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Original Research Articles

Case Report

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Original Research Article

TELENCEPHALON VASCULARITY IN DOG (*Canis lupus* f. *familiaris*)

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Abstract: The studies of the vascularization of the cerebrum in dog were performed on 80 cerebral hemispheres. It was found that the middle cerebral artery is the strongest vessel supplying blood to the cerebrum. The artery gets divided into ten permanent branches. Two olfactory arteries supply the region of the cerebrum located on the border between the old and the new cortex. The other eight supply the region of the new cortex: three branches aiming at the frontal lobe, two branches at the parietal lobe and three temporal branches aiming at temporal area. The frontal, parietal and temporal branches descended independently from the main trunk of the middle cerebral artery or formed a common trunk. Common trunks for respective groups of branches have been described as the rostral, dorsal and caudal middle cerebral artery. In 2.5% of cases there were two independent branches of the middle cerebral artery extending from the rostral cerebral artery.

Key words: brain arteries; dog; vascularity; variability

Introduction

A review of the literature shows that the basic morphological publications in the field of vascularization of the brain were provided by Hofman (1) and Jenke (2), where one may find the first information on the construction of the middle cerebral artery in the dog. More information about the construction of the middle cerebral artery and its branches in the dog are found in the publication of Hebermehl (3). The author dealt with the construction of the artery only and discussed the topography of its branches on the surface of the telencephalon, ignoring its variability. The literature concerning the blood supply to the brain describes the dog (4,5). These authors mention that the middle cerebral artery is one of the vessels departing from the arterial circle of the brain. In other predatory species similar studies were carried out in the cat (6), in the raccoon dog (7). In the literature, there are publications outlining in detail the cortical branches of the middle cerebral artery. This problem was described by Chadzypanagiotis in cat (8), the author gives nomenclature for individual cortical branches of the artery. Structured descriptions of the construction and the course of the cortical branches of the middle cerebral artery in some predatory species were presented by Wiland (9). In recent years there have been numerous studies

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that discuss the construction of the middle cerebral artery in various mammalian species. This applies to vessels which isolate as a single branch, for example, in red squirrel (10), in ground squirrel (11), in otter (12) and multiple arteries presented in domestic pig (13). These publications stated that cortical branches of the middle cerebral artery in examined species attained the same areas of the telencephalon. The differences occur in the pattern of descent and division of respective cortical branches of the middle cerebral artery. The pattern of division of the middle cerebral artery is affected by how the species has been classified and the pattern of groove-coverage of the cortex. In mammals on the surface of the cortex there is a different pattern of sulci, which can affect the structure of the cortical branches of the middle cerebral artery (14). In vast literature there seem to be missing a paper on the cortical branches of the middle cerebral artery in dog. Considering the discrepancy resulting from respective descriptions and considering new studies, one has decided to investigate the pattern, the division and variation of cortical branches of the middle cerebral artery in dog and to compare the results with the data reported by other authors.

Materials and methods

The research was performed on 40 brains in dog, namely a total of 80 cerebral hemispheres received from animal shelters in Bydgoszcz. Ethics approval was not required since animals died because of natural reasons. The animal heads were cut off at the height of the 3rd – 4th cervical vertebrae. The arteries were filled with latex introduced with medical syringe into the common carotid artery. This method was described by Godynicki (15). The heads were fixed in a 5% formalin solution for 3 months, and then decalcified in hydrochloric acid, the skull cavity was opened and brains were taken out. The cerebral hemispheres were photographed and the following were being described: the anatomy, the division pattern and the course of cortical branches of the middle cerebral artery.

Results

In dog the blood is supplied to the brain with internal carotid arteries (Fig. 1-a) and vertebral arteries.

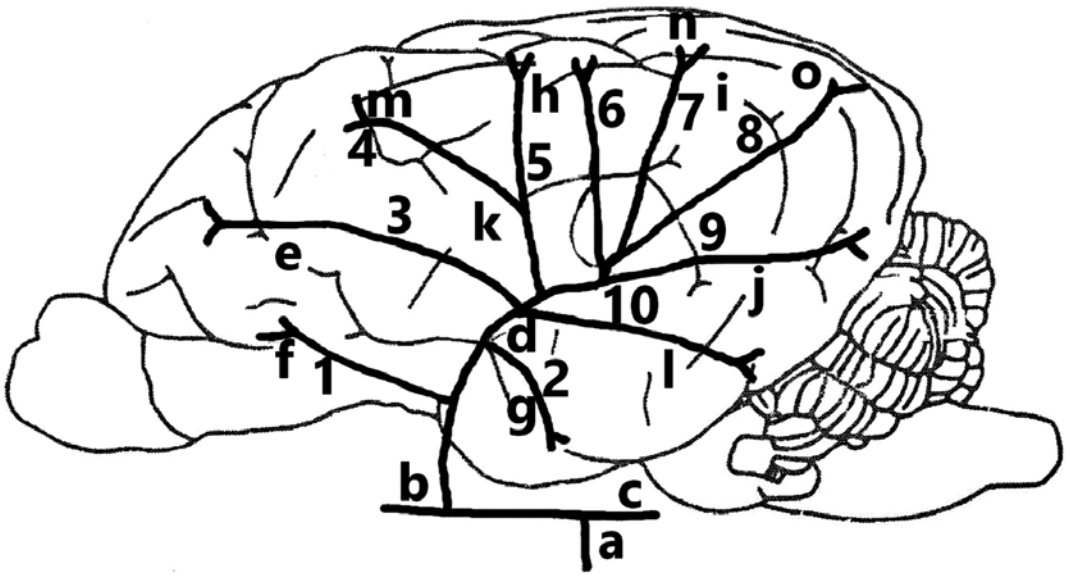

Figure 1: Diagram of the division of the middle cerebral artery on the surface of the cortex in dog

1 – rostral olfactory artery, 2 – caudal olfactory artery, 3 – orbital branch, 4 – inferior frontal branch, 5 – dorsal frontal branch, 6 – rostral parietal branch, 7 – caudal parietal branch, 8 – dorsal temporal branch, 9 – middle temporal branch, 10 – ventral temporal branch, a – internal carotid artery, b – rostral cerebral artery, c- caudal communicating artery, d – Sylvian fissure, e – Presylvian sulcus, f – rostral lateral olfactory sulcus, g – caudal lateral olfactory sulcus, h – rostral Suprasylvian sulcus, i – middle Suprasylvian sulcus, j – caudal Suprasylvian sulcus, k – caudal external Sylvian sulcus, l – middle external Sylvian sulcus, m – coronary sulcus, n – marginal sulcus, o – external marginal sulcus.

The internal carotid artery, having entered the skull cavity and penetrated the dura mater, bifurcates into the rostral cerebral artery (Fig. 1-b) and caudal communicating artery (Fig. 1-c) which, together with their symmetrical vessels form an arterial circle of the brain.

From the initial section of the rostral cerebral artery towards the cortex there separates the middle cerebral artery.

The middle cerebral artery is the strongest vessel supplying blood to the cerebrum. The initial section of the main trunk of the middle cerebral artery goes along the dorsal surface of the optic tract. Then the section gets bended around the piriform lobe and goes through its rostral margin. Further on it runs to the lateral olfactory sulcus and, having passed it, it gets divided. From the initial section of the main trunk of the middle cerebral artery there descend minor central branches supplying blood to olfactory tracts and the piriform lobe. The main trunk of the middle cerebral artery gets divided into a number of cortical branches which run to the specific region of the cerebral hemisphere, supplying blood to specific regions of the brain.

The first permanent branches of the middle cerebral artery which supply both the old and the new cortex are olfactory arteries.

The rostral olfactory artery (Fig. 1-1), having separated from the main trunk of the middle cerebral artery it creates an arch and runs to the rostral part of the lateral olfactory sulcus it can ascend into in various places. Its terminal branches can also appear again from under the lateral olfactory sulcus and then ascend under the cortex surface.

The caudal olfactory artery (Fig. 1-2) ascends into the caudal part of the lateral olfactory sulcus and its terminal branches supply the area of the cortex found under the sulcus.

The other branches of the middle cerebral artery supply the areas of the cortex over the lateral olfactory sulcus. On the cortex towards the frontal lobe there spread three thick branches. As the first one there separates the orbital branch (Fig. 1-3) which is located lowest and it goes towards the region of the Presylvian sulcus where its terminal branches reach the coronary sulcus.

The ventral frontal branch (Fig. 1-4) vascularizes the middle part of the frontal lobe. The vessel goes through the rostral external Sylvian sulcus and the rostral Suprasylvian sulcus towards the coronary sulcus it passes towards the fornix.

The dorsal frontal branch (Fig. 1-5), having separated from the middle cerebral artery at the height of the rostral external Sylvian sulcus, goes up to the region of the cruciate sulcus. The vessel supplies blood to the upper part of the medial surface of the frontal lobe.

The next vessel which runs towards the parietal lobe bifurcates into two branches.

The rostral parietal branch (Fig. 1-6) runs towards the middle external Sylvian sulcus to the marginal sulcus. The terminal twigs of that vessel supply blood to the area of the cortex found under the ansiform sulcus.

The caudal parietal branch (Fig. 1-7) also runs to the region of the marginal sulcus and further on it branches out into smaller vessels. Some of them ascend into the medial Suprasylvian sulcus.

The lateral-caudal surface of the cerebral hemisphere is supplied by the branches of the middle cerebral artery which descend from at various heights and they are referred to as temporal branches.

The dorsal temporal branch (Fig. 1-8) having left the Sylvian fissure, it runs towards the middle Suprasylvian sulcus and further to the upper margin of the cerebral hemisphere. It is uasually the strongest cortical branch of the middle cerebral artery. The branch supplies blood to the upper part of the cortex.

The middle temporal branch (Fig. 1-9) descends a small distance away from the previous branch. The branches of that vessel spread towards the external marginal sulcus. Its terminal branches go onto the surface of the occipital lobe.

The ventral temporal branch (Fig. 1-10) runs to the end of the caudal external Sylvian sulcus. Having passed the caudal part of the sulcus, its branches spread towards the caudal Suprasylvian sulcus. Its terminal branches take part in the supply of a part of the occipital lobe.

Considering the general pattern of the spread the cortical branches of the middle cerebral artery in dog, one shall note that respective sections of those branches can run inside respective sulci and divide, always running towards the cortex areas described.

Analysing the pattern of descent of the cortical branches of the middle cerebral artery in the dog, it was found that from the rostral cerebral artery on 78 (97,5%) cerebral hemispheres there descended a single independent vessel - the middle cerebral artery. Among them on 8 (10%) hemispheres from the main trunk there descended rostrally with a

common trunk: the orbital branch, the ventral frontal branch and the rostral olfactory artery. The main trunk of the middle cerebral artery, got onto the surface of the cerebral cortex and formed a common descent for the dorsal frontal branch as well as rostral and caudal parietal branches. Caudally from the main trunk of the middle cerebral artery, with a common trunk there separated the dorsal, middle and ventral temporal branches and the independent caudal olfactory artery. (Fig. 1).

In another 12 (15%) cases there descended rostrally an independent rostral olfactory artery and a common trunk for the orbital, rostral and dorsal frontal branches. The main trunk got onto the surface of the cerebral cortex from the Sylvian fissure and formed a common descent for rostral and caudal parietal branches. Caudally from the main trunk of the middle cerebral artery, with a common trunk there separated the dorsal, middle and ventral temporal branches, whereas the caudal olfactory artery got separated independently from the main trunk of the middle cerebral artery.

On another 12 (15%) hemispheres from the main trunk of the middle cerebral artery there separated rostrally a common trunk for the rostral olfactory artery and for the orbital branch as well as the common descent for the ventral and dorsal frontal branch. The main trunk separated caudally the caudal olfactory artery with a common descent with the ventral temporal branch. The main trunk, having ascended into the Sylvian fissure, on the surface of the cortex it showed a common trunk for rostral and caudal parietal branches as well as for the middle and dorsal temporal branches.

On another 14 (17,5%) cerebral hemispheres from the main trunk of the middle cerebral artery departed the independent rostral olfactory artery and a common departure for the ventral and dorsal frontal branches. Caudally from the main trunk there descended the middle and ventral temporal branch through the common trunk with the caudal olfactory artery. The main trunk, having descended into the Sylvian fissure, got onto the surface of the cortex with a common descent for rostral and caudal parietal branches as well as the rostral and dorsal temporal branch.

On another 12 (15%) hemispheres from the main trunk the following separated rostrally with a common trunk: the rostral olfactory artery, common trunk for the orbital branch, the ventral frontal branch. The caudal branch was a common descent for the ventral temporal branch and the caudal olfactory artery. The main trunk, having descended into the Sylvian fissure, got onto the surface of the cortex with a common descent for the dorsal frontal branch, rostral and caudal parietal branches as well as the middle and dorsal temporal branch.

On yet another 6 (7,5%) cerebral hemispheres from the main trunk rostrally there separated, with a common descent, the orbital branch, the ventral and dorsal frontal branch and the rostral olfactory artery. Caudally from the main trunk of the middle cerebral artery the following separated with a common descent: rostral and caudal parietal branches as well as the ventral, middle and dorsal temporal branches. The caudal olfactory artery departed independently from the main trunk.

On another 14 (17,5%) cerebral hemispheres from the main trunk of the middle cerebral artery the following departed rostrally with the common trunk: the orbital branch, the ventral frontal branch and the rostral olfactory artery. Caudally from the main trunk of the middle cerebral artery there descended with the common descent: the dorsal frontal branch, the rostral and caudal parietal branches and the dorsal, middle and ventral temporal branches. The caudal olfactory artery departed independently from the main trunk.

On the other 6 (7,5%) hemispheres it was found that from the main trunk of the middle cerebral artery there departed the common trunk for the rostral olfactory artery and the orbital branch, then the common descent for the ventral and dorsal frontal branch. The main trunk got onto the surface of the cortex with a common descent for rostral and caudal parietal branches as well as the dorsal, middle and ventral temporal branch. The caudal olfactory artery departed caudally from the main trunk of the middle cerebral artery as an independent vessel.

On the other 2 (2,5%) hemispheres it was found that from the rostral cerebral artery in dog there bifurcated two independent branches of the middle cerebral artery. Among them the first independent branch from the rostral cerebral artery was the rostral olfactory artery, while the second branch was the main trunk of the middle cerebral artery from which there descended rostrally independently: the orbital branch, the ventral and dorsal frontal branch. Caudally from the main trunk there separated an independent caudal olfactory artery and the ventral temporal branch. The main trunk, having descended into the Sylvian fissure, got onto

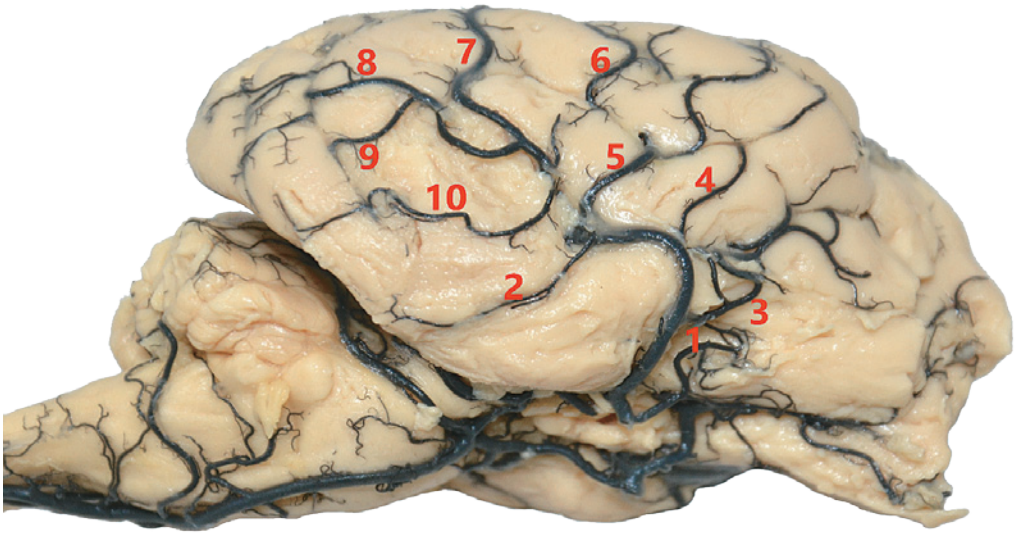

Figure 2: Independent departure of the anterior olfactory artery and the main trunk of the middle cerebral artery from the rostral cerebral artery

1 - rostral olfactory artery, 2 - caudal olfactory artery, 3 - orbital branch, 4 - ventral frontal branch, 5 - dorsal frontal branch, 6 - rostral parietal branch, 7 - caudal parietal branch, 8 - dorsal temporal branch, 9- middle temporal branch, 10 - ventral temporal branch.

the surface of the cortex with a common descent for rostral and caudal parietal branches as well as the common trunk for dorsal and middle temporal branch (Fig.2).

On the other 2 (2,5%) hemispheres it was found that from the rostral cerebral artery in dog there bifurcated two independent branches of the middle cerebral artery. Among them the first independent branch from the rostral cerebral artery was the rostral olfactory artery, while the second branch was the main trunk of the middle cerebral artery from which there descended rostrally independently: the orbital branch, the ventral and dorsal frontal branch. Caudally from the main trunk there separated an independent caudal olfactory artery and the ventral temporal branch. The main trunk, having descended into the Sylvian fissure, got onto the surface of the cortex with a common descent for rostral and caudal parietal branches as well as the common trunk for dorsal and middle temporal branch (Fig.2).

Discussion

The middle cerebral artery supplies blood to the greatest region of the cerebrum and is the most shaped branch extending from the rostral cerebral artery. In dog the middle cerebral artery supplies the same areas of the brain as in the mammalian species studied so far. The discrepancies concern mostly its division into respective branches. Chadzypanagiotis (8), describing the cortical branches in cat, differentiated between the branches supplying the old cortex, the branches on the border of the old and the new cortex as well as the branches for the new cortex. In dog the arteries supplying the old cortex are minor branches onto the piriform lobe and olfactory tracts. On the border of the old and the new cortex there are found the rostral and caudal olfactory arteries. In dog the rostral olfactory artery in 2.5% of the cases was a vessel which descended independently from the rostral cerebral artery. On the other cerebral hemispheres it was a vessel which got separated independently from the main trunk of the middle cerebral artery in 32.5% of the cases. In 22.5% of the cases it formed a common departure with the orbital branch. On the 35% of the cerebral hemispheres it was one of the branches descending from the common trunk of the middle cerebral artery which gave rise to the orbital branch and the ventral frontal branch. In the other 7.5% cases the rostral olfactory artery demonstrated a common descent with the orbital, ventral and dorsal frontal branches.

The caudal olfactory artery, on the other hand, in 45% of the cases was a vessel which descended independently from the rostral cerebral artery. On 37.5% the caudal olfactory artery separated with a common descent with the ventral temporal branch. In the other 17.5% hemispheres the caudal olfactory artery was one of the branches of a common trunk for ventral temporal branch. In another 20% of the cases it was one of the branches of the common trunk for the ventral and middle temporal branches.

The other cortical branches of the middle cerebral artery can be divided into a group of frontal, parietal and temporal branches. In dog, similarly as in other Carnivora species there occur eight main vessels which supply blood to the area of the new cortex of the cerebrum.

Besides, respective cortical branches can descend from the main trunk of the middle cerebral artery with a common descent. Such cases of descent were reported by Wiland (9), Skoczylas et al. (12) as the rostral, dorsal and caudal middle cerebral artery. In dog the rostral middle cerebral artery has been presented as a common trunk for frontal branches and it occurred in 32.5% of the cases investigated, the dorsal middle cerebral artery was described as a common trunk for parietal branches, which was observed in 17.5% of the cases. The caudal middle cerebral artery as a common trunk for temporal branches was found in 25% of the cases.

In dog the dorsal middle cerebral artery occurred as the lowest percentage of the cases, however, here the rostral middle cerebral artery dominated. Making a comparison of the present results with those reported by Wiland (9) in American mink, and by Skoczylas et al. (12) in otter one can state the dorsal middle cerebral artery was reported as the lowest percentage of the cases. In dog, similarly as in the other animal species studied, the parietal branches have developed poorest. On the surface of the cerebrum the best developed are the frontal branches of the middle cerebral artery.

From the description of the structure of the middle cerebral artery in the publications by Aydin et al. (11), Ozudogru et al. (16), Skoczylas et al. (12) in the ground squirrel, common fox and otter one can see that it is usually a single vessel descending from the rostral cerebral artery. The vessel, having passed the lateral olfactory sulcus, gets divided along its course into respective cortical branches. In the material investigated such a pattern of division of the middle cerebral artery was found in 97.5% of the cases. In dog there were identified the cases of descent from the rostral cerebral artery of two independent arterial trunks in 2.5% of the cases. The second independent branch from the rostral cerebral artery was the rostral olfactory artery. In other mammalian species the presence of two independent descents of the branches of the middle cerebral artery was found in rabbit in 31.4% (17), in wild rabbit (18) in 36.5% of the cases, in raccoon dog (7) in 18.6% of the cases.

The present research show that observed in dog the division of the middle cerebral artery into the same branches or their groups, like in the other mammalian species investigated so far is, according to Wiland (19), a result of genetic limitations.

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OŽILJENOST TELENCEFALONA PRI PSIH *(Canis lupus* f. *familiaris)*

K. Kirkiłło-Stacewicz, W. Nowicki, J. Wach

Izvleček: : Študije ožiljenosti možganov pri pseh so bile izvedene na 80 možganskih poloblah. Ugotovljeno je bilo, da je srednja možganska arterija najmočnejša žila, ki dovaja kri v možgane. Arterija se razdeli na deset stalnih vej. Dve vohalni arteriji napajata predel možganov, ki se nahaja na meji med staro in novo možgansko skorjo. Ostalih osem arterij oskrbuje področje nove skorje: tri veje, ki potekajo do prednjega režnja, dve veji, ki potekata v parietalni reženj in tri temporalne veje, usmerjene v temporalno področje. Čelne, parietalne in temporalne veje so se razvejale neodvisno od glavnega debla srednje možganske arterije, ali pa so tvorile skupno deblo. Običajna debla za posamezne skupine vej so opisana kot rostralna, dorzalna in kavdalna srednja možganska arterija. V 2,5 odstotkih primerov sta obstajali dve neodvisni veji srednje možganske arterije, ki izhajata iz rostralne možganske arterije.

Ključne besede: možganske arterije; pes; ožiljenost; raznolikost

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Original Research Article

COMPARISON EFFICACY OF SYNTHETIC CHEMICALS AND PLANT EXTRACTS FOR TICK CONTROL

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Abstract: Ticks are considered as harmful and economically important ectoparasites because their infestation seriously affects the cattle worldwide. Tick control with synthetic acaricides is not only dangerous for animal and human health but also causes environmental pollution. The present study was designed to evaluate the plant extracts in comparison with synthetic acaricides to control *Hyalomma anatolicum*. Five different concentrations (50, 100, 250, 500 and 750 ppm) of methanolic plant extracts and acaricides, were employed to evaluate the mortality of ticks after 2, 4, 6, 12, 24 and 48 hrs. Mortality data was analyzed through Probit analysis to calculate the median lethal concentration (LC_{50}) and the median lethal time (LT_{50}). Methanolic extract from *Azadirachta indica* demonstrated the highest mortality (LC_{50} = 38.3 ppm) of ticks as compared to *Dalbergia sissoo* (LC_{50} = 58.76 ppm) and *Morus alba* (LC_{50} = 92.95 ppm). Amongst acaricides, fipronil exhibited highest mortality (LC_{50} = 35.01 ppm) when compared with emamectin (LC_{50} = 46.87 ppm) and cypermethrin (LC_{50} = 37.83 ppm). Higher concentration (750 ppm) of acaricides (fipronil, emamectin and cypermethrin) displayed quicker mortality (LT_{50} = 6.53-8.95 hrs) as compare to the plant extracts (LT_{50} = 8.49-29.17 hrs). Effects of these treatments were also studied on egg masses and reproductive index (RI) of the surviving ticks. The results revealed a significant, concentration-dependent variation among the egg masses treated with plant extracts and acaricides; and subsequently, their reproductive index values also decreased significantly. Phytochemical analysis of the tested plant extracts revealed presence of flavonoids, steroids, terpenoids, saponins, tannins and phenols in variable quantities. Conclusively, our results describe a significant scope of environment friendly plant extracts for ticks' management.

Key words: plant extracts; synthetic acaricides; tick mortality

Introduction

Ticks are blood sucking ectoparasites that act as vectors of diseases like rickettsiosis, anaplasmosis, tularemiosis, babesiosis and theileriosis in meat and dairy animals (1,2). The ectoparasites harm the hosts both directly (blood loss and reduction in weight gain) and indirectly (act as vectors for a wide range of viral, bacterial and protozoan pathogens to humans and domestic animals) (3,4). Their infestation leads to weaken the animals with poor growth and result in substantial economic loss (5,6). An estimated loss of 14-19 billion USD per year is reported because of tick borne diseases with a worldwide infection of 80% cattle population (7). The most important pathogen observed is Crimean Congo hemorrhagic fever virus usually associated with ticks of genus *Hyalomma*. Many outbreaks of this disease have been recorded from Pakistan (7).

Various chemical acaricides (chlorinated hydrocarbons, synthetic pyrethroids, organophosphates, formamidines and macrocyclic lactones) are used by pest exterminators to control ticks. But many problems are associated with these acaricides such as acaricidal resistance in ticks and long residual effects in milk and meat that cause health hazards for human beings. These acaricides also contaminate environment and water, so cause harmful effects to nontarget organisms (8). The chemical

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pesticides are much expensive products and are concerned with ecological threats. So, pesticide usage has forced scientists to find out less harmful and inexpensive chemicals. They have made great contributions to develop a substitute and found natural products as alternative source of synthetic acaricides (9). The naturally occurring plants are used as ethno-veterinary medicine. Botanical products when applied show insufficient adverse effects on non-target organisms as well as on the environment (10) as compared to the synthetic insecticides due to their low toxicity. Pesticidal products of plant origins have been found remarkably effective in the form of antifeedants, repellents, protectants and growth disrupting hormones as other biocides (11). Neem (*Azadirachta indica*) is extensively distributed in Africa, Asia and other tropical areas of the world. A variety of chemicals (azadirachtin, salannin, meliantriol, nimocinolide, isonimocinolide and triterpenoids) are present in neem extract. The neem seed extract usage was recognized for poor farmers as a potential source to control ticks particularly in cattle (12). *A. indica* is effective to be used for tick control in both dry and humid areas (13). *Dalbergia* genus has 300 species of which almost 25 species exist in India. Many species of *Dalbergia* are considered as vital timber trees, appreciated for their attractive and fragrant wood and are rich in aromatic oils (14). Bark of this tree is employed as antihelmintic, aphrodisiac, antipyretic, abortifacient, expectorant and is also used for treatment of blood diseases, dysentery and leukoderma, whereas seeds' oil is employed to treat scabies and the leaves extract has analgesic, antihelmintic and antipyretic properties (15). *Morus* contains over 150 species and among these, *Morus alba* L. is dominant and indigenous to Pakistan, Nepal, India, China and Japan (16). It is widely cultivated all over the plains of both Pakistan and India, and also on the mountains of Himalaya up to 3,300 m altitude for the purpose of its foliage, as a source of food for silkworms. Its extract represented a strong activity against gram-negative, gram-positive bacteria and fungi due to high pesticidal activity (17). Many reports provide information of different plant extracts possessing pesticidal properties and thus could be used against ticks. Plant extracts carry phytochemical constituents that have potential to control ticks population as effectively and equally as synthetic acaricides. Besides these, plant products (botanical pesticides) are considered environment friendly, safe to non- target organisms, and are inexpensive to be used by livestock owners and farmers. So, the present study was carried out to compare the efficacy of some selected synthetic acaricides and plant extracts.

Materials and methods

Collection of plant materials

Fresh leaves of *Azadirachta indica*, *Dalbergia sissoo*, and *Morus alba* were collected and identified by a botanist. The collected leaves were washed thoroughly with tap water and dried under shade for a span of one month. The dried leaves were then chopped and ground to powder form using an electric grinder (Anex Germany, TS-639).

Preparation of plants extract

The plant extracts were prepared by dissolving 500 grams of powdered material of each selected plants individually in methanol in a beaker at room temperature. The beaker was covered with aluminium foil and stirred daily for seven days. Afterwards, the material was filtered by Whatman No.1 filter paper and the solvent (methanol) was evaporated in rotary evaporator for 30 minutes at 60 ºC. After evaporation, the material was placed overnight in the incubator set at 40 ºC to evaporate remaining methanol.

Preparation of stock solution and dilutions

Stock solutions of each plant extract was prepared by dissolving 0.5 mg of the extracted material in a few drops of Dimethyl sulfoxide (DMSO) and then topped up with saline to make solutions of 0.25 mg/ml. The stock solution was then used for preparing different concentrations (50, 100, 250, 500 and 750 ppm) (5). Three different acaricides; emamectin (Tycon 1.9% EC, Four Brothers Group, Pakistan), fipronil (Regent 50 SC, Bayer Pakistan (Pvt.) Ltd.) and cypermethrin (Bulletin 10% EC, Ali Akbar Group, Pakistan) were purchased from the market and different concentrations (50, 100, 250, 500 and 750 ppm) were prepared for bioassay tests (18).

Collection and storage of ticks

The ticks were collected from rural area of Samundri (31°03′45″N 72°57′15″E), district Faisal-

abad from buffaloes with forceps having gloves on hands. Ticks were stored in sterile glass bottle with muslin cloth on the top (2). Ticks were then shifted in the department of Zoology, Government College University, Faisalabad for identification (19) and rearing. *Hyalomma anatolicum* were reared on rabbits for bioassay tests (8). For reproductive index calculation, a total of 20 *H. anatolicum* were weighed individually after washing with distilled water and drying with filter paper, and were placed in glass tubes covered with muslin cloth. Each glass tube, containing a single mated female *H. anatolicum* was kept in an incubator set at 28 ºC and 90% RH for oviposition. After 20 days, the eggs laid within the first 3-4 days were counted, weighed and transferred into 5-ml sterile glass tubes for hatching under same conditions (28 ºC and 90% RH). After 25-27 days, the hatched larvae were counted again (20).

Adult immersion test (Bioassay)

Adult immersion test (AIT) was performed as per the protocol described by Drummond et al. (21). Five replicates of each treatment were used for bioassay tests with 20 ticks in each treatment. Ticks were immersed in the solution (10 ml) at room temperature for two minutes in a 25 ml beaker with gentle agitation. Water was used as control treatment and the treated ticks were recovered from the solution, dried with absorbent paper and were placed in separate plastic specimen tubes (25 mm×50 mm). These tubes were incubated at 28±1°C and 85±5 per cent relative humidity in a biological oxygen demand (BOD) incubator. Ticks treated with different concentrations of the plant extracts and commercial acaricides were compared with the control ticks and the mortality of ticks was observed after 2, 4, 6, 12, 24 and 48 hrs period. The glass tubes with survivors were placed in an incubator (28 ºC and 90% RH) to determine the reproductive index of the ticks. After 20 days, the eggs laid within the first 3-4 days were counted, weighed and transferred into 5-ml sterile glass tubes for hatching under same conditions (28 ºC and 90% RH). After 25-27 days, the hatched larvae were counted again (20). The reproductive index was calculated by following formula according to FAO guidelines,

RI = (weight of eggs in milligrams/weight of female in milligrams) x % hatch

Phytochemical analysis

Phytochemical analysis of the three plant extracts viz., *Azadirachta indica* (Neem), *Dalbergia sissoo* (Sheesham) and *Morus alba* (White mulberry) was carried out for alkaloids, flavonoids, steroids, terpenoids, saponins, tannins and phenols by employing the methods as described by Rosenthaler (22). The powdered leaves were extracted using suitable solvent and necessary reagent added to the right quantity of the extract.

Statistical Analysis

The percent efficacy (mortality) was calculated by the formula explained by Holdsworth et al. (1);

$$\text{Efficacy (\%)} = N_0 - N / N_0 * 100$$

Where,

N_0 is the number of ticks before treatment.

N is the number of ticks after treatment.

Mortality data was analyzed through ANOVA followed by the post-hoc Tukey's test to significant factors and probit analysis was employed to calculate median lethal concentration (LC_{50}) and median Lethal time (LT_{50}) by using Minitab – 17 statistical software (23).

Results

The results of our bioassay experiments describe significant variation among the toxicity values (LC_{50}) of plant extracts and chemical acaricides (non-overlapping confidence intervals) against *H. anatolicum* and are displayed in table 1.

Table 1: LC_{50} of plant extracts and synthetic acaricides at different exposure periods against *H. anatolicum*

Plants	Time (hrs)	LC_{50} (95.0% Fiducial Cl)	SE	$\mathbf{x}^2$ (df=4)	p-value
Azadirachta indica	2	431.42(398.01–515.02)	0.909	2.123	0.000
	4	320.75(299.19–438.02)	0.863	2.596	0.000
	6	252.20(199.26–304.26)	0.727	5.477	0.000
	12	128.55(80.25–192.80)	0.569	0.161	0.038
	24	93.98(0.051–230.92)	0.567	0.235	0.068
	48	38.30(0.39–94.08)	0.585	0.454	0.068
Dalbergia sissoo	2	492.5(385.24–552.5)	1.242	0.537	0.001
	4	343.69(373.43–451.5)	1.168	0.772	0.000
	6	293.80(170.32–352.5)	0.848	2.699	0.000
	12	192.50(120.51–292.5)	0.590	0.201	0.010
	24	164.68 (103.8–209.5)	0.565	0.202	0.098
	48	58.76(20.5–79.2)	0.565	0.184	0.218
Morus alba	2	501.50(402.79–592.2)	6.209	0.177	0.116
	4	395.90 (299.8 – 435.2)	2.256	0.339	0.009
	6	339.80(279.8–435.2)	0.909	2.123	0.000
	12	230.94(192.8–301.5)	0.616	0.148	0.014
	24	198.50 (113.5 – 259.5)	0.572	0.018	0.111
	48	92.95(25.60 –135.5)	0.561	0.020	0.260
Fipronil	2	289.12(201.78–368.51)	0.900	0.166	0.000
	4	201.109(172.2–290.4)	0.637	0.150	0.008
	6	131.68(82.01–159.02)	0.584	0.070	0.021
	12	82.54(50.44–102.11)	0.569	0.299	0.024
	24	38.30(0.39–94.08)	0.585	0.454	0.068
	48	35.01(10.84–59.02)	0.757	1.472	0.002
Emamectin	2	302.79(207.6–389.9)	0.888	0.061	0.001
	4	231.78(190.76–298.5)	0.687	0.174	0.003
	6	150.72(88.62–201.76)	0.599	0.293	0.007
	12	98.12(50.71–150.45)	0.575	0.219	0.005
	24	81.85(12.98–156.55)	0.576	0.031	0.011
	48	46.87(17.73–75.60)	0.684	3.377	0.000
Cypermethrin	2	299.14(211.78–392.9)	1.242	0.537	0.001
	4	219.80(162.8–307.57)	0.706	0.062	0.001
	6	142.82(102.8–216.8)	0.607	0.077	0.008
	12	95.74(49.9–122.54)	0.572	0.313	0.028
	24	81.85(12.98–156.55)	0.568	0.544	0.088
	48	37.83(06.14–74.15)	0.625	0.487	0.010

At minimum exposure time (2hrs), LC_{50} values of *A. indica, D. sissoo* and *M. alba* were 431.42, 492.50, 501.5 ppm and that of fipronil, emamectin and cypermethrin were 289.12, 302.79, 299.14 ppm while after maximum exposure time (48hrs), plant extracts showed 38.30, 58.76, 92.95 ppm and synthetic acaricides showed 35.01, 46.87, 37.83 ppm respectively. The LC_{50} value 38.30 ppm shown by *A. indica* after exposure period of 48h was significantly very close to the LC_{50} values of synthetic acaricides that caused significant mortality of *H. anatolicum* as compared to *D. sissoo* and *M. alba* as shown in table 1.

Table 2: LT_{50} of plant extracts and synthetic acaricides at various concentrations against *H. anatolicum*

Plants	Concentration (ppm)	LT_{50} (95.0% Fiducial Cl)	SE	$\mathbf{x}^2$ (df=4)	p-value
A. indica	50	32.31(21.57-63.99)	0.3022	3.644	0.000
	100	19.71(12.92-38.10)	0.2489	0.920	0.001
	250	16.20(10.61-29.69)	0.2416	0.657	0.000
	500	10.91(7.36-16.85)	0.2378	0.602	0.000
	750	8.49(5.76-12.30)	0.2365	0.308	0.001
D. sissoo	50	44.44(29.17-94.92)	0.3614	2.199	0.000
	100	30.24(20.60- 56.25)	0.3000	0.815	0.0001
	250	25.84(17.38-48.82)	0.2736	0.541	0.000
	500	20.76(13.95-38.07)	0.2565	0.880	0.000
	750	16.97(11.30-30.46)	0.2450	0.401	0.000
M. alba	50	49.50(32.67-106.49)	0.4119	3.019	0.000
	100	44.44(29.17-94.92)	0.3614	2.199	0.000
	250	38.90(25.73-80.23)	0.3312	2.210	0.000
	500	34.85(22.58-75.00)	0.2950	0.923	0.000
	750	29.17(18.88-61.94)	0.2721	0.733	0.000
Fipronil	50	22.43(15.67-38.15)	0.2767	1.522	0.0001
	100	15.28(11.10-22.74)	0.2634	1.056	0.000
	250	11.60(8.68-16.01)	0.2610	0.793	0.000
	500	8.31(6.37-10.80)	0.2640	1.659	0.000
	750	6.53(4.96-8.42)	0.2599	2.584	0.0001
Emamectin	50	31.01(20.56-62.13)	0.2901	1.546	0.000
	100	21.27(14.82-36.10)	0.2694	0.375	0.000
	250	14.03(10.19-20.65)	0.2581	0.302	0.0001
	500	9.37(7.16-12.35)	0.2629	0.794	0.000
	750	7.37(5.65-9.50)	0.2636	3.703	0.000
Cypermethrin	50	29.17(20.31-51.52)	0.3106	2.639	0.000
	100	20.66(14.93-32.39)	0.2845	1.420	0.0001
	250	15.96(11.67-23.70)	0.2684	1.205	0.000
	500	11.60(8.68-16.01)	0.2610	0.793	0.000
	750	8.95(6.77-11.85)	0.2591	0.781	0.000

Table 2 represented LT_{50} values of plant extracts and synthetic acaricides at various concentrations against *H. anatolicum*. At minimum concentration (50 ppm), LT_{50} values of plant extracts (*A. indica, D. sissoo* and *M. alba*) were 32.31, 44.44, 49.5 hrs and that of acaricides (fipronil, emamectin and cypermethrin) were 22.43, 31.01, 29.17 hrs while at maximum concentration (750 ppm), LT_{50} values of plant extracts were 8.49, 16.97, 29.17 hrs and that of acaricides were 6.53, 7.37, 8.95 hrs respectively. Results also revealed that *A. indica* could kill *H. anatolicum* in minimum duration among the employed plant extracts that was statistically similar to the synthetic chemicals.

To check the statistical significance of the plants, chemical acaricides, different time intervals (2, 4, 6, 12, 24 and 48 hrs) and concentrations (50, 100, 250, 500 and 750 ppm), the analysis of variance (ANOVA) was applied.

Table 3: Analysis of variance (ANOVA) results

Source	df	Sum of Squares	Mean Square	F	p-value
Time	5	5198.921	1039.784	938.619	<0.001
Chemicals	2	166.980	83.49	78.16	<0.001
Plant	2	185.311	92.655	75.041	<0.001
Concentration	5	8710.566	1742.113	1618.86	<0.001
Error	735	953.949	1.298		
Total	749	15215.727			

Table 4: Reproductive parameters of control and treated groups of *Hyalomma anatolicum*

Treatments	Conc. ppm	Tick wt. (mg) (mean ± SD)	Egg mass (mg) (mean ± SD)	Fecundity = Egg mass/tick wt.	% Hatch	RI = Fecundity x % hatch
Control	dH_2O	198.8±9.06	75.8±10.6	0.38	80	30.4
A. indica	50	196.6±10.93	59±8.9	0.300	54	16.2
	100	195.4±9.74	40±7.5	0.204	43	8.77
	250	187.9±8.96	38±7.1	0.202	32	6.46
	500	202.3±11.07	32±7.1	0.158	24	3.79
	750	194.6±9.86	25±6.4	0.128	10	1.28
D. sissoo	50	196.6±10.71	63±9.9	0.320	56	17.92
	100	195.4±9.76	42±7.4	0.214	43	9.202
	250	193.3±8.67	40±7.2	0.206	33	6.80
	500	197.1±9.75	38±6.5	0.192	27	5.18
	750	194.8±9.58	37±6.2	0.189	13	2.46
M. alba	50	202.8±9.45	65±6.5	0.320	59	18.88
	100	197.3±8.51	46±6.6	0.233	46	10.72
	250	195.4±9.85	44±6.5	0.225	32	7.2
	500	201.1±11.09	43±6.1	0.213	30	6.39
	750	196.1±8.91	40±5.8	0.204	14	2.856
Fipronil	50	198.3±10.94	21±8.9	0.105	25	2.63
	100	197.7±9.76	16±7.5	0.080	17	1.36
	250	188.6±8.96	9±7.1	0.047	8	0.376
	500	201±10.97	0±7.1	0	0	0
	750	196.4±9.95	0±6.4	0	0	0
Emamectin	50	197.8±10.95	29±9.9	0.146	29	4.23
	100	199.6±8.77	18±7.4	0.090	18	0.162
	250	189.9±8.65	12±7.2	0.063	4	0.252
	500	198.3±10.91	0±6.5	0	0	0
	750	198.9±9.55	0±6.2	0	0	0
Cypermethrin	50	202.9±10.48	45±6.5	0.221	29	6.409
	100	193.9±9.67	32±6.6	0.165	17	2.805
	250	192.5±9.73	23±6.5	0.119	8	0.952
	500	201.7±10.44	10±6.1	0.049	3	0.147
	750	197.6±9.86	0±5.8	0	0	0

The results presented in Table 3 revealed that all the three plants, three synthetic acaricides, times and concentrations were significantly different (p-values <0.001). Then we applied Tukey's test to see which plant and acaricide provide highest mortality. The results revealed that *A. indica* was statistically significant (p-value = 0.04) from *M. alba* and *D. sissoo* and it provided highest mortality. Tukey's test revealed that fipronil was statistically insignificant (p-value = 0.99) from cypermethrin and significant (p-value = 0.02) differences were noted from emamectin. Tukey's test was also applied to time intervals, the mortality was found to be statistically significant (p-value = 0.02). For the concentrations with the control, Dunnet's test was applied. The results of Dunnet's test showed that the mortality in all the concentrations (50, 100, 250, 500 and 750 ppm) was significantly higher than the control group (water only).

The reproductive parameters of ticks treated with different concentrations of plant extracts and synthetic acaricides were shown in table 4. This table showed that all plant extracts and synthetic acaricides showed excellent results in lowering reproductive index at high concentrations (250, 500 and 750 ppm). All three acaricides even with low concentrations were as effective in tick mortality as higher concentrations, however plant extracts were not very effective at lower concentrations (50 and 100 ppm) as shown in table 4.

Table 5: Phytochemical analysis of plants extracts tested against *H. anatolicum*

Phytochemical	Plant extracts		
	Azadirachta indica	*Dalbergia sissoo*	*Morus alba*
Alkaloids	_	_	_
Flavonoids	+++	+	+++
Steroids	++	_	++
Terpenoids	+	++	++
Saponins	+++	+	+
Tannins	++	++	++
Phenols	_	+++	++

(-) Not detected, (+) Low in concentration, (++) Moderate, (+++) High in concentration

The phytochemical analysis of plant extracts used in our bioassay experiments revealed that saponins and flavonoids showed highest scoring in neem extract while tannins and steroids indicated moderate scores (Table 5). Phenols represented highest scores in *Dalbergia sissoo* extract, while tannins and terpenoids showed moderate scoring. Phytochemical analysis revealed highest scoring of flavonoids and moderate scoring of steroids, terpenoids, tannins and phenols. Alkaloids were not detected in the extracts of selected plants.

Discussion

In the present study methanolic extracts of three locally existing plants *Azadirachta indica* (Neem), *Dalbergia sissoo* (Sheesham), *Morus alba* (Shehtoot) and three synthetic acaricides fipronil, emamectin and cypermethrin were employed to evaluate the mortality of *H. anatolicum* under laboratory conditions. Maximum mortality of *H. anatolicum* was observed after exposing these ticks for a period of 48h at 750 ppm concentration and minimum mortality was recorded at least time duration (2h) and concentration (50 ppm).

Magadum et al. (24) evaluated the efficacy of *Azadirachta indica* and *Annona squamosa* extracts against *Rhipicephalus* (syn. *Boophilus*) *microplus* in India. They observed 71 % efficacy with *A. squamosa* extracts against the *R. microplus* by in vivo but in vitro methods showed more efficacy of *A. indica* extracts than the extracts of *A. squamosa*. These results are related to the present in vitro outcomes in which *A. indica* was highly effective against *H. anatolicum* than *D. sissoo and M. alba.*

In our study, *A. indica* showed lethal effects against *H. anatolicum* and these results are related to the investigations of Zaman et al. (25) who evaluated the anti-tick efficacy of combined aqueous herbal extracts of *A. indica* leaves, *Nicotiana tabacum* leaves, *Calotropis procera* flowers and *Trachispermum ammi* seeds against the *Rhipicephalus* (*Boophilus*) *microplus* using adult immersion, larval packet and ear bag method. They

stated that the extract exhibited lethal effects on egg laying, hatching and total larval mortality.

Parte et al. (26) screened the acaricidal activity of aqueous extracts of *Azadirachta indica, Mangifera indica, Polyalthia longifolia, Annona squamosa* and *Ficus benghalensis* against the *Rhipicephalus* (*Boophilus*) *microplus*. They observed that the combination of five plant extracts showed 100 percent mortality as compared to individual plant extracts. Furthermore, they concluded that extended exposure of the target pest to individual plant extract is required to obtain 100 percent mortality. Increased mortality of ticks was also observed in present studies with the increase of exposure time at the same concentration of employed plant extract.

Results of *M. alba* leaves extract exhibited a moderate acaricidal activity against *H. anatolicum*. Percentage mortality of the test ticks evaluated for a concentration of 750 ppm after periods of 24h and 48h were 50% and 66.67%, respectively. Data represented that mortality of ticks was time and concentration dependent. Mortality increased with the increase of concentration and time of exposure. The results obtained in this study are supported by Dantas et al. (27) who studied the acaricidal activity of crude ethanolic extract and fractions from the leaves of *Morus nigra* on female cattle ticks *Rhipicephalus microplus*, using the adult immersion test. The mortality and fertility of females exposed to different concentrations of hexane, chloroform and ethyl acetate fractions, as well as ethanolic extract of *M. nigra.* The chloroform extract of leaves of *M. nigra* (25 mg/mL) showed the best results, obtaining 62.6% of inhibition of oviposition, 39.3% of eggs eclosion average and 65.4% of effectiveness.

D. sissoo leaves extract showed mean mortality of ticks after 24h and 48h periods as 48.67% and 58.67% respectively. Highest mean mortality of ticks (42.22%) was observed at 750 ppm concentration. Mortality of ticks was found to be dependent on exposure time and concentration of extract applied. These results are in line with those obtained by Singh et al. (28). They evaluated mortality and fecundity of *Rhipicephalus* (*Boophilus*) *microplus* exposed to Sheesham leaf aqueous (SLA) and ethanolic (SLE) extracts. Higher acaricidal activity was recorded in SLA with a lower LC_{50} (95% CL) value of 1.58% than SLE (5.25%).

Synthetic acaricide, cypermethrin showed a remarkable efficacy against *H. anatolicum* and the mortality was increased with increase in acaricides concentration and exposure periods. The mean mortality of ticks observed after 24h period was 58.00% and mortality recorded after 2 days (48h) was 73.33%. Similar investigation was performed by Khalaf-Allah (29), who reported 100% effectiveness of cypermethrin against *R. annulatus* up to 7 weeks of post-treatment after which the efficacy was dropped to 98 %. Sajid et al. (30) also investigated the pour-on preparations of cypermethrin which showed a higher in vivo efficacy compared to ivermectin against *Hyalomma anatolicum anatolicum* at 15 days post-treatment interval.

Burridge et al. (31) employed eight acaricides (amitraz, carbaryl, chlorpyrifos, cyfluthrin, fipronil, permethrin, pyrethrin, and selamectin) for their efficacy in the rapid killing of *Rhipicephalus sanguineus* (Acari: Ixodidae). *R. sanguineus* was most sensitive to fipronil, carbaryl and cyfluthrin. Our findings also suggest that fipronil possesses greater potential to control ticks and has caused 84% mortality of *H. anatolicum* when compared with emamectin and cypermethrin.

Our results indicated that all plant extracts inhibit oviposition and reduce hatching percentage depending on concentration of extracts. These results are in line with those of Rawani et al. (32) who also noted these deterrent properties in *Carica papaya* against ticks. Roobakkumar et al. (33) used garlic extract and noted more than 70% mortality in ticks with reduced oviposition and hatching percentage of surviving parents. These results are different from Kalakumar et al. (34) and Borges et al. (35) who noted mortality (more than 60%) but failed to record the inhibition of oviposition and reduction in hatching percentage of ticks with neem extract treatment. Our results are also at par with the results of Shyma et al. (36) who noted 0 to 50% reduction in hatching with neem, calotropis, datura, garlic and papaya plant extracts.

Conclusion

Plants presented a significant mortality of *H. anatolicum* but less than that of synthetic chemicals. From above observations it has been concluded that plant extracts could effectively control ticks population when applied on house hold animals as well as farm animals. As synthetic acaricides cause toxicity in environment, affect animal health and may develop resistance in ticks against these chemicals. It is recommended

to encourage the use of plant extracts instead of synthetic chemicals to control ticks population on animals. These botanical pesticides could efficiently control ticks population without posing any health risk to the animals.

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PRIMERJAVA UČINKOVITOSTI SINTETIČNIH KEMIKALIJ IN RASTLINSKIH EKSTRAKTOV ZA NADZOR NAD KLOPI

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Izvleček: Klopi veljajo za škodljive in ekonomsko pomembne ektoparazite, kajti njihova okužba po vsem svetu hudo prizadane govedo na paši. Zatiranje klopov s sintetičnimi akaricidi ni nevarno samo za zdravje živali in ljudi, temveč povzroča tudi onesnaževanje okolja. Študija je bila zasnovana z namenom ovrednotenja rastlinskih izvlečkov v primerjavi s sintetičnimi akaricidi za nadzor nad *Hyalomma anatolicum*. Za oceno umrljivosti klopov po 2, 4, 6, 12, 24 in 48 urah je bilo uporabljenih pet različnih koncentracij (50, 100, 250, 500 in 750 ppm) metanolnih rastlinskih izvlečkov in akaricidov. Podatki o smrtnosti so bili analizirani z analizo Probit za izračun srednje smrtne doze (LC_{50}) in srednjega časa smrti (LT_{50}). Metanolni ekstrakt iz *Azadirachta indica* je pokazal najvišjo umrljivost (LC_{50}=38,3 ppm) klopov v primerjavi z *Dalbergia sissoo* (LC_{50} =58,76 ppm) in *Morus alba* (LC_{50}=92,95 ppm). Med akaricidi je imel fipronil največji učinek smrtnosti (LC_{50}=35,01 ppm) v primerjavi z emamektinom (LC_{50}=46,87 ppm) in cipermetrinom (LC_{50}=37,83 ppm). Višja koncentracija (750 ppm) akaricidov (fipronil, emamektin in cipermetrin) je pokazala hitrejšo smrtnost (LT_{50}=6,53-8,95 ur) v primerjavi z rastlinskimi ekstrakti (LT_{50}=8,49-29,17 ur). Učinke zdravljenj so preučevali tudi na jajčnih masah in obravnavali reproduktivni indeks (RI) preživelih klopov. Rezultati so pokazali pomembno, koncentracijsko odvisno variacijo med jajčnimi masami, obdelanimi z rastlinskimi izvlečki in akaricidi. Posledično so se vrednosti njihovega reproduktivnega indeksa znatno zmanjšale. Fitokemijska analiza preizkušenih rastlinskih izvlečkov je razkrila prisotnost flavonoidov, steroidov, terpenoidov, saponinov, taninov in fenolov v spremenljivih količinah. Rezultati opravljene raziskave opisujejo pomembne lastnosti okolju prijaznih rastlinskih izvlečkov pri preprečevanju napadov klopov.

Ključne besede: rastlinski izvlečki; sintetični akaricidi; smrtnost klopov

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Original Research Article

THE PRESENCE OF PUTATIVE VIRULENCE DETERMINANTS, TETRACYCLINE AND β - LACTAMS RESISTANCE GENES OF *Aeromonas* SPECIES ISOLATED FROM PET TURTLES AND THEIR ENVIRONMENT

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Abstract: This study aimed to characterize *Aeromonas* spp. isolated from ten popular species of pet turtles and their environment to evaluate the potential risk of pet turtles as a source of virulence-associated genes, and tetracycline and β-lactams resistance determinants. Presence of eight virulence genes (*ser, aer, exu, lip, fla, ascV, ahyB* and *gcat*), and tetracycline (*tetA, tetB* and *tetE*) and β-lactams (bla_{TEM}, bla_{SHV}, bla_{OXA} and bla_{CTX-M}) resistance genes were evaluated by conventional PCR assays. The *aerA* gene showed the highest frequency of occurrence (92%), followed by *fla* (75%), *gcaT* (68%), *ahyB* (59%), *ser* (39%), *lip* (37%) and *ascV* (25%) genes. None of the isolates carried amplicon of DNase-associated *exu* gene. *A. hydrophila, A. dharkensis, A. veronii* and *A. caviae* were carried seven tested virulence genes except for exu while only four virulence genes were detected in *A. enteropelogenes*. Among the 75 tetracycline-resistant isolates, *tetA, tetE* and *tetB* genes were detected in 38, 26 and 6 isolates, respectively. Among the tested β-lactam resistance genes, bla_{OXA} and bla_{TEM} genes were detected in 54% and 36% of β-lactam resistant isolates, respectively. No bla_{CTX-M} and bla_{SHV} genes were detected. Our results indicate that pet turtle-associated aeromonads, exhibiting potential virulence and antimicrobial (tetracycline and β-lactams) resistance genes, may pose a serious health risk to pet turtle owners, particularly to immunocompromised individuals.

Key words: *Aeromonas* spp.; virulence-associated genes; tetracycline resistance; β-lactams resistance; pet turtle

Introduction

Mesophilic aeromonads are ubiquitous bacteria that are a component of the normal microbiota of many aquatic animals such as fish, amphibians, and reptiles (1). They can cause ulcerative stomatitis, pneumonia, dermatitis, and septicemia in reptiles under stressful conditions such as trapping, handling and temperature variations of rearing environment (2, 3). Over the years, many studies have been investigated to evaluate the prevalence of *Aeromonas* species in aquatic animals, mainly food-producing animals (4, 5). However, a limited number of studies evaluating the distribution of aeromonads in pet turtles have been published up to date (6, 7).

The pathogenesis of *Aeromonas* species involves various virulence factors including cytotoxic heat-labile enterotoxin (*act*), cytotonic heat-labile enterotoxin (*alt*) and cytotonic heat-stable enterotoxin (*ast*), aerolysin (*aer*), lipase (*lip*), serine protease (*ser*), elastase (*ahyB*), DNase (*exu*), glycerophospholipid-cholesterol acyltransferase (*gcaT*), flagellar system (*fla*) and Type III secretion system (TTSS) effector (*ascV*). These genes encoding virulence factors have been broadly used in determining the potential pathogenicity of *Aeromonas* species isolated from the environment, foodstuffs, and human clinical samples (1, 8-10).

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Recently, antibiotic-resistant aeromonads have been recognized as a serious concern due to their potential health risks to animals and humans (11, 12). Especially, the dissemination of tetracycline and β-lactams resistance aeromonads in the aquatic environment has been widely documented (12, 13, 14). Among many tetracycline resistance genes, the *tetE, tetA* and *tetB* genes were frequently identified from *Aeromonas* species in the aquatic environment (12, 15, 16). *Aeromonas* species can produce numerous β-lactamases for conferring resistance to β-lactams. According to isolation sources, the previous studies have shown the different prevalence of genes encoding β-lactamases in *Aeromonas* species. In the aquatic environment, the bla_{TEM}, bla_{SHV}, bla_{OXA} and bla_{CTX-M} β-lactams genes were frequently detected from *Aeromonas* species (17-19).

These resistance genes containing plasmids and transposons are known as mobile genetic elements that can be transferred horizontally among distantly related lineages. Particularly, The aquatic environment is more favorable for the transmission of resistant bacteria, thus, *Aeromonas* species as opportunistic pathogens might be dangerous vectors for the spreading of antibiotic resistance genes through the aquatic environment (18, 20). Hence, the present study was conducted to determine the occurrence of antimicrobial resistance genes (tetracyclines and β-lactams) and virulence-associated genes of *Aeromonas* species isolated from pet turtles and their environment.

Materials and methods

Bacterial isolates

One hundred and two *Aeromonas* species isolates obtained from ten commercially popular pet turtles species (Chinese stripe-necked turtles *Ocadia sinensis*, yellow belly sliders *Trachemys scripta scripta*, river cooters *Pseudemys concinna concinna*, northern Chinese softshell turtles *Pelodiscus maackii*, western painted turtles *Chrysemys picta belli*, peninsula cooters *Pseudemys peninsularis*, African sideneck turtles *Pelusios castaneus*, common musk turtles *Sternotherus odoratus*, red belly cooters *Pseudemys rubriventris* and alligator snapping turtles *Macroclemys Temminckii*) and their rearing environment was screened to investigate the presence of putative virulence, and β-lactams and tetracycline resistance genes. These isolates have been previously characterized for their antimicrobial susceptibilities, enterotoxin (*act*, *alt* and *ast*) genes and quinolone resistance determinants (7, 21).

Detection of antibiotic resistance genes

Twenty-eight and seventy-five isolates were selected (21) for the detection of β-lactams and tetracycline resistance determinants, respectively. These isolates were tested by PCR assays to detect the genetic determinants associated with resistance to β-lactams (bla_{TEM}, bla_{SHV}, bla_{OXA} and bla_{CTX-M}), and tetracyclines (*tetA, tetB* and *tetE*). The primer sets used in PCR amplification are summarized in table 1. PCR amplifications were conducted in 20 µL volumes consisting of 10 µL of Quick Taq® HS DyeMix (Toyobo, Japan), 1 µL of 10 pmol/µL each primer and 1 µL of the template under standard conditions. The PCR products were analyzed by electrophoresis on 2% (wt/vol) agarose gels. Positive controls were implemented with previously characterized enterobacterial strains that harbored the corresponding genes (21, 22).

Detection of virulence-associated genes

All isolates were subjected to PCR assays to detect the 8 tested virulence genes including *ser, aer, exu, lip, fla, ascV, ahyB* and *gcat*. The PCR amplification of the virulence-associated genes was carried out according to the PCR primers and conditions reported previously (Table 1). The PCR mixture of 20 µL contained 10 µL Quick Taq HS DyeMix (Toyobo, Japan), 7 µL PCR water, 1 µL template and 1 µL of each primer. The PCR products were examined by electrophoresis on 1.5% (W/V) agarose gel.

Results

Bacterial isolates

One hundred and two *Aeromonas* species isolates were isolated from the feces, skin and rearing environments of pet turtles and identified by biochemical and *gyrB* sequence analyses. *Aeromonas enteropelogenes* was the predominant species among the isolates (52.9%) followed by *A. hydrophila* (32.4%), *A. dharkensis* (5.9%), *A. veronii* (4.9%) and *A. caviae* (3.9%) [7].

Presence of resistance genes

Among the tested β-lactam resistance genes, bla_{OXA} and bla_{TEM} genes were detected in 54% and 36% of β-lactam resistant isolates, respectively. No bla_{CTX-M} and bla_{SHV} genes were detected (Table 2). Among the 75 tetracycline-resistant isolates, *tetA, tetE and tetB genes* were detected in 38, 26 and 6 isolates, respectively (Table 3).

Table 1: Oligonucleotide primers and PCR conditions [a] used to amplify virulence and antibiotic resistance genes of *Aeromonas* spp.

Gene	Target	Nucleotide Sequence (5'-3')	Size (bp)	Annealing temperature (°C)	Reference
aerA	Aerolysin	F: CTATGGCCTGAGCGAGAAG	431	62	30
		R: CAGTTCCAGTCCCACCACT			
ser	Serine protease	F: ACCGAAGTATTGGGTCAGG	350	55	13
		R: GCTCATGCGTAACTCTGGT			
fla	Flagella	F: CCAACCGTYTGACCTC	608	56	36
		R: MYTGGTTGCGRATGGT			
ahyB	Elastase	F: CACGGTCAAGGAGATCAAC	513	58	13
		R: GCTGGTGTTGGCCAGCAGG			
lip	Lipase	F: ATCTTCTCCGACTGGTTCGG	382	62	36
		R: CCGTGCCAGGACTGGGTCTT			
exu	DNase	F: AGACATGCACAACCTCTTCC	323	59	13
		R: GATTGGTATTGCCTTGCAAG			
gcaT	Glycerophospholipid-cholesterol acyltransferase	F: TCCTGGAATCCCAAGTATCAG	237	65	13
		R: GCAGGTTGAACAGCAGTATCT			
ascV	Type III Secretion System	F: AGCAGATGAGTATCGACGG	891	58	38
		R: AGGCATTCTCCTGTACCAG			
bla_{TEM}	β - lactams resistance	F: ATAAAATTCTTGAAGACGAAA	1080	60	22
		R: GACAGTTACCAATGCTTAATC			
bla_{SHV}		F: TTATCTCCCTGTTAGCCACC	795	60	
		R: GATTTGCTGATTTCGCTCGG			
bla_{CTX-M}		F: CGCTTTGCGATGTGCAG	550	52	
		R: ACCGCGATATCGTTGGT			
bla_{OXA}		F: TCAACTTTCAAGATCGCA	591	60	
		R: GTGTGTTTAGAATGGTGA			
tetA	Tetracycline resistance	F: GTAATTCTGAGCACTGTCGC	1000	62	22
		R: CTGCCTGGACAACATTGCTT			
tetB		F: CTCAGTATTCCAAGCCTTTG	400	57	
		R: CTAAGCACTTGTCTCCTGTT			
tetE		F: GTGATGATGGCACTGGTCAT	1100	62	
		R: CTCTGCTGTACATCGCTCTT			

[a] PCR thermocycle conditions for each reaction; initial denaturation of 94 °C for 2 min followed by a total of 35 cycles of amplification. Each cycle consisted of 94 °C denaturation for 30 s, annealing for 50 s and 72 °C extension for 10 min.

Table 2: β-lactams resistance profiles of turtle-associated *Aeromonas* spp.

Isolate	Host [a]	β-lactam resistance [b]	β-lactam resistance genes
Aeromonas caviae			
AD14	CSN	AMP, AMX, CEP, FOX	bla_{TEM}
AC50	RC	AMP, AMX, CEP, CRO, FOX, IMI	bla_{OXA}, bla_{TEM}
A. dharkensis			
AD17	RC	AMP, AMX, CEP, CRO, FOX, CTX	bla_{OXA}, bla_{TEM}
AD18	RC	AMP, AMX, CEP, CRO, FOX, CTX	bla_{OXA}, bla_{TEM}
AD19	RC	AMP, AMX, CEP, CRO, FOX	bla_{OXA}
AD15	CSN	AMP, AMX, CEP, FOX, CTX	bla_{OXA}
A. enteropelogenes			
AC2	RC	AMP, AMX, CEP	bla_{OXA}
AC6	RC	CEP, FOX	-
AC15	NCS	AMP, AMX, CEP, FOX	bla_{OXA}
AC30	CM	CEP, CTX, ATM	-
AC31	WP	CEP, CTX, ATM	-
AC32	WP	CEP, CTX, ATM	-
AC35	RC	CEP, CTX, ATM	-
AC44	YB	AMP, AMX, CRO	bla_{TEM}
AC45	RC	CEP, CRO, ATM	-
AC53	WP	AMP, AMX, CEP, FOX	bla_{OXA}
AV4	RC	AMP, AMX, CEP, FOX	bla_{OXA}
AD1	CM	AMP, AMX, CEP, FOX	bla_{OXA}
A. hydrophila			
AH1	RC	AMP, CEP	-
AH11	CSN	AMP, AMX, CEP, CRO	bla_{OXA}, bla_{TEM}
AH13	CSN	CEP, CRO, FOX, IMI	-
AH19	NCS	AMP, AMX, CEP, CRO	bla_{OXA}
AH20	NCS	AMP, AMX, CEP	bla_{TEM}
AH22	YB	AMP, AMX, CEP	-
AH23	YB	AMP, AMX, CEP, CRO	bla_{OXA}
AH25	CM	AMP, AMX, CEP, FOX	bla_{TEM}
AD10	AF	AMP, AMX, CEP, FOX	bla_{OXA}, bla_{TEM}
A. veronii			
AC52	SN	AMP, AMX, CEP, FOX	bla_{OXA}, bla_{TEM}

[a]**Host:** CSN= Chinese stripe-necked turtle, YB= yellow belly slider, RC= river cooter, PC= peninsula cooter, NCS= northern Chinese softshell turtle, CM= common musk turtle, WP= western painted turtle, AF= African sideneck turtle, SN= Alligator snapping turtle.
[b]**β-lactams resistance:** AMX=Amoxicillin (10 µg), AMP=Ampicillin (10 µg), CEP=Cephalothin (30 µg), CRO=Ceftriaxone (30 µg), FOX=Cefoxitin (30 µg), CTX=Cefotaxime (30 µg), IMI=Imipenem (10 µg)

Table 3: Distribution of tetracycline resistance genes among tetracycline resistant Aeromonas species isolated from pet turtles and their environment

Species	Number of positive isolates (Subtotal %)		
	tetA	*tetB*	*tetE*
Aeromonas enteropelogenes (*n* = 50)	32 (64)	-	8 (2)
A. hydrophila (*n* = 17)	6 (35)	-	12 (71)
A. dharkensis (*n* = 4)	-	2 (50)	3 (75)
A. veronii (*n* = 3)	-	3 (100)	2 (66)
A. caviae (*n* = 1)	-	1 (100)	1 (100)
Total (%) (*n* = 75)	38 (51)	6 (1)	26 (35)

Table 4: Prevalence of virulence-associated genes in *Aeromonas* species isolates from pet turtles and their environment

Species	Number of positive isolates (Subtotal %)							
	aerA	*lip*	*ahyB*	*ser*	*exu*	*fla*	*gcat*	*ascV*
Aeromonas enteropelogenes (*n* = 54)	46 (85)	0	21 (39)	0	0	51 (94)	23 (43)	0
A. hydrophila (*n* = 33)	33 (100)	30 (91)	28 (85)	31 (94)	0	19 (58)	33 (100)	15 (47)
A. dharkensis (*n* = 6)	5 (83)	3 (50)	4 (67)	4 (67)	0	2 (33)	6 (100)	4 (67)
A. veronii (*n* = 5)	5 (100)	3 (60)	5 (100)	3 (60)	0	2 (40)	4 (80)	3 (60)
A. caviae (*n* = 4)	4 (100)	2 (50)	2 (50)	2 (50)	0	3 (75)	3 (75)	4 (100)
Total (%) (*n* = 102)	93 (92)	38 (37)	60 (59)	40 (39)	-	77 (75)	69 (68)	26 (25)

Distribution of virulence-associated genes

The occurrence and frequencies of virulence genes are shown in Table 4. The *aerA* gene showed the highest frequency of occurrence (92%), followed by *fla* (75%), *gcaT* (68%), *ahyB* (59%), *ser* (39%), *lip* (37%) and *ascV* (25%) genes. None of the isolates carried amplicon of the DNase-associated *exu* gene.

Discussion

The *Aeromonas* spp. under study were multi-drug-resistant turtle-associated bacteria which carried quinolone resistance determinants, as well as enterotoxin genes (7, 21). The isolates were highly resistant to β-lactams especially amoxicillin, ampicillin and cephalothin. β-lactam antibiotics have used for the treatment of *Aeromonas* infection during the last decade. However, their efficacy has significantly declined due to the production of β-lactamases by resistant bacterial strains (14, 17, 23). The *Aeromonas* spp. are naturally resistant to β-lactams because of the expression of chromosomal β-lactamases (24).

In this study, twenty-eight aeromonads isolates were resistant to the more than one β-lactam antibiotics. Among them, 54% and 36% of isolates harbored bla_{OXA} and bla_{TEM} genes. Several previous studies have documented the detection of the bla_{OXA} and bla_{TEM} genes in *Aeromonas* isolates recovered from the environment (14, 25) and clinical samples (26) and the prevalence of gene detection varies according to the isolation sources. In Korea, a previous study reported that the bla_{OXA} and bla_{TEM} genes were detected in 3% and 100% of *Aeromonas* isolates from aquaculture fish [14]. However, a different trend was observed in this study which the bla_{OXA} and bla_{TEM} genes were detected in *Aeromonas* isolates from pet turtles that suggest a wide distribution of β-lactamase genes in *Aeromonas* isolates from various sources.

A much higher level of tetracycline resistance was observed amongst aeromonads in our previous study (7) and 78 of tetracycline-resistant isolates were selected to detect their tetracycline resistance determinants (*tetA, tetB* and *tetE*). *A. enteropelogenes* and *A. hydrophila* harbored *tetA* and *tetE* genes while other *Aeromonas* species harbored *tetB* and *tetE* genes. Previous reports indicate that the *tetA* and *tetE* determinants are the predominant tetracycline resistance genes in the aquatic environment (16, 27) and both genes code for an efflux pump that eliminates the drug from the cell [28]. The *tetA, tetB* and *tetE* genes are located on the plasmid as well as *tetA* in the transposon (Tn*1721)* and *tetE* is adjacent to the integrons (15). Han et al. (27) has reported that *tetE* gene was the predominant tetracycline determinant in *Aeromonas* spp. isolated from Korean fish farms and aquariums. However, Kim et al. (29) reported that *tetA* was the most frequent gene in *A. salmonicida* strains isolated from salmonid farms and private aquariums in Korea. The *tetB* gene was detected at a low frequency, while Jacobs and Chenia. (12) reported a lower prevalence of *tetB* genes among *Aeromonas* spp. isolated from the South African aquaculture system.

Detection of virulence encoding genes of *Aeromonas* spp. have been widely applied for evaluating their potential pathogenicity (30, 31). However, the prevalence of virulence-associated genes has rarely been reported in *Aeromonas* strains from pet turtles (7). In the current study, *Aeromonas* isolates were found to possess genes *aerA, lip, ahyB, ser, fla, gcat* and *ascV*, while genes for DNase (*exu*) was not identified. Especially, none of *A. enteropelogenes* isolates harbored *lip, ser, exu* and *ascV*

genes. Previous studies have revealed that multiple virulence-associated genes are present in *Aeromonas* isolates and having high heterogeneity in the distribution of virulence-associated genes (10, 30, 31). The pore-forming aerolysin/hemolysin encoded *aer* gene was the most prevalent in this study which was detected in 92% of the total isolates representing all species of the genus. Several studies have reported the high prevalence of the *aer* gene in clinical and environmental *Aeromonas* isolates (30, 32).

The three enterotoxins *act, alt*, and *ast* have been implicated as major virulence factors in diarrhoeal disease which had been investigated in our previous study (7). However, the presence of these toxins might not be enough for virulence (31). The temperature-stable metalloprotease with elastolytic activity (*ahyB*) and serine protease (*ser*) play an important role in the invasiveness and establishment of infection (1). In the current study, the *ahyB* and *ser* genes were detected in 59% and 39% of isolates, respectively. None of the *A. enteropelogenes* isolates harbored *ser* gene. The flagella are important appendages for the initial attachment of bacteria to the gastrointestinal epithelium and involve in the subsequent adherence process and biofilm formation (33, 34). The *fla* gene-encoded polar flagella were common among the *Aeromonas* isolates from the aquatic environment. The *fla* gene was detected in 99% of *Aeromonas* isolates from diseased eel in Korea (10). The *gcaT* gene plays a coherent, integrated role in the establishment of pathogenicity of *Aeromonas* spp. by involving in the regulation and secretion of extracellular glycerophospholipid-cholesterol acyltransferase (13). The *gcaT* gene was detected in 68% of *Aeromonas* isolates.

Lipases play a role as virulence factors by interacting with leukocytes or by disturbing several immune system functions through free fatty acids produced by the lipolytic activity. Extracellular lipases secreted by *Aeromonas* spp. actively involve in the alteration of the host plasma membrane and thus increase the severity of infection (35). Among *Aeromonas* strains isolated in the present study, 91% of *A. hydrophila*, 60% of *A. veronii*, 50% of *A. dharkensis* and 50% of *A. caviae* isolates were found to have *lip* gene. Several previous studies reported a high prevalence of *lip* gene among the *Aeromonas* isolates from the aquatic environment (10, 36). Type III secretion system (T3SS) plays a crucial role in host-pathogen interactions by injecting effector toxins directly into the cytosol of host cells (37). The *acsV* gene encodes the T3SS and which was detected in 59% of *Aeromonas* spp. except for *A. enteropelogenes* isolates. The presence of *ascV* gene was previously detected in 68% of *Aeromonas* spp. isolated from diseased farmed fish and farm environment (38). Besides, the high frequency of *ascV* gene was reported in human clinical isolates (37).

The *exu* gene is responsible for DNA hydrolysis which was not detected in this study. The absence of *exu* gene was also reported by Nawaz et al. (13) in *A. veronii* isolated from catfish in the USA. In contrast, the high prevalence of *exu* gene was observed in *Aeromonas* spp. isolated from freshwater lakes in Malaysia (39) and diseased eel in South Korea (10). The specificity of the host or environmental source could be the possible reasons for the absence of *exu* gene in this study.

According to the available literature, this is the first description of these virulence-associated genes in *Aeromonas* of pet turtle origin. Most of *Aeromonas* strains isolated from pet turtles and their environment harboring multiple virulence-associated genes have the potential to be pathogenic. Turtle born aeromonads carrying tetracycline and β-lactams resistance determinants can disseminate through the environment. Collectively, which may pose a public health risk to pet turtle owners, particularly to immunocompromised individuals.

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Authors declare that no any conflict of interest exists.

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PRISOTNOST DETERMINANT ZA DOLOČITEV DOMNEVNE VIRULENCE TER GENOV ZA ODPORNOST NA TETRACIKLIN IN β-LAKTAM VRST *Aeromonas* IZOLIRANIH IZ LJUBITELJSKIH VRST ŽELV IN IZ NJIHOVEGA OKOLJA

S.H.M.P Wimalasena, G-J. Heo

Izvleček: Namen študije je bil določiti bakterije *Aeromonas* spp., izolirane iz desetih priljubljenih vrst hišnih želv in njihovega okolja, z namenom ocenjevanja potencialnega tveganje hišnih želv kot vira genov, povezanih z virulenco, ter determinante odpornosti proti tetraciklinom in β-laktamom. Prisotnost osmih virulentnih genov (*ser, aer, exu, lip, fla, ascV, ahyB in gcat*) ter genov za odpornost na tetracikline (*tetA, tetB in tetE*) in β-laktame (bla_{TEM}, bla_{SHV}, bla_{OXA} in bla_{CTX-M}) je bila ocenjena s konvencionalnimi testi PCR. Najbolj pogost je bil Gen *aerA* (92 %), sledili so geni *fla* (75 %), *gcaT* (68 %), ahyB (59 %), ser (39 %), lip (37 %) in *ascV* (25 %). Nobeden od izolatov ni imel pomnoženega gena *exu*, povezanega z DNAzo. *A. hydrophila*, *A. dharkensis*, *A. veronii* in *A. caviae* so vsebovali sedem testiranih genov virulence, razen *exu*, medtem ko so bili v *A. enteropelogenih* odkriti le štirje virulenčni geni. Med 75 izolati, odpornimi na tetracikline, so bili geni *tetA*, *tetE* in *tetB* odkriti v 38, 26 oziroma 6 izolatih. Med preizkušenimi geni za odpornost proti β-laktamu so bili geni bla_{OXA} in bla_{TEM} odkriti pri 54 % oziroma 36 % izolatov, odpornih proti β-laktamu.

V nobenem vzorcu nista bila zaznana gena bla_{CTX-M} in bla_{SHV}. Rezultati študije kažejo, da bakterije *Aeromonas spp.* iz hišnih želv lahko imajo potencialne virulenčne gene in gene za odpornost proti tetraciklinu in β-laktamom, in lahko potencialno ogrožajo zdravje lastnikov hišnih želv, zlasti imunsko oslabljenih posameznikov.

Ključne besede: *Aeromonas* spp.; geni povezani z virulenco; odpornost na tetracikline; rezistenca na β-laktami; ljubiteljske vrste želv

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Case Report

PALLIATIVE JAW-SPARING TREATMENT OF A NON-RESECTABLE CANINE ORAL FIBROSARCOMA USING COMBINATION OF ELECTROCHEMOTHERAPY WITH BLEOMYCIN AND IL-12 GENE ELECTROTRANSFER

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Abstract: A 15-year- old male castrated English setter was presented for evaluation of a rapidly growing oral mass. Patient's history was otherwise unremarkable, except of moderate proteinuria of 5 years duration. Clinical examination findings were within normal limits, except of an ulcerated lesion located at the left mandibular canine tooth, which was histologically confirmed as a high grade infiltrative fibrosarcoma with high mitotic index (61/10 HPF) and multifocal necrotic areas. The client declined full staging, so only hematological and biochemistry examinations of blood were performed, which were within normal limits. Furthermore a fine needle aspiration biopsy of regional lymph nodes was performed, which revealed reactive lymphadenopathy without signs of metastases. After declining other more invasive therapeutic procedures, the clients elected treatment with combination of electrochemotherapy and IL-12 electrogene therapy. Four consecutive treatment sessions were performed, resulting not only in complete response of the primary tumor, but also in regression of untreated distant metastases, which were diagnosed approximately one month after the initial examination. Furthermore, the percentage of circulating CD8+ cells was increased after each therapy session, indicating possible systemic induction of immune response by IL-12 gene therapy. This case shows that this type of therapy can represent an alternative type of both local and systemic treatment in selected tumor cases, where clients seek a less invasive nonsurgical treatment.

Key words: dog; fibrosarcoma; electroporation; electrochemotherapy; electrogene therepy; interleukin-12

Introduction

Malignant neoplasms of the oral cavity represent approximately 6% of all canine tumors, with fibrosarcoma (FSA) being one of the three most common malignant oral tumors in dogs (1). The most common site of presentation is gingiva, followed by lip, cheek and tongue (1). FSAs are reported to be primarily locally aggressive with a low potential for distant metastases. Gingival tumors can invade the bone and spread to the palate. Early diagnosed tumors (i.e., smaller tumors) are mostly treated by curative-intent surgery (2), which can be combined with radiotherapy in selected cases. However, oral tumors often develop unnoticed until the tumor reaches an advanced stage, when it is often inoperable without causing significant dysfunction and cosmetic changes to the animal. In such cases, different palliative therapeutic approaches may be used, depending on the sensitivity of the tumor. Outcome of any such therapy is usually short-lived, with reportedly high recurrence rates, even over 50% and short survival times (3). Wide margin surgical excision of oral FSA as a sole therapy results in mean survival time (MST) of 12-24 months (3, 4).

Electrochemotherapy (ECT) and gene electrotransfer are techniques, where electric pulses are

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used to increase cell membrane permeability to enhance entry of the cytotoxic agents (i.e., electrochemothrapy, ECT) and plasmid DNA (i.e., gene electrotransfer or electrogene therapy, EGT) into the cells. ECT combined with EGT with plasmid encoding interleukin-12 (IL-12) has already been described for the treatment of a small number of naturally occurring spontaneous canine oral neoplasms with encouraging results (5, 6).

Important feature of ECT and EGT with plasmid encoding IL-12 is the elicitation of the immune response, which is believed to have systemic effects. IL-12 stimulates cytotoxic T lymphocytes, which are crucial in cancer elimination (7) as they target the tumor cells and eliminate them by the action of perforin and granzyme B (8). Perforin is involved in formation of pores on the membrane of tumor cells, thus enabling the granzyme B to enter the tumor cells where it causes apoptotic tumor cell death (9). Such induction of immune response can be detected using different techniques, including flow cytometry, which utilizes gating different types of peripheral blood mononuclear cells using specific antibodies.

The purpose of this case report is to describe a case of a large grade II mandibular gingival FSA in a 15-year-old dog successfully treated with ECT and EGT with plasmid encoding IL-12.

Case presentation

A 15-year- old male castrated English setter, weighing 22 kg, was presented to the Small animal clinic of Veterinary faculty Ljubljana for evaluation of a rapidly growing oral mass. History of the patient was unremarkable, except persistent moderate proteinuria of 5 years duration, which has been well controlled with appropriate diet and enalapril. Clinical examination findings were within normal limits, apart the large mass at the left mandibular canine tooth. The client declined full staging including head CT scan and diagnostic imaging for possible distant metastases. Therefore only partial staging was performed, including complete blood count with white blood cell count (performed using an automated laser hematology analyzer with species-specific software Advia 120, Siemens, Munich, Germany) and detailed biochemistry panel (performed using automated chemistry analyzer RX-Daytona, Randox, Crumlin, UK). Biochemistry panel consisted of serum concentrations of glucose, urea, creatinine, Na, K, Cl, Ca, total proteins, albumin and serum activity of alkaline phosphatase, alanine aminotransferase and creatine kinase. All parameters were within normal limits except moderate, clinically

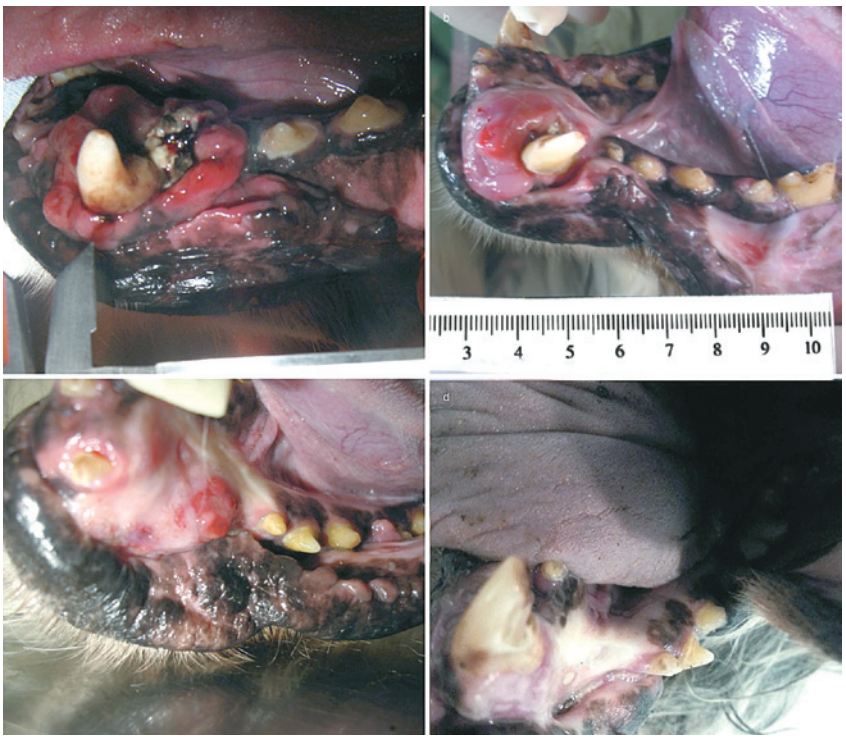


Figure 1: The figure is showing regression of the tumor mass at different time points. (A) tumor mass at the time of the first therapy. (B) clinical regression of the tumor at 4 weeks after the first therapy and before the second therapy. (C) four months after the first of the tumor therapy the size of the tumor significantlly decreased. (D) seven months after initial therapy complete regression could be seen

irrelevant thrombocytosis (platelets 575 x 10-9/L, reference value 143-400 x 10-9/L).

A detailed oral examination and dental radiographs with the dog under general anesthesia were performed, revealing a 4cm x 3cm x 3cm partly ulcerated proliferative mass at the left mandibular canine tooth (Figure 1). Geographic bone loss at the left mandibular incisor and canine teeth was visible on dental radiographs, and permeative pattern of bone loss in the symphyseal region suggested bony involvement of both rostral mandibles (T3b) (Figure 2). At the same time incisional biopsy was performed, and the mass was histologically confirmed as a high grade infiltrative FSA with high mitotic index (61/10 HPF) and multifocal necrotic areas. Fine needle aspiration biopsy of regional lymph nodes was performed, revealing reactive lymph nodes.

After discussing possible treatment options, including bilateral rostral mandibulectomy in combination with radiotherapy, the client elected ECT and EGT. During the next four months, four therapy sessions were performed (Graph 1). Each session was performed with the dog under short (approximately 30 min) general anesthesia, starting with ECT using intravenous application of bleomycin (Blenoxane, Bristol-Myers, Princeton, USA; 3 mg/ml) at the dose 0.3 mg/kg, followed by delivery of electric pulses with electric pulses generator Cliniporator™ (IGEA s.r.l., Carpi, Italy). Train of 8 pulses was applied, each pulse of 100 μs duration and amplitude to electric distance ratio of 1300 V/cm and frequency of repetition 1 Hz, using two parallel stainless steel plate electrodes with 6 mm distance between them. This procedure was followed

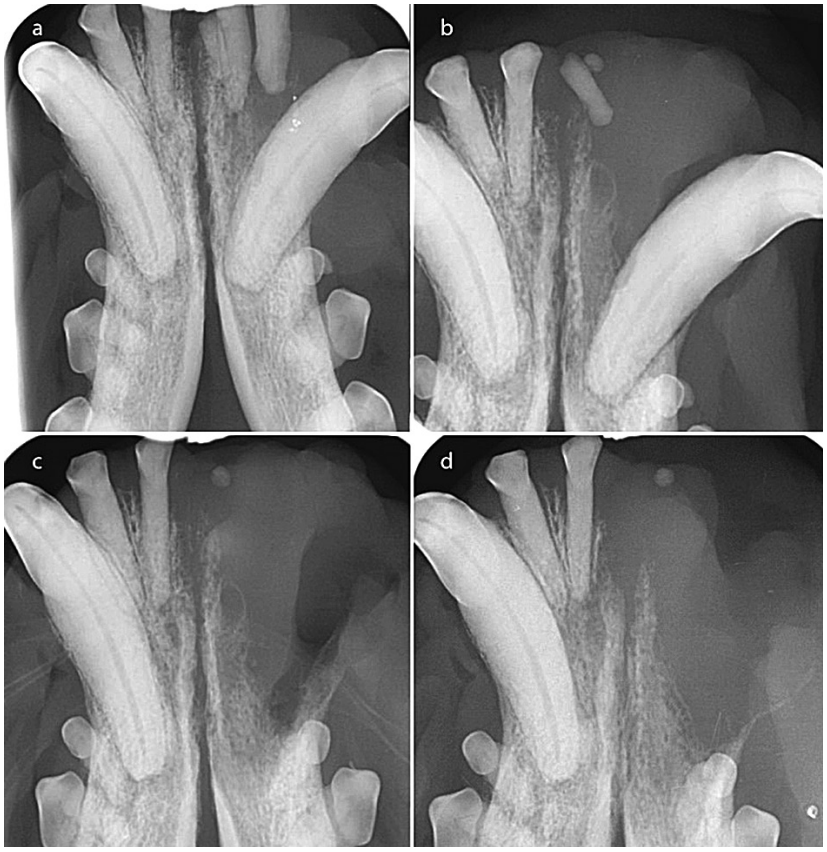
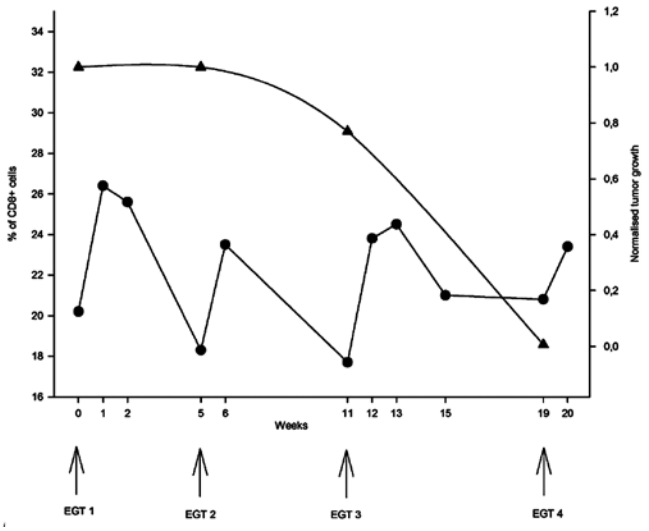


Figure 2: (A) Dental radiograph, occlusal view of the rostral mandibles at initial presentation. Geographic bone loss at the left mandibular incisor and canine teeth was visible on dental radiographs, and permeative pattern of bone loss in the symphyseal region suggested bony involvement of both rostral mandibles (T3b). Clinical assessment of radiographically abnormal (fractures or abrasion) left mandibular incisor teeth was impossible as they were embedded in the tumor mass. Right mandibular third incisor tooth is missing. (B) Dental radiograph, occlusal view of the rostral mandibles at 4 weeks. Radiographically visible severe progression of the osteolysis. Left mandibular second and third incisor teeth had exfoliated since the last visit. There is a total loss of attachment at the left mandibular first incisor tooth and near total loss of attachment at the left mandibular canine tooth, therefore these two teeth were removed (C). The round poorly mineralized structure remained, as it was hidden in the soft tissues of the tumor. (D) Dental radiograph, occlusal view of the rostral mandibles at 10 weeks. Radiographically subjectively decreased osteolysis progression

by EGT with peritumoral application of 2 mg of plasmid encoding canine IL-12 (pCMVcaIL-12) into the mucosa. The same generator of electric pulses was used to deliver electric pulses for gene delivery. Two sets of pulses were used, one high- and four low-voltage. High-voltage pulses consisted of 100 μs duration and amplitude to electric distance ratio of 600 V/cm and low-voltage pulses consisted of 100 ms and amplitude to distance ratio of 200 V/cm. Plate electrodes with 6 mm distance between them were used.

Tumor regression was observed 4 weeks after the first therapy (Figure 1, Graph 1), although dental radiographs revealed progression of the osteolysis, requiring removal of the left mandibular incisor and canine teeth. After the second session, subjective decrease in osteolysis progression was also noted on dental radiographs. However, one month after the first therapy a subcutaneous metastases in the intermandibular region was diagnosed by cytopathologic examination of fine needle aspirate. In the course of the next three months tumor burden steadily decreased (Graph 1) and re-check at four months after the initial therapy revealed only ongoing mild-moderate necrosis of the treated area. Client reported minimal side effects and an improved quality of life of the dog. At this time point complete remission of both intraoral tumor as well as subcutaneous metastases was reached. The dog was euthanized 8 months after the initial combined therapy due to causes unrelated to the oral tumor and at that time point the dog was without any macroscopic evidence of either oral tumor or metastases in surrounding tissue. Necropsy was declined by the client.

At different time points following each therapy (1, 2, 4 weeks and then monthly after remission was achieved), detailed bloodwork was performed, including the same hematological and biochemical parameters as at the initial staging. In addition, flow cytometry was performed on frozen whole blood collected at each visit. The lymphocytes were gated for CD45 (leukocytes), CD3 (lymphocytes T), CD4 (helper T lymphocytes) and CD8 (cytotoxic T lymphocytes) markers and the percentage of each cell population was calculated. The percentage of CD8+ cells temporarily increased after each therapy and decreased in the following weeks (Graph 1). We did not observe any change in the percentage of other cell populations. Furthermore, we used quantitative polymerase chain reaction (qPCR) with specific primers for the pCMVcaIL-12 plasmid to detect the presence of plasmid DNA in the urine, stool and oral mucosa swab samples in order to determine possible shedding of plasmid into the environment. Urine and stool samples were collected one week after each therapy and no plasmid DNA was detected in these samples. Oral mucosa swabs from the area of plasmid injection were collected immediately after the therapy and at different time points thereafter (1, 2 and 4 weeks). In the first sample maximal concentration of IL-12 plasmid detected at the site of injection was 100 ng/mL of plasmid DNA. After one week, the concentration already dropped to 0,5 pg/mL and at later time points no quantity of plasmid could be detected.



Graph 1: Percentage of CD8+ cells (●) detected by flow cytometry and tumor growth rate normalised on tumor size at first visit. (▲) The percentage of CD8+ cells temporarily increased after each therapy and decreased in the following weeks after EGT therapy

Discussion

In this case report we describe effective use of ECT with bleomycin and EGT with canine IL-12 in the treatment of a canine oral FSA with subcutaneous metastases, resulting in complete regression of primary treated tumor as well as untreated distant metastases, which enabled marked prolongation and improved quality of the dog's life. Oral FSA in dogs are usually fast growing neoplasms, prone to aggressive infiltrative growth. Therefore, the treatment of choice is surgical excision. However, in cases of advanced oral tumors, wide excision may cause significant cosmetic changes and, more importantly, impaired

function in the treated animal (e.g., mandibular drift after a segmental or total mandibulectomy with glossoptosis and drooling) (2). Prolonged recovery after large surgical resections, combined with usually high costs and poor outcome (high recurrence rate due to infiltrative tumor growth), makes the owners often reluctant to elect such invasive procedures, especially in older animals, as was also the case in the presented case. Based on our previous experience with treating oral and superficial cutaneous tumors in both dogs (10, 11) and cats (12) with either ECT or EGT or combined therapy, we offered this treatment combination as an alternative treatment option.

The dog received four therapy sessions in the course of 4 months and was euthanized 8 months after the first session due to tumor unrelated causes. At that time, there was no evidence of local tumor growth (oral cavity, intermandibular area, regional lymph nodes). Given the rapid tumor growth upon presentation (the mass approximately doubled in size in the course of one week before initiation of the treatment), the combined treatment resulted in slower progression of the tumor, with the first evidence of tumor regression 4 weeks after instituting ECT and EGT. With subsequent treatments we were able to induce complete regression of primary lesion, as well as subcutaneous metastases, which resulted in highly improved quality of the remaining life of the animal.

The pronounced cytoreductive effect of the combined treatment resulting in remission of described FSA can mainly be attributed to the ECT, since previous studies showed excellent local antitumor effect of this type of therapy, resulting in even up to 100% of complete response (CR) rates in certain human tumor types (13). In veterinary medicine, a number of studies employed ECT as an antitumor treatment, in which CR rates around 70-80% were accomplished (10,11, 14). On the other hand, experience with IL-12 EGT as a single treatment in veterinary patients is lacking. The only published study (15) reported less pronounced cytoreductive effect. In contrast to preclinical studies on laboratory animals in which IL-12 EGT as a single therapy resulted in even 100% eradication of different tumor types (16, 17), study in canine mast cell tumors resulted in only 36% of CR rate (15).

It should be emphasized that the presented case exhibited one important distinction to other reports of ECT efficacy, namely distant effect on subcutaneous untreated metastases, which, according to the current knowledge, cannot be attributed to any antitumor effect of ECT. Although it is known, that ECT causes tumor antigen shedding into surrounding tissue and blood due to ECT-induced immunogenic cell death, this is considered inadequate for prevention of the growth of distant tumors, thus resulting in antitumor effect only at local level (18, 19, 20). Therefore the distant effect of the combined therapy in our case can be attributed to immunotherapeutic effects of the procedure. It was already established, both on preclinical level as well as human clinical study, that IL-12 EGT exhibits antitumor effect on distant untreated tumors, distant metastases and even elicits long-term resistance to regrowth of tumors (17, 21). Similar distant effect on lymph node metastases was also seen in other studies, employing *IL-12* EGT with or without ECT component (5, 15, 22).

Immunological response to therapy was followed by flow cytometry, measuring different population of T lymphocytes. It was shown that dogs with tumors have decreased percentage of circulating CD8+ cells in comparison with healthy dogs (23). In our case the percentage of circulating CD8+ cells was increased after each session, which lasted up to 2 weeks. Therefore, the fluctuation of CD8+ cells in blood could serve as a guideline for timing repetition of the therapeutic procedure. In other studies, CD8+ cells were measured only in tumors, and the results showed the correlation between the increase of these cells in the tumor and better response of these tumors to the treatment in comparison to tumors without the increased infiltration of CD8+ cells (24, 25). It is proposed that intratumoral cytotoxic T lymophocytes migrate into the circulation reaching distant metastases where they can exert their immunological mediated tumor cell death (20). Therefore, the systemic increase of CD8+ lymphocytes could have abscopal effect on distant metastases, which was observed in our case. Therefore, the increase of CD8+ cells in blood could serve also as a predictive factor for the abscopal effect of the therapy.

One of the most important aspects of any gene therapy is environmental safety of the procedure. Namely, therapeutic plasmid contains antibiotic resistance gene, which can be horizontally transferred into commensal bacteria present on either treated patient's skin or in gastrointestinal tract when treating oral tumors (26). To our best knowledge, only our research group addressed this aspect of safety of IL-12 EGT in clinical setting (11). In the presented case, persistence of plasmid DNA was

monitored, at the site of injection (mucosa) as well as in the feces and urine. Similar to the larger study (11), majority of the plasmid in oral mucosa swabs was detected immediately after application, with abrupt decline of its quantity in the first week and complete disappearance in the course of the second week after the procedure. Furthermore, no plasmid was detected in feces and urine, further confirming the results of our previous study (11), that the IL-12 plasmid cannot be horizontally transferred into the culturable bacteria from the patients, therefore the possibility of any environmental shedding of antibiotic resistance gene is negligible.

Conclusion

In conclusion, the combination of ECT with bleomycin and EGT with IL-12 plasmid in the presented case of canine oral FSA exhibited very good local and systemic antitumor effect and was safe for the treated patient. Furthermore, potential for any environmental hazard of this type of gene therapy is negligible. Therefore, this treatment modality can represent an alternative type of therapy in selected cases, where clients seek a less invasive nonsurgical treatment of these tumors.

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PALIATIVNO ZDRAVLJENJE NERESEKTABILNEGA FIBROSARKOMA SPODNJE ČELJUSTI PRI PSU S KOMBINACIJO ELEKTROKEMOTERAPIJE Z BLEOMICINOM IN GENSKEGA ELEKTROPRENOSA IL-12

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Izvleček: Petnajstleni kastrirani angleški seter je bil na našo kliniko sprejet z namenom pregleda hitro rastoče novotvorbe v ustni votlini. Pes ni imel pomembnejših sočasnih bolezni, razen zmerne proteinurije zadnjih pet let. Fizikalni pregled ni pokazal nikakršnih odstopanj z izjemo ulcerirane novotvorbe na področju levega mandibularnega grabilca. Histološka diagnoza novotvorbe je bila infiltrativni fibrosarkom visoke stopnje z visokim mitotičnim indeksom (61/10 v polju visoke povečave) in multifokalnimi nekrotičnimi področji. Skrbnik psa je zavrnil popolno določitev stadija bolezni, zato smo izvedli le osnovne hematološke in biokemijske preisakve, ki niso pokazale pomembnejših odstopanj. Izvedli smo tudi tankoligelno biopsijo regionalnih bezgavk, ki je pokazala reaktivno limfadenopatijo brez znakov prisotnosti zasevkov. Po predstavitvi vseh možnosti zdravljenja se je lastnik odločil za zdravljenje s kombinacijo elektrokemoterapije in genskega elektroprenosa IL-12. Izvedli smo štiri zaporedne cikluse kombinirane terapije, s katero smo dosegli ne le popolni odgovor primarnega tumorja, pač pa tudi regresijo nezdravljenih oddaljenih podkožnih metastaz, ki so se pojavile približno mesec dni po začetku terapije. Poleg tega smo po vsaki terapiji ugotovili povečan delež cirkulirajočih CD8+ celic, kar lahko nakazuje na to, da je genska terapija z IL-12 sprožila sistemski imunski odziv. Predstavljeni primer kaže, da lahko kombinacija elektrokemoterapije in genskega elektroprenosa IL-12 predstavlja alternativno obliko tako lokalnega kot sistemskega zdravljenja določenih novotvorb, zlasti v primerih, ko skrbnik živali želi manj invazivne terapevtske postopke.

Ključne besede: pes; fibrosarkom; elektroporacija; elektrokemoterapija, genski eletroprenos; interlevkin-12

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