>
<td>R</td>
</tr>
<tr>
<td>16.</td>
<td>Trimethoprim</td>
<td>6</td>
<td>R</td>
</tr>
</tbody>
</table>

S= susceptible, MS= moderate susceptible, R= resistant.

The Gah IDSA 161 strain was isolated from ovaries, also.

Figure 1. Gallibacterium anatis biovar haemolytica IDSA 161 strain on nutrient agar supplemented with 5% sheep blood, after 48 hours of aerobic incubation at 37º C.
G. anatis infection is, until recently, an under-diagnosed or ignored cause of economic losses and mortality in poultry (Krishnegowda et al., 2020), with a global distribution in Europe, Asia, Africa, Australia, North America and South America. So far, from our knowledge, there isn’t any report of G. anatis infection in Romania.

The Gah IDSA 161 strain, isolated from a hen from a 30 birds flock, Ilfov county, Romania, could be phenotypically identified only by ABIS on line software, Pasteurellaceae Database version, unifying the results of API 20 E, API STAPH, API 20 NE and API ZYM kits, and other biochemical tests. The indications that Gah IDSA 161 belongs to the Pasteurellaceae family are some morphologically (Gram-negative non-motile rods), culturally (facultative anaerobic) and biochemically (ability to reduce nitrate, oxidase- and phosphate-positive tests) characters (Christensen et al., 2007). For the Gallibacterium genus taxonimic classified of the isolated strain, they were important catalase positive test, urease negative, indole negative, ONPG positive, H2S negative, Simmon’s citrate negative, Voges-Proskauer positive, arginin dihydroxalase negative, lysine decarboxilase negative, ornithine decarboxilase negative, gelatinase negative, acid production positive from D-glucose, D-mannitol, D-fructose, mannos, sucrose, acid production negative from amygdalin, and negative reactions for β-glucuronidase, β-glucosidase, α-mannosidase and α-fucosidase. Gah IDSA 161 belongs the G. anatis biovar haemolytica being strong β-haemolytic, L-arabinose negative, m-inositol positive, D-sorbitol positive, D-maltose negative, D-xylose positive and D-trehalose positive (Christensen et al., 2003). Comparing with the key characters for separation of the biovars of the avian [P. haemolytica]-A. salpingitidis’ complex, Gah IDSA 161 matches only with biovar 4 strains (L-arabinose negative, m-inositol positive, D-sorbitol positive, D-maltose negative, D-xylene positive and D-trehalose positive). The three strains of bv. 4 described, belonging G. anatis, were all associated with lesions at different birds [10672/9 Salp. (=F114), chicken, Denmark; Gerl. 2396/79 (=F465), chicken, Germany; 5821/88, parrot, Germany] (Christensen et al., 2003). However, there are, also, another strains of bv. 4, belonging G. melopsittaci species (F 416 = HIM778-3, isolated from a parakeet with septicemia, G. salpingitidis (1998/2, from a goose with salpingitis and peritonitis) and Gallibacterium genomospecies 3 (F298, from a duck with septicemia) (Bisgaard et al., 2009). So, all the strains from bv. 4, apart of host, were associated with lesions.

Gah IDSA 161 differs from the emended description of the genus Gallibacterium Christensen et al., 2003 (Bisgaard et al., 2009) by test positive for production of acid from L-rhamnose. So, this character could be a phenotypic marker for the strain, together with above fermentative characters which include it in biovar 4 of former avian [P. haemolytica]-A. salpingitidis’ complex.

As for antibiotic susceptibility, Gah IDSA 161 has a similar profile, generally, with isolates from Germany (El-Adawy et al., 2018), which were susceptible to sulfomethoxazole/trimethoprim (80% of strains), gentamicin (73%), neomycin (100%), apramycin (100%), florphenicol (100%), and all strains were resistant to oxitetracycline, clindamycin, penicillin, sulfathiazole, sulfamethoxim, spectinomycin and tyllosin. Also, generally, Gah IDSA 161 has a similar profile with the isolates from Austria (Hess et al., 2019), which were susceptible to amoxicillin/ clavulanate (99% of strains), ampicillin (79%), amoxicillin (69%), gentamicin (93, 9%), streptomycin (96,7%), cefotaxime (82%), colistin (75%), imipenem (83,5%), neomycin (59,6%), and were resistant to oxacillin (98%), tyllosin (94%), tetracycline (89%), nalidixic acid (77%), sulfamethoxazole (77%), sulfomethoxazole/ trimethoprim (61%), enrofloxacin (58%). The isolates of G. anatis from Denmark and Mexico were resistant to tetracycline (92% of strains) and sulfamethoxazole (97%) and were susceptible to quinolones (Bojesen et al., 2011). Multi-drug resistant isolates of this species are frequently reported, with resistance to clindamycin, sulfonamides, novobiocin, tyllosin, penicillin, and tetracycline (Krishnegowda et al., 2020). So, since antimicrobial susceptibility of isolates constantly changes, in vitro testing of the strains is often needed.

Conclusion

Has been isolated and phenotypically identified a strain of G. anatis biovar haemolytica from a hen with hemorrhagic ooforitis. From our knowledge, this is the first reporting in Romania of isolation and identification of G. anatis biovar haemolytica. This isolate, Gah IDSA 161, differs from the emended description of the genus Gallibacterium Christensen et al., 2003 (Bisgaard et al., 2009) by test positive for production of acid from L-rhamnose. So, this character could be a phenotypic marker for the strain, together with some fermentative characters which include it in biovar 4 of former avian [P. haemolytica]-A. salpingitidis’ complex (L-arabinose negative, m-inositol positive, D-sorbitol positive, D-maltose negative, D-xylene positive and D-trehalose positive). The Gah IDSA 161 strain showed a multi-drug resistant profile to tetracyclines (tetracycline, oxitetracycline, doxycyclin), florquinolones (enrofloxacin, ciprofloxacin), ampicillin, trimethoprim, nalidixic acid, clindamycin, and it was susceptible to sulfonamide, sulfomethoxazole/trimethoprim, gentamicin, cephalothin, streptomycin, amoxicillin/clavulanic acid. This profile was similar, generally, with the one of isolates from Germany (El-Adawy et al., 2018) and Austria (Hess et al., 2019).

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References


