Original Article

Isolation and identification of Klebsiella aerogenes from bee colonies in bee dysbiosis

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Abstract

This article presents modern methods for obtaining and isolating pure culture from bee colonies (from honeycombs with infected broods and contaminated by faeces of bees), and the identification and indication of pathogenic bacteria of Klebsiella aerogenes species in honey bees during the winter, spring, summer and the autumn. The purpose was to isolate and identify the pure culture of enterobacteria from the pathological material of diseased bee colonies. The study of their basic biological properties for the possible use of the obtained strains, which should be used as indicator bacterial cultures in the study of the biological properties of drugs for the treatment and prevention of enterobacteriosis of bees was undertaken. The etiological factor for the origin of the collapse of bee colonies belongs to the Family Enterobacteriaceae, the genus being Klebsiella – Klebsiella aerogenes (previously named Enterobacter aerogenes), which was established by its cultural properties (Endo – light pink, small, flat, mucous, matte colonies; Levin – light pink, flat, mucous, matte colonies); by morphological properties (short, sticks, small, placed together and singly, without capsules, mobile); by tinctorial (gram negative) properties and by results of biochemical typing of microorganisms (Kligler medium: glucose – negative, acid-gas H_2S negative; Simons medium – positive, acetate Na – positive, malonate Na – negative; Phenylalanine – negative: Indole –negative; Urease – negative), which were isolated from the intestines of bees and their faeces from the frames of bee colonies.

Keywords: bee colonies, indication, identification, Klebsiella aerogenes

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Introduction

Honey bees play an important role in polluting natural and managed ecosystems and understanding the biological causes of honey bee loss will improve management and breeding strategies aimed at improving bee health (Cornman *et al*., 2012).

In recent years, mass deaths of bees have been reported periodically in the world and in the Ukraine (Galatiuk, 2014). It is found out that the collapse of bee families has been observed due to complex factors: the decrease or absence of a honey plant base; the low genetic resistance of bee colonies; the impact of *Varroa jacobsoni* ticks on family resistance; the action of electromagnetic waves and radioactive radiation; pesticides (Glenny *et al*., 2017); mass reproduction of conditionally pathogenic microflora of bee intestines; and the spread of pathogens in the hive and in the apiary as a result of reduced resistance in some families. Bacteria have a pathogenic effect on the bee organism which is manifested in acute diarrhea and general weakness of the bee colony (Galatiuk *et al*., 2016). However, there is no single factor recognized as the cause of the high annual loss of honeybee colonies (Tentcheva *et al*., 2004; Cox-Foster *et al*., 2007; Nguyen *et al*., 2010; Genersch *et al*., 2010; Runckel *et al*., 2011; Evans *et al*., 2011; Cornman *et al*., 2012; Ravoet *et al*., 2013; Lee *et al*., 2015; Traynor *et al*., 2016; Seitz *et al*., 2016).

According to recent works by domestic and foreign authors, bacterial diseases of bees are becoming widespread in the apiaries of the Ukraine and some countries in Europe and America (Galatiuk *et al*., 2016; Glenny *et al*., 2017), causing significant economic loss to beekeepers.

In the United States, high annual losses of honeybee colonies have averaged ~ 33% annually since 2006 and have increased approximately 12%. (Lee *et al*., 2015; Traynor *et al*., 2016).

Bee diseases caused by enterobacteria were reported in 2015 in the Krasnodar Territory (Russian Federation) for the first time in Eastern Europe (Nikolayenko, 2015). Literature sources have reported on upper respiratory and intestinal tract disease in cattle and pigs with enterobacteriosis (Zolotukhin *et al*., 2018). There are also many reports of enterobacter-induced intestinal diarrhea in humans (Miró *et al*., 2013). We have not found any more reports about the disease caused by bee enterobacteriosis in the literature available to us.

The microbiota of the intestine plays an important role in the health and nutrition of the host. Evidence has been identified of a phylogenetically grouped shift in the bacterial association of honey bees, causing a decrease in bifidobacteria and alpha-proteobacteria. (Gilliam, 1997). It has been found that the bee intestine contains representatives of at least 10 genera of bacteria belonging to the families *Enterobacteriaceae*, genera *Klebsiella, Enterobacter, Providencia, Proteus, Citrobacter, Hafnia, Escherichia, Pantoea, Morganella* and *Serratia* (Chechotkina *et al*., 2011). At the same time, the violation of quantitative and qualitative microbial contents of the intestine towards pathogenic representatives leads to the disease (Galatiuk *et al*., 2016; Galatiuk *et al*., 2018). These pathogens penetrate into the deeper cells of the digestive tract of bees and cause dysbiosis. It should also be noted that they have expanded their range of habitation to include hemolymph, ovaries, salivary glands, etc. (Galatiuk *et al*., 2016; Forsgren *et al*., 2018; Rivera *et al*., 2018).

Our study addresses the current issues of isolation and identification of pathogenic enterobacteria (*Klebsiella aerogenes*) in honey bees (Galatiuk *et al*., 2019) in the event of massive enterobacteriosis in winter, spring, summer and the autumn (Evans *et al*., 2011; Rivera *et al*., 2018).

Our studies of the epizootic situation regarding the collapse of bee families in the Ukraine have shown that this syndrome manifests itself in bee colonies in winter and spring. In this case, the disease is more often developed on industrial apiaries, which have brought in southern breeds of bee - Italian, Buckfast and others. Other factors, such as pesticide effects, lack of honey plants and pollen diversity, high levels of invasion by varatosis contribute to the reduction of resistance in bee colonies, which contributes to the manifestation of the collapse in the number of bees (Galatiuk, 2014). Bacteriological studies of diseased bees and affected broods allowed us to isolate the 2 cultures of *Klebsiella Aerogenes* and *Klebsiella Pneumoniae* enterobacteria, which cause factor infectious diseases in bees. Clinical features of the disease are described depending on the time of year and the treatment and prevention measures performed in apiaries (Galatiuk *et al*, 2018).

This article, for the first time addresses, the current issues of isolation and identification of pathogenic enterobacteria (*Klebsiella aerogenes*) in honey bees in the occurrence of massive enterobacteriosis.

The purpose - isolate and identify the pure culture of enterobacteria from the pathological material of diseased bee colonies. The study of their basic biological properties is for possible use of the obtained strains, which should be used as indicator bacterial cultures in the study of the biological properties of drugs for the treatment and prevention of enterobacteriosis of bees.

Materials and Methods

According to the reports of beekeepers of the Ukraine, during the spring flight, a lot of dead bees were found at the bottom of hives and near the flyers. Colonies of bees were usually weakened, the abdomen in sick bees was thickened, filled with fecal masses and the bees move slowly. The frames and the walls of the hives were polluted with defecation and the strength of the colonies declined sharply. Initially, gaps were found among the evenly sealed brood, then the brood became uneven and separate cells were formed with holes in the wax lids, in which dead pupae were found with traces of fecal masses near them.

Sampling and transportation: 50 sick bees and a part of a honeycomb (10x15cm) with affected brood contaminated with faeces were sent to the laboratory from each sick family. We also washed 5 honeycomb control points (for each control point using a separate sterile swab moistened with saline solution, which from each control point was placed in a sterile flask 20 cm^3 with saline solution, closed with a sterile stopper

and shaken for 20-40 mins). Pathological material was packaged and sent to: sick living bees - in glass jars with openings in the lid with feed in an amount sufficient for the time of transportation; dead bees - in paper bags. Samples were sent to the Research Laboratory of the Department of Microbiology, Pharmacology and Epizootology, Faculty of Veterinary Medicine, ZhNAEU on the day of selection.

Selection of pure cultures: Culturing was carried out in the thickness of the culture medium by the deep method. The control of the nutrient media was carried out in accordance with DSTU ISO\TS 11133-1:2000 IDT "Microbiology of food and animal feed: guidelines for the preparation and production of nutrient media" Parts 1 and 2. We made diagnostic suspensions according to conventional methods before culturing the honeycombs with faeces (Galatiuk, 2010). A sterile graduated pipette was filled with 0.5 or 1.0 cm3 of material and poured into sterile Petri dishes. After that, the material was poured 15-20 cm3, melted and cooled to 45-50°C Endo medium. Carefully the cup was shaken. The cup was left closed until the agar was completely solidified and then turned upside down and placed in a thermostat. Incubation was carried out at 37 °C for 24-36 hours. Common bacteriological regimens have been used to isolate pure bacterial cultures (Pul'cherovskaya *et al*., 2017). For the isolation of bacteria, a number of conditions were considered: early culturing of the selected pathological material; selection of optimal nutrient media for primary culturing; using the Gold method (Hudz *et al*., 2014). At culturing we provided growths of isolated colonies (Fig. 2-b), followed by staining and microscopy of smears from the investigated suspected colonies by Gram method and Burri method for the presence of a capsule (Dykyy *et al*., 2002).

The identification of the selected crops: To establish and confirm the generic identity and species affiliation of the cultures, biochemical typing reactions were carried out together with physicians-bacteriologists of the State Institution "Zhytomyr Regional Laboratory Center of the Ministry of Health of Ukraine" and Zhytomyr Regional State Laboratory of the State Service of Ukraine on Food Safety in 2019.

Our study was approved according to the guidelines and roles of the Animal Researches Committee of Zhytomyr National Agroecological University.

Results

Re-passage of similar microbial colonies was performed by Gold's method for isolating the pure culture of differential diagnostic environments for enterobacteria (Zakon. Rada, 2005; Sybirna, 2009).

After preparation of the bacteriological suspension, primary culturing was carried out on Endo medium, cultivation was performed in a thermostat at 37 °C for 24-36 hours (Fig. 1-a,b).

From different bee colonies, the microbial landscape differed somewhat but in all the Petri dishes studied, the S-shaped colonies were similar in cultural terms - small, round, matte, flat, with a pink tinge, without metallic luster, placed in clusters or singly.

Colonies of microorganisms that were in the Petri dishes were superficial, brown, matte, without staining the medium below on bismuth-sulphite agar after 48 hours (Fig. 2-a), similar to the colonies of *Escherichia coli* but other than the colonies of *Salmonella typhimurium* (Fig. 2-b).

Figure 1

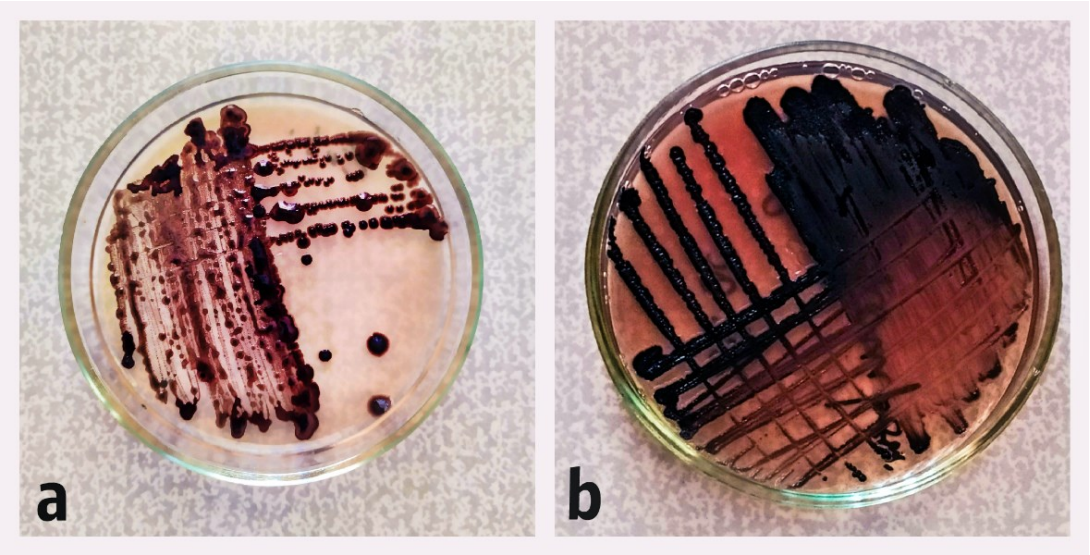

Figure 2

On purple-red bile agar with glucose, colonies were formed in the form of a purple color with or without precipitation.

On Levin's medium, colonies were light pink, convex, matt, formed at different densities (from fused to single colonies), very different from the growth of *Escherichia coli* colonies on the same medium.

The bacteria of each typical colony were gram-negative, short sticks, small, placed together and singly, without capsule (magnification of the microscope × 1000) (Fig. 3-a).

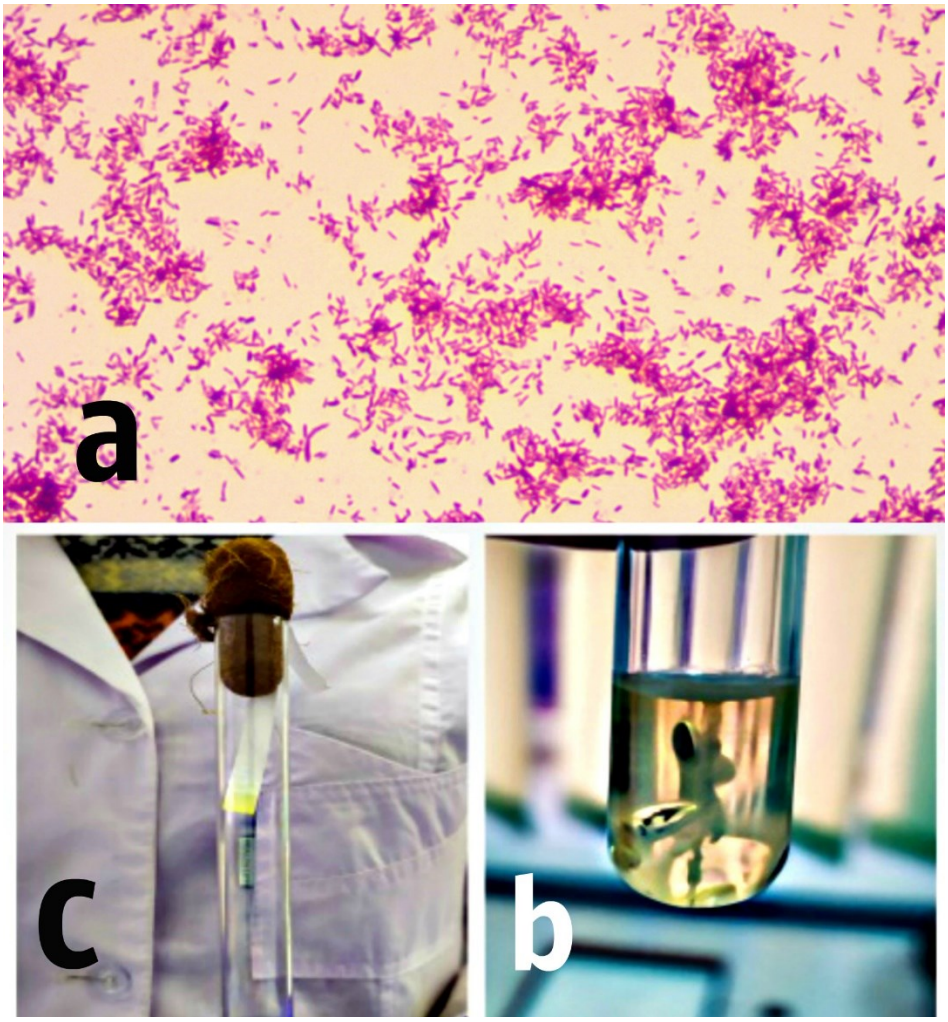

Figure 3

Isolated characteristic colonies were transferred to a disk moistened with a reagent to determine oxidase. After 10 seconds, the contact point of the microbial material remained colorless; the test bacteria were oxidase-negative. According to the totality of cultural, morphological and tinctorial features, all the strains studied by us, isolated from the intestines of bees and feces of the tainted frame, belonged to the *Enterobacteriaceae* Family.

The isolated colonies were subjected to further study in order to establish ancestral and species identity. Biochemical typing on Cligler medium showed that the bacteria ferment glucose without gas, as evidenced by the change in the color of the lower column of the medium from orange to yellow (Fig. 4-a, b); lactose-negative - beveled agar red-pink; hydrogen sulfide was not formed - no black ring.

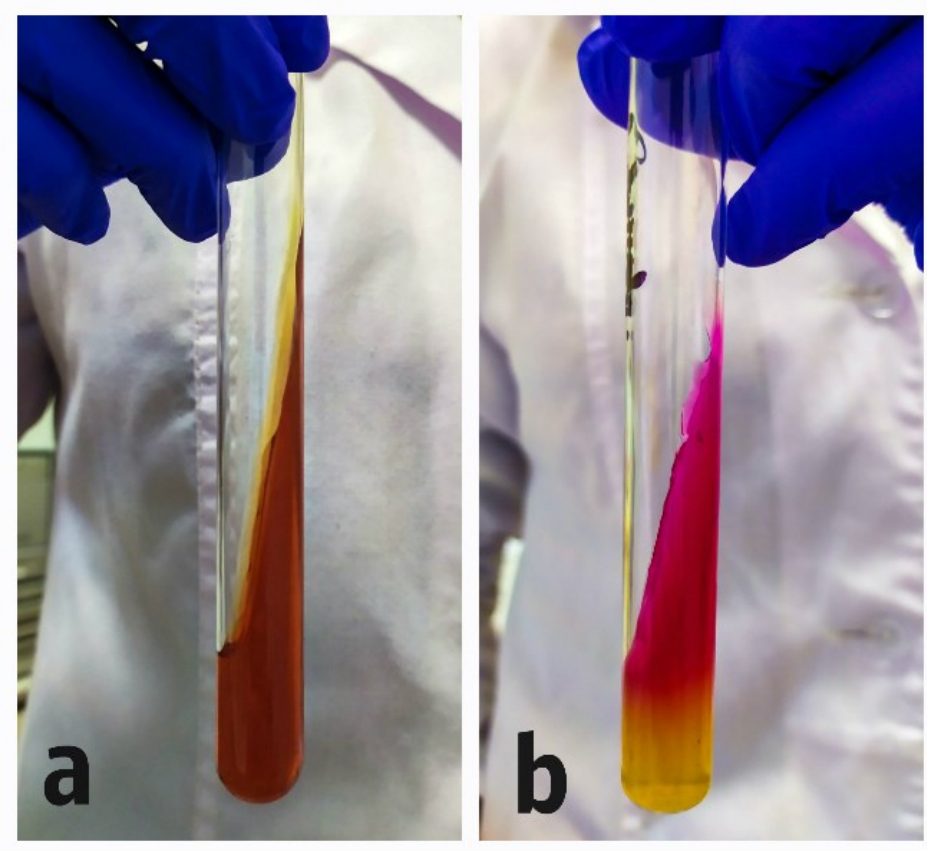

Figure 4

Microorganisms are active in relation to the acetate of natrium, promote the pH medium of Symons's (discoloration is from green on blue); nonactive in relation to the malonate of natrium – the medium remains green (Fig. 5.).

Peptolytic properties were studied using an alcohol solution of paradimethylamidobenzaldehyde - the color indicator did not change (Fig. 3-c).

Enterobacteria do not form urease, so hydrolysis of urea to form ammonia is impossible - the solution remains colorless. In the phenylalanine test, the result was negative - yellow. Culturing with a prick of 0.3% meat-peptone agar (MPA) observed the branching of bacteria into the thicker medium from the prick after 24 hours (Fig. 3-b). Culturing the isolated microorganisms onto blood meat-peptone agar, we observed their hemolytic activity (ß-hemolysis of ram erythrocytes) as one of the pathogenicity factors.

According to the results of biochemical typing, all isolated microorganisms (from the diseased bees and washes from honeycombs) belonged to the *Enterobacteriaceae* family, *Klebsiella* genus and *Klebsiella aerogenes (Enterobacter aerogenes)*. Every 15 days it was cultured on the MPA medium (meat-peptone agar) with the help of the deep method. The cultures were stored in the research laboratory of the Department of Microbiology, Pharmacology and Epizootology, Faculty of Veterinary Medicine, ZhNAEU. *Klebsiella aerogenes* antigen was washed with saline (NaCl 0.9%) at a rate of 2 cm^3 per tube and cooled to + 5 ° C in all other tubes.

Discussion

The *Enterobacteriaceae* family encompasses a large set of bacteria that have a number of properties and form large genera, united into a family, grouped by similar biochemical and morphological, cultural traits. Biochemical activity and epitope changes characterize species and the intraspecific differences of enterobacteria. However, some non-generic representatives go beyond traditional means of classification.

The name *Enterobacter aerogenes* was created by Hormaeche and Edwards in 1960 (Hormaeche *et al*., 1960) to name the species formerly known as "*Aerobacter aerogenes*" (Hormaeche *et al*., 1958). Recognizing the fact that non-stationary strains of

"*Aerobacter aerogenes*" were part of the *Klebsiella pneumoniae* species until 1886, it was proposed in 1887 to distinguish a separate genus "*Aerobacter*" that could be distinguished from the genus *Klebsiella* and to retain the use of the name "*Aerobacter aerogenes*" as microflora of the respiratory tract. Realizing that this decision was also not reasonable, Hormaeche and Edwards withdrew the proposal and made an alternative proposal to name a taxonomic entity - *Enterobacter aerogenes* (Bascomb *et al*., 1971) and published a study of 177 strains, which included, among others, representatives of the *Klebsiella* and *Enterobacter* genera. Having come to the conclusion that the three species of the genus *Enterobacter* they had studied do not belong to one genus, they proposed to attribute the genus *Enterobacter aerogenes* to the genus *Klebsiella* and named it *Klebsiella mobilis*, because of its ability to move as a heterotypical synonym. *Properties* are shown in the studies of Bascomb et al., (Bascomb *et al*., 1971) for *Klebsiella mobilis*, namely the sequence in the DNA genome of the strain contained in the Korean collection for crop types was similar in this percentage - 54.8% (Shin *et al*., 2012). The genome sequence of this strain is stored in the GenBank database under accession number CP002824 but the name *Klebsiella mobilis* Bascomb was taxonomically illegitimate and was replaced by the oldest legal epithet "*Enterobacter aerogenes*" (Tindall *et al*., 2017).

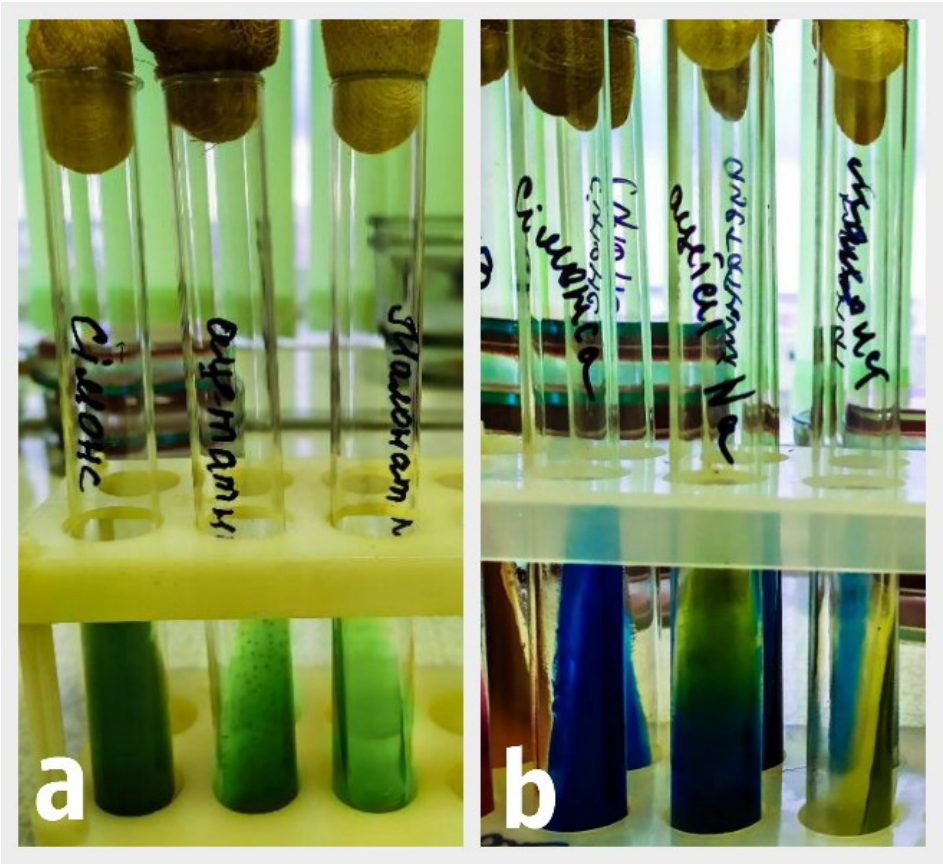

Figure 5

Genome-based comparative bacterial phylogenetics have led to the enterobacter aerogens being renamed *Klebsiella aerogenes* (Wesevich *et al*., 2019). Currently, according to research by Tindall *et al*., In 2017, *Enterobacter aerogenes* was given the binomial name - *Klebsiella aerogenes* with the preservation of synonyms *Klebsiella mobilis* (Bascomb *et al*., 1971) and *Klebsiella aerogenes* (Tindall *et al*., 2017).

Klebsiella aerogenes has been known to be an important conditionally pathogenic and highly resistant bacterial pathogen for humans over the last three decades in hospital settings. This Gram-negative bacterium has been reported in several outbreaks of hospital-acquired infections in Europe and, in particular, in France (Davin-Regli, 2015).

In 2019, Ming-Huang Chang found that, in addition to domestic animals and humans, companion dogs can serve as mcr-1 gene reservoirs, adding another level of complexity, resulting in the rapidly developing epizootic process in populations. Therefore, scientists believe it is advisable to continue to study the resistance of macroorganisms to these bacteria. Ongoing microbiological and molecular surveillance is important for the early detection and minimization of mcr-1 distribution (Cang *et al*., 2019).

Interestingly, microorganisms of the *Klebsiella* genus affect mainly immunocompromised patients, are widespread in the environment and are important among nosocomial pathogens (Qiu *et al*., 2017).

Klebsiella aerogenes is an important causative agent of health-related infections in both human and veterinary medicine. Compared to other clinically important pathogens, Enterobacter aerogenes population structure, genetic diversity and pathogenicity remain poorly understood, especially in bees.

Clinical signs (aroma with unpleasant odor in the spring-winter and summer period, decrease in activity and insect death, fly-out of beehives) are associated with activation of the main virulence factors, increase in the concentration of pathogenic bacteria in the intestines and reduced concentrations of lactobacilli.

The spread of *Klebsiella aerogenes* is associated with the presence of excess regulatory cascades in the cell that are capable of controlling membrane permeability, providing bacterial protection and the expression of detoxifying enzymes involved in the inactivation of biologically active substances in the environment. In addition, these bacteria are capable of forming numerous genetic motifs (flagella) that facilitate the rapid colonization of multiple organism systems and different hosts and can rapidly and effectively adapt the metabolism and physiology of the host cells to external conditions and environmental loads (Davinli, Davli 2015).

We have identified the isolated microorganisms from the bee and feces-contaminated frames on the basis of the study of morphological, cultural, tinctorial features and biochemical properties, which are based on differences in the composition of the enzymes of isolates. Analyzing the scientific works of domestic and foreign authors, we have found that there is no complete information on the biochemical properties of the agents of bees' enterobacteriosis. Therefore, we used conventional tests from human medicine (Marievsky, 2011) and DSTU ISO 21528-1: 2014 "Microbiology of food and animal feed; the horizontal method for the detection and calculation of enterobacteriaceae (*Enterobacteriaceae*). Part 1. Detection and calculation by pre-enrichment of NICs (ISO 21528-1: 2004, IDT) in sufficient numbers to determine the generic identity and species membership of isolated microorganisms (Golovko *et al*., 2007).

The key test for determining the generic identity of the studied cultures was the reaction to the presence of the oxidase enzyme. Some bacteria are known to have an iron-containing hemoprotein protein that provides biological oxidation to produce energy, that is, respiration. Named protein (cytochrome oxidase or indophenol oxidase) catalyzes the transfer of electrons from the donor substance to the recipient substance. During the oxidase test, an artificial electron recipient (colorless N, N-dimethyl-para-phenylenediamine) is oxidized with the participation of microbial oxidase to produce indophenol in a bright blue color. This shows that the isolates we isolate are optional anaerobes and receive energy through substrate phosphorylation (Smirnova, 2010).

The enzymatic activity of the studied microorganisms was detected by the classical method of inoculating the culture into tubes containing the necessary substrates and indicators (Marievsky, 2011).

It was found that all of the moving gram negative rod-shaped bacteria that we selected - did not form spores and capsules. Microorganisms are able to use citrate as the sole carbon source and ammonium hydrophosphate as the sole source of nitrogen, propagating on the Simons medium, the enterobacteriaceae produced alkaline metabolites that cause the color of the indicator to change due to changes in the concentration of ions. A similar mechanism is triggered in differentiating the ability of bacterial cells to ferment sodium acetate. Since acetate in the acetate agar is a source of carbon, the reproduction of bacteria that are not capable of its disposal is inhibited. In turn, our identified *Klebsiella aerogenes* isolate utilizes sodium acetate in the process of vital activity, with the release of alkaline metabolic products, the presence of the latter in the medium changes its color from green to blue.

The effects of metabolic acids can only be neutralized by bacteria that simultaneously utilize sodium malonate and ammonium sulfate to produce sodium hydroxide, whereby the pH remains neutral and the color does not change. Glucose, which is a minimal part of the medium, supports the growth of microbes that are unable to utilize malonate or ammonium salts and cannot maintain alkaline pH, because the acid formed during glucose fermentation neutralizes the sudden alkalinity of the medium (Marievsky, 2011).

To differentiate *Klebsiella aerogenes* from common *Salmonella enterobacteria* and *Klebsiella pneumoniae* species, the isolates were injected with Kligler's beveled agar, which included glucose, lactose, iron sulfate and the phenolic red indicator. Bacteria of the *Klebsiella pneumonia* species form gas bubbles in the thickness of the medium due to glucose fermentation. Enterobacteria of the genus *Salmonella* do not ferment lactose but they break down glucose to produce hydrogen sulfide, which turns out to be blackened in a column of agar.

These microorganisms did not form indole and did not disinfect phenylalanine. Deamination of the amino acid phenylalanine leads to the formation of phenylpyruvic acid, which, when reacted with chlorine iron, forms compounds of a green color, which differ from our results.

Isolated enterobacteria from bees have a wide range of enzymatic properties, which allows the digestion and absorption of carbohydrates and proteins in the insect digestive tract at different times of the year.

According to Serdyuchenko I. V. (2017) it became known that in the beginning of winter young bees never participated in the process of honey harvesting and did not fly out of the hive. In adult bees that go to winter after the autumn collection, in the intestinal tract there is a pathogenic microflora, and, in the adult bees, the quantitative presence of isolated bacteria is much greater than in young bees. This is due to the fact that the bees do not empty during the wintering process and accumulate fecal matter in the thick intestine, which leads to a sharp change in the picture of the microbial landscape (Serdyuchenko, 2017).

The scientific work justified and confirmed our opinion about the dependence of the beginning of the infectious process not only on the abiotic factors but also on the quantitative and qualitative microbial composition of the intestines of bees. In the presence of favorable conditions of existence for bacteria, the concentration of conditionally pathogenic microflora in the gut, including *Klebsiella Aerogenes*, increases. The investigated isolate is aggressive and virulent and can cause disease.

In conclusions: 1. The etiological factor of origin of collapse of bee colonies belongs to the Family *Enterobacteriaceae*, the genus *Klebsiella* – *Klebsiella aerogenes* (previously named *Enterobacter aerogenes*), which was established by the cultural properties (Endo – light pink, small, flat, mucous, matte colonies; Levin – light pink, flat, mucous, matte colonies); by morphological properties (short, sticks, small, placed together and singly, without capsule, mobile); by tinctorial (gram negative) properties and by results of biochemical typing of microorganisms (Kligler medium: glucose – negative, acid-gas H_2S negative; Simons medium – positive, acetate Na – positive, malonate Na – negative; Phenylalanine – negative: Indole –negative; Urease – negative), which were isolated from the intestines of bees and feces from the frames of bee colonies.

2. Isolates of selected cultures were stored in tubes with beveled agar at a temperature of +5°C. Every 15 days they were cultured on the MPA (meat-peptone agar). The cultures were stored in the research laboratory of the Department of Microbiology, Pharmacology and Epizootology, Faculty of Veterinary Medicine, ZhNAEU.

3. This strain of *Klebsiella aerogenes* should be used to study the antagonism of probiotics and disinfectants in relation to this laboratory culture when organizing therapeutic and preventive measures in apiaries in the event of collapse of bee colonies.

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