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MICROFLORA OF INTERNAL ORGANS AND MUSCLES OF LAMBS AND PIGS IN SPONTANEOUS INFECTION WITH *CYSTICERCUS TENUICOLLIS*

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Abstract

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Microbiological investigations for a carrier of pathogenic microorganisms were performed in internal organs of lambs and pigs spontaneously infected with *Cysticercus tenuicollis*, the larval form of *Taenia hydatigena* (Pallas, 1766). Studies were conducted in 2010 and first half of 2011. From the livers of the studied lambs were isolated *Enterobacter agglomerans, Citrobacter diversus, Klebsiella pneumoniae* and *Dichelobacter nodosus*, and of the pigs - *Proteus mirabilis, Pseudomonas fluorescens, Bacillus* sp. and *Clostridium* sp. From the livers of both species were isolated *Pseudomonas cepacia, Campylobacter coli, Enterococcus faecalis, Staphylococcus haemolyticus, Listeria innocua* and *Candida albicans*. Lowest number of species was isolated from the lungs of the tested animals - *E. faecalis* in the lambs and *Streptococcus pneumoniae* in the pigs. Most bacterial species were found in muscles of lamb: *Escherichia coli, Citrobacter freundii, Campylobacter coli*, *Staphylococcus sciuri, E. faecalis* and *Bacillus* sp. The data show that the parasites make prerequisites for penetration and growth of pathogenic microorganisms in internal organs of affected animals. Results from the studies of extracts of infested and healthy livers of lambs for antibacterial effect strongly suggest that *Cysticercus tenuicollis* has inhibitory effects on microorganisms isolated from infested organs. Even more pronounced was that effect on bacteria that were not in contact with this parasite. Microorganisms growing in organs with invasion of *Cysticercus tenuicollis*, to some extent develop resistance and less influenced by inhibiting factors emanating from it.

Key words: Cysticercus tenuicollis, lambs, pigs, microflora, antibacterial effect

Introduction

Bacterial and parasitic liver diseases in farm animals cause losses associated with decreased productivity of affected animals, and forfeiture of damaged organs. Entrance to the microorganisms that cause liver abscesses, necroses and generalized infections is often created from migrating parasites through the liver parenchyma such as *Cysticercus tenuicollis* and *Fasciola hepatica* (Kamburov et al., 1994; Soulsby, 1982). Bacterial diseases of the liver often can lead to disturbances in bile secretion and hepatic functions. In addition, the internal organs and meat from infected animals may be source of food borne infections and intoxications of the consumers (El-Dakhly et al., 2007; Roberts et al., 2005).

Liver damages caused by parasites, including *C. tenuicollis*, create favorable conditions for penetration and growth of pathogenic microorganisms. For instance, the development of necrotic hepatitis caused by *Clostridium novyi* type B, to which are sensitive the small ruminants, often is seen in animals with liver parasites (Abu-Samra et al., 1984). These authors and others (Hamid et al., 1992) have isolated *Clostridium novyi* type B in Sudan from liver samples of goats that died with signs of necrotic hepatitis at once infected

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with *C. tenuicollis* and *Fasciola gigantica*. The authors recommend the introduction of routine vaccination for prevention of necrotic hepatitis, whose development is obviously easier at liver infestations.

Literature data show that traumatically and functional damages of the liver by the migration of young *C. tenuicollis*, can create favorable conditions for local growth of some pathogenic microorganisms. On the other hand, competitive interactions between parasites and microorganisms are possible which would impede the free development of the microbial population. Therefore, this work is focused on conducting research to establish the nature of the microflora, growing jointly with *C. tenuicollis* in the livers of spontaneously infected lambs and pigs, as well as on research for a possible inhibitory effect of products of this larval form to some bacteria.

Material and Methods

Organs. Microbiological tests on 12 samples of internal organs of clinically healthy lambs and pigs with spontaneous infection with *Cysticercus tenuicollis* were performed. Nine of them were from livers, 2 from lungs and one of the muscles. All the animals originated from the same farm near Pernik city.

Of all the samples after burning on their surface and incision with sterile instruments slopes on elective and selective nutrient media for different groups of microorganisms were made.

Nutrient media. 1. Solid: Blood agar, agar in Tsaysler, Folic-azide medium for enterococci, Bordet-Gengou (BUL BIO NCIPD Ltd. – Sofia); Cetrimide, Chapman Stone, Sabouraud (Antisel, Sharlau Chemie S. A., Spain); Chromocult® Coliform agar for Gram-negative bacteria, Campylobacter agar (Merck); Listeria Selective agar (Biolab Zrt., Budapest). 2. Liquid: Soy-casein medium for blood cultures; broth in Mosel, medium of Tarotsi (BUL BIO NCIPD Ltd. – Sofia); Listeria Enrichment Broth (Merck).

Cultures were grown at 37°C for 24-72 h in aerobic, microaerophilic and anaerobic conditions (by anaerob packs with palladium catalyst – $H_2 + CO_2$ – BUL BIO NCIPD Ltd. – Sofia).

Identification of the isolated microorganisms was made by microscopic examination of native (for motility) and stained by Gram and Klet preparations, as well as by reading of cultural characteristics on solid and in liquid media and of biochemical properties using Polymicrotest (BUL BIO NCIPD Ltd. – Sofia) and tests for oxidase and catalase. Isolation and identification of bacteria was conducted in accordance with the international identifier of Bergey (Holt et al., 1994) and of fungi - according Dictionary of the Fungi (Hawksworth et al., 1983).

Extracts. Microbiological tests were performed to determine the antimicrobial activity of extracts from livers of lambs with and without parasites. The organs were homogenized 1:2 with sterile saline solution in a tissue mortar, then filtered successively through a sieve with pores 0,2 and 0,1 mm and sterilized mechanically through bacterial filter with a pore size 0,2 μm (VWR N.A. PN: 28145-477, Europe PN: 514-0061).

Determination of the sensitivity of isolated bacteria to organ extracts, as well as to antimicrobial means was done through the classic agar-gel diffusion method of Bauer et al. (1966). The bacterial suspensions in the exponential growth phase were inoculated at a dose of 2.10^6 cells/ml on Mueller – Hinton agar (Scharlau - Antisel, Bulgaria) with pH 7,2 - 7,4 and thickness of 4 mm in Petri dishes 9 cm in diameter. The extracts were applied through instillation of 0,1 ml in 9-mm wells in agar, and antibiotics - in the form of standard antibiotic discs (BUL BIO NCIPD Ltd. – Sofia) and such prepared by us. Incubation was performed at 37° C for 24 hours. The results were interpreted by the three-degree system of Bauer et al. (1966) after measuring the diameters of inhibitory zones in mm.

In order to study the antimicrobial action of tissue extracts the following nine bacterial strains isolated from the organs tested were used: by one strain of *Pseudomonas fluorescens, P. cepacia, Enterobacter agglomerans, Citrobacter diversus, Staphylococcus haemolyticus, Listeria innocua* and 3 strains of *Campylobacter coli*. The same strains were tested for susceptibility to antimicrobial agents. For comparison of the results about the effects of the infected organs on the bacteria, in the research were included 5 more strains isolated from other animals (dogs) with pyodermatitis: *P. aeruginosa 514*, *P. cepacia*, *S. aureus 319*, *Streptococcus pyogenes 321*, *S. pyogenes 321* and *Enterococcus* sp.

Statistical analysis of the results was made using the classical method of Student – Fisher.

Results

The results of the performed microbiological tests are summarized in Table 1.

As seen from the data presented in the organs tested prevalent Gram-negative bacteria, which are 10 in number. Half of them belong to the family *Enterobacteriaceae* and refer to genera *Enterobacter, Citrobacter, Klebsiella, Proteus* and *Escherichia*. Species of the genus *Pseudomonas*, as well as *Campylobacter coli*, were found in the livers of animals of both species. Out of Gram-positive bacteria dominated *Listeria innocua*, streptococci (*Enterococcus faecalis, Streptococcus pneumoniae*), staphylococci (*S. haemolyticus* and *S. sciuri*) and some bacilli and clostridia. Out of fungi, only *Candida albicans* was isolated, found in the livers of pigs and two lambs.

The results of the tests for antibacterial activity of extracts of the infected and the healthy livers of lambs to isolated microorganisms are summarized in Table 2.

From the data in the table it is seen that at the first group of bacteria isolated from infected organs, areas of growth inhibition around the wells with the extract of infected livers were larger than those around the wells, in which was applied extract from healthy organ.

Table 1
Results of microbiological examinations of internal organs of lambs and pigs spontaneously infected with *Cysticercus tenuicollis*

Sample N	Origin	Isolated microorganisms
1	5 lambs, livers	*Pseudomonas cepacia, Enterococcus faecalis*
2	5 lambs, livers	*Enterobacter agglomerans 3, Campylobacter coli, Dichelobacter nodosus, Listeria innocua, Candida albicans*
3	5 lambs, livers	*Pseudomonas cepacia, Staphylococcus haemolyticus, Listeria innocua, Candida albicans*
4	Pig, liver	*Campylobacter coli, Staphylococcus haemolyticus, E. faecalis*, *Listeria innocua, Clostridium* sp., *Bacillus* sp.
5	Pig, liver	*Pseudomonas cepacia, Campylobacter coli, Enterococcus faecalis*, *Clostridium* sp.
6	Lamb, liver	*Klebsiella pneumoniae, Citrobacter diversus, Listeria innocua*
7	Lamb, lung	*Enterococcus faecalis*
8	Pig, liver	*Pseudomonas fluorescens, Proteus mirabilis, Candida albicans*
9	Pig, liver	*Candida albicans*
10	Pig, lung	*Streptococcus pneumoniae*
11	Lamb, liver	*Dichelobacter nodosus, Listeria innocua*
12	Lamb, muscle	*Escherichia coli, Citrobacter freundii, C. coli, Staphylococcus sciuri, E. faecalis, Bacillus* sp.

Table 2
Results from studies of extracts of spontaneously infected and healthy livers of lambs for antibacterial action

Groups of strains tested	Number	Diameters of inhibitory zones in mm of extracts from the lamb livers	
		Infected	Healthy
I. Isolated from organs infected with *C. tenuicollis*	9	12.5 ± 0.5	11.3 ± 0.3
II. Isolated from other animals (dogs with pyodermatitis)	5	21.5 ± 3.2	-

Although the differences were not big, they were statistically significant (P<0.01). Considerably larger were the diameters of sterile areas at studied bacteria isolated from other animals that have not been in contact with the parasite. Differences in results obtained in bacteria from the first group had high statistical significance (P<0.001).

Data obtained in studies of susceptibility to antibacterial agents of isolates from infected organs are presented in Table 3. They make clear that the studied microorganisms show high sensitivity to aminoglycoside antibiotics, to chemotherapeutics (potentiated sulfonamides Sulfamethoxazole+Trimethoprim and the quinolones Ciprofloxacin and Enrofloxacin), and most also to the broad spectrum Tetracycline and Thiamphenicol. Resistance is seen to the β-lactam antibiotics (penicillins and cephalosporins), to the lincosamides Lincomycin and Clindamycin, and to Novobiocin.

Discussion

The microorganisms isolated by us from the organs of lambs and pigs spontaneously infected with *Cysticercus tenuicollis*, are potential pathogens and under favorable conditions associated with demotion of the total or local resistance of hosts can cause purulent infections of different localization, gastroenteritis, respiratory, urogenital, visceral and other diseases in animals and humans. At the same time, they are characterized by easy development of resistance to antimicrobial drugs. This applies not only to the isolated bacteria of the family *Enterobacteriaceae*. Similar pathogenic and even higher adaptive properties have the representatives of the genus *Pseudomonas*, which were located in the liver of animals of both species. Interestingly, from none of the samples *Escherichia coli* was not isolated, to which seems is difficult to adapt to coexistence with *C. tenuicollis*. *Campylobacter coli* often is part of the normal microflora of the intestinal tract of sheep and pigs, but can also cause enteritis. In the liver of sheep, also *Dichelobacter nodosus* was found. This species is involved in the etiology of ungulate rot, which is problematic disease in these animals. As an obligate anaerobe, it is apparently favorable in this case. Found streptococci and staphylococci are also characterized by high resistance to adverse physical

Table 3
Sensitivity of nine bacterial strains isolated from infected organs to antimicrobial agents *in vitro*

Antimicrobial agent	Content of the disk, μg	Sensitivity of the strains, %		
		S	I	R
Thiamphenicol	30	67	0	33
Tetracycline	30	78	11	11
Lincomycin	15	33	56	11
Clindamycin	2	22	22	56
Penicillin G	10 u	0	22	78
Oxacillin	1	0	0	100
Amoxycillin	20	45	22	33
Cefuroxim	30	33	11	56
Streptomicin	10	78	22	0
Novobiocin	30	0	0	100
Gentamicin	10	100	0	0
Kanamycin	30	100	0	0
Amikacin	30	100	0	0
Ciprofloxacin	5	100	0	0
Enrofloxacin	5	100	0	0
Sulfamethoxazole+Trimethoprim	23.75/1.25	89	0	11
Penicillin+Streptomicin	10 u/10	0	55	45

S-sensitive; I-intermediate; R-resistant

and chemical factors, when the enterococci are the most resistant and can even grow under extreme environmental conditions (high and low pH, high concentrations of salts, bile acids and chemicals). Bacilli and clostridia are even able in form of spores to survive the most adverse impacts of a different nature. The presence of listeria had likely exogenous origin. *Listeria innocua* is meeting in the environment (plants, feed, soil, water) and falls in the digestive tract mainly by silage, in which can be propagated. It also has a high resistance to adverse effects, but is not a health hazard (Buchrieser et al., 2003; Jemmi and Stephan, 2006). The lactic acids bacteria to which relate listeria too, are often the main microorganisms that develop in the livers of slaughtered animals (Roberts et al., 2005). *Candida albicans* in small quantities is considered as a part of the normal microflora, but during disbakteriosis it can cause severe deep mycoses and abscesses in internal organs. Similar to these bacteria were found in muscles of lamb. Other authors also establish microorganisms from these groups as part of the microflora in the carcasses of lambs (Roberts et al., 2005; Feyzulah, 2011).

El-Dakhly et al. (2007) in Egypt also established bacterial infections in most of the tested samples of infected livers of ruminants, some of which - with *C. tenuicollis*, and others - with *Fasciola gigantica*. They most frequently isolated *Enterococcus* sp., followed by staphylococci and bacteria of the genera *Escherichia* and *Lactobacillus* and occasionally *Enterobacter, Micrococcus, Citrobacter, Aerococcus, Pseudomonas, Chrysomonas* and *Streptococcus*. According to them, the control of parasitic diseases reduces the risk of some important bacterial infections in animals.

In our studies were not found pathogenic clostridia such as *Clostridium perfringens* and *C. oedematiens*. For this obviously an important role has the regular use of combination vaccines with the participation of these agents in the farm from which the test animals originate.

Karasev and Stepanova (1980) reported that after experimental invasion with *C. tenuicollis* there was no decrease in quality of meat, except after administration of high doses above 500 and 1000 ova / kg body weight. However, in our opinion the microorganisms can be a significant factor affecting adversely in this direction, for which penetration and development conditions are created by parasites in damaged organs.

The results of the antibacterial activity of extracts of infected and healthy livers of lambs to the isolated microbial species clearly show that *C. tenuicollis* emits chemical substances that inhibit bacterial growth adjacent to it. This is especially clear from the data obtained in the second group studied microorganisms that have not been in contact with this parasite. However, obviously with *C. tenuicollis* cohabitate bacteria that develop resistance and less influenced by inhibiting factors emanating from it. Perhaps with the parasite develop only those microorganisms that succeed to adapt to antibacterial substances emitted from it. This is evident from the results received during the study of sensitivity of microbial strains with different origin, which had no contact with *C. tenuicollis*.

The established similarity in susceptibility to antimicrobial means of bacteria isolated from animals apparently because the animals come from the same farm and have been subjected to therapeutic treatment with the same agents to which bacteria have developed resistance. This result gives reason to believe that the microorganisms isolated from infected organs are not an incidental finding, but are part of the microflora of the farm. It comes to microorganisms with greater resistance to chemical agents, including a number of antibiotics and capable to withstand adverse chemical effects such apparently broadcast cysticerci, probably as waste products of their metabolism.

Conclusions

During its penetration and development in the liver of intermediate hosts the larvae of *Taenia hydatigeha* create a gateway for microorganisms. *Cysticercus tenuicollis* releases substances with antimicrobial action that restrict the development of microflora in the infected organs. The extract from the livers of sheep with *Cysticercus tenuicollis* has an antimicrobial effect. It is most likely due to inhibitors released by larvae or to competitive relationship between microorganisms and parasites in terms of nutrients and living space at simultaneous penetration in the organs.

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