Cellular and Molecular Biology

Abd El-Hamid *et al. Cell. Mol. Biol.*2016, 62 (10): 49-54
ISSN: 1165-158X
doi: 10.14715/cmb/2016.62.10.8

Original Research

Protective potency of clove oil and its transcriptional down-regulation of *Aeromonas sobria* virulence genes in African catfish (*Clarias gariepinus* L.)

M. I. Abd El-Hamid[1], N. K. Abd El-Aziz[1*], H. A. Ali[2]

[1] Department of Microbiology, Faculty of Veterinary Medicine, Zagazig University, Zagazig, Sharkia, Egypt
[2] Department of Biochemistry, Faculty of Veterinary Medicine, Zagazig University, Zagazig, Sharkia, Egypt

Abstract: Disease episodes of fish caused by *Aeromonas* species are moved to the top list of limiting problems worldwide. The present study was planned to verify the *in vitro* antibacterial activities as well as the *in vivo* potential values of clove oil and ciprofloxacin against *Aeromonas sobria* in African catfish *(Clarias gariepinus)*. The *in vitro* phenotypic virulence activities and the successful amplification of aerolysin and hemolysin genes in the precisely identified *A. sobria* strain were predictive for its virulence. In the *in vivo* assay, virulence of *A. sobria* strain was fully demonstrated based on constituent mRNA expression profile of tested virulence genes and typical septicemia associated with high mortalities of infected fish. Apparent lower mortality rates were correlated well with both decrescent bacterial burden and significant down-regulated transcripts of representative genes in the treated groups with clove oil, followed by ciprofloxacin as a prophylactic use for 15 days ($P < 0.0001$); however, the essential oil apart from ciprofloxacin significantly enhanced different hematological parameters ($P < 0.05$). In addition, administration of antibiotic may be considered as a pronounced stress factor in the fish even when it used in the prophylactic dose. In conclusion, medicinal plants-derived essential oils provide a virtually safer alternative to chemotherapeutics on fish, consumers and ecosystems.

Key words: Clove oil, *Aeromonas sobria*, African catfish, gene expression, blood chemistry.

Introduction

Aeromonas sobria infection in catfish is fatal causing hemorrhagic septicemia, ascites and ulcer formation (1). The occurrence of septicemia is mainly contributed by the release of two important virulence factors; extracellular hemolysin and aerolysin (2).

At present, the interest in protection against fish diseases has grown enormously. The use of antibiotics has become an urgent need to solve the problem of progressing and persistence of the serious bacterial diseases` outbreaks, particularly when used as early as possible. However, several difficulties are often encountered by antibiotics regarding the cost of drugs, accumulation of antimicrobial hazardous residues in fish flesh and subsequently in human tissues, environmental pollution and the risk of developing bacterial resistance (3). In order to cope with these negative impacts, several attempts have been made to develop the disease control measures using natural plant-derived extracts with multi–antimicrobial properties as alternative therapeutic and/or prophylactic agents against several fish pathogens (4).

Considering the importance of *A. sobria* as a lethal pathogen in fish farms, many practical and experimental studies were reported to investigate the antimicrobial potential values of the essential oils against the considered pathogen (1, 5); but, there were no previous reports explored their impact on virulence genes` expressions in such aeromonad. Therefore, the present work was conducted to determine the *in vitro* antibacterial activities of clove oil and ciprofloxacin against *A. sobria*. Further, a possible *in vivo* approach was performed in African catfish challenged with *A. sobria* for demonstrating the efficacy of clove oil and ciprofloxacin on the expression profiles of virulence-associated genes of the considered bacterial pathogen using quantitative real time reverse transcriptase PCR (qRT–PCR).

Materials and Methods

Bacterial strain

A presumptive strain of *Aeromonas* species isolated from naturally infected freshwater fish during a previous microbiological survey was kindly obtained from Department of Fish Diseases, Faculty of Veterinary Medicine, Zagazig University, Egypt. The putative isolate was identified on the basis of Vitek® 2 system (bioMérieux, Inc., Hazelwood, MO, USA), then molecular characterization was verified by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of 16S rRNA gene following the methods described previously (6). The bacterial isolate was passed twice in a healthy African catfish to enhance its virulence then re-isolated from moribund, freshly dead or sacrificed fish to be used for experimental challenge (7).

Virulence properties of the bacterial strain

The confirmed *A. sobria* strain was tested for β-hemolytic activity on blood agar base (Oxoid, Hampshire, England, UK) supplemented with 5% of sheep erythrocytes and its ability to take up Congo red dye (Sigma-Aldrich, Co., St. Louis, MO, USA) using the methods reported previously (8). Further, it was scree-

Received June 13, 2016; **Accepted** August 17, 2016; **Published** August 31, 2016

*** Corresponding author:** Norhan K Abd El-Aziz, Lecturer of Microbiology, Faculty of Veterinary Medicine, Zagazig University, Zagazig, Sharkia, Egypt. Email: norhan_vet@hotmail.com; nourhan_vet@zu.edu.eg; pdrahmedpdrahmed@yahoo.com

ned for the presence of genes encoding aerolysin and extracellular hemolysin by PCR using primers and conditions already mentioned previously (9-11).

In vitro antibacterial assays

The susceptibility testing of *A. sobria* strain against clove oil emulsion (20%) in Tween-20 (1%) (Sigma-Aldrich, Co., St. Louis, MO, USA) (12) and several antibiotics including nalidixic acid (30 µg), ciprofloxacin (5 µg), norfloxacin (5 µg), ampicillin (10 µg), amoxicillin/clavulanic acid (20/10 µg), ceftriaxone (30 µg), chloramphenicol (30 µg), trimethoprim/ sulfamethoxazole (1.25/23.75 µg), gentamicin (10 µg), erythromycin (15 µg) and colistin sulphate (10 µg) (Oxoid, Hampshire, England, UK) was carried out using disc diffusion method (13). Moreover, minimum inhibitory concentrations (MICs) of clove oil and ciprofloxacin (Sigma-Aldrich, Co., St. Louis, MO, USA) were determined using the reference broth microdilution method as the protocols described elsewhere (14-16). The susceptibility breakpoints were defined in accordance with the interpretive criteria published in the Clinical and Laboratory Standard Institute (CLSI) document M45 (15).

Fish, ethics and consent

A total of 240 apparently healthy African catfish (*C. gariepinus*) obtained from Abbassa Fish Hatchery, Egypt with an average body weight of 15 ± 5 g were transported alive to the laboratory for an experimental study. The health status of fish was examined immediately upon arrival for any abnormal behavioral changes, external signs and gross lesions before the onset of the experiment. Fish were acclimatized to laboratory conditions for two weeks providing adequate feed and better aeration. They were maintained at 25-28ºC in glass aquaria with dimensions of 30 x 80 x 40 cm, supplied with chlorine free tap water. During this time and throughout the experiment, they were fed a commercial balanced diet containing 30% crude protein twice daily. Handling of fish was conducted in strict accordance with the recommendations set up by the Canadian Council on Animal Care (CCAC) following the guide to the care and use of experimental animals (17). The protocol was approved by the Committee on the Ethics of Animal Experiments of the Faculty of Veterinary Medicine, Zagazig University, Egypt.

Experimental design

Eight triplicate groups each of 10 healthy African catfish were allocated for the experiment. Group one (infected non-treated) was consistently intra-peritoneal (I/P) inoculated with 0.2 ml of 24 h broth culture of *A. sobria* ($2.5x10^8$ CFU) (1); group two (non-infected non-treated) was injected I/P with 0.2 ml of sterile tryptic soy broth; the next four groups represented infected treated ones and comprising the following: group three was supplied by a fish diet supplemented with 3% clove oil for five successive days, infected similarly on the 6^{th} day then reared for additional 10 days with the assigned fish diet as a prophylactic use (18), group four was administered by a prophylactic dose of ciprofloxacin (15 mg/kg body weight) orally, five times every 72 h intervals (19), then infected similarly, group five received the bacterial infection then treated by a therapeutic dose of clove oil (13.25 µl/l) as a bath and group six was infected and treated by a therapeutic dose of ciprofloxacin (25mg/l) (1), both therapeutic treatments were continued for five successive days without water renewal. Last 2 groups (non-infected treated) received either clove oil or ciprofloxacin only, and assigned as groups seven and eight, respectively. Importantly, clove oil concentration has no sedative effect in African catfish and was chosen to avoid behavioral side effects during the long exposure time of the experiment protocol (20).

All the experimentally treated fish were monitored twice daily for any abnormal clinical signs and mortalities. Besides, the dead, moribund and clinically diseased fish were subjected to re-isolation and identification of the challenged bacterium adopting the methods described formerly (5).

Biochemical and serum analysis

Serum samples were used to evaluate aspartate aminotransferase (AST), alanine aminotransferase (ALT), total proteins, malondialdehyde (MDA), reduced glutathione (GSH), urea, creatinine and immunoglobulin (IgM) levels two weeks post challenge (21).

RNA extraction and qRT–PCR analysis

Total RNA extraction was accomplished using QIAamp RNeasy Mini kit (Qiagen, Germany, GmbH(. Quantitative RT–PCR was performed to determine the expression patterns of aerolysin and hemolysin genes of *A. sobria* in tissue specimens of fish in triplicates using one-step RT-PCR kit with SYBR green (Qiagen, Germany, GmbH). Reaction mixtures were incubated at 50°C for 30 min, followed by one cycle of 94°C for five min and 40 cycles of 94°C for 15 s, 52°C for 30 s, 72°C for 30 s for aerolysin gene and 94°C for 45 s, 55°C for 30 s, 72°C for one min for hemolysin gene. Dissociation curves were generated by a cycle of 94°C for one min, 52°C for one min (aerolysin); 55°C for one min (hemolysin) and 94°C for one min. The relative quantitation of mRNA expression in each sample was normalized to the constitutive expression of the 16S rDNA housekeeping gene. The transcription levels were analyzed using the comparative $\Delta\Delta C_t$ method (22).

Statistical analysis

Results were analyzed by One-way analysis of variance (ANOVA), followed by Tukey's Honestly Significant Difference (HSD) as post hoc test using Statistical Package for Social Sciences (SPSS) version 22.0 (IBM Corp., Armonk, NY, USA). The data were showed in means ± Standard Error of Mean (SEM) and significance was indicated at P value < 0.05.

Results

Identification and *in vitro* virulence traits of *A. sobria* strain

Typical aeromonad morphological and biochemical characteristics delivered a preliminary identification of presumptive Aeromonas strain. Further, PCR- RFLP analysis of the 16S rRNA gene resulting in DNA fingerprints (ranging from 33 to 207 bp) constituting specific patterns accurately identified the bacterial strain to the level of phylogenetic species. It is worthy to mention

that our strain showed a positive hemolytic reaction, strongly bound with Congo red and possessed aerolysin and hemolysin genes encoding virulence traits as revealed in simplex PCR assays, hence assumed to be more virulent.

In vitro antibacterial activities

In vitro sensitivity results using disc diffusion method indicated a strong inhibitory activity of clove oil against *A. sobria* strain (zone diameter = 40 mm). Various susceptibility patterns to 11 antibiotics of eight different groups were reported. Concerning this point, ciprofloxacin exhibited the highest efficacy against *A. sobria* (36 mm) than other quinolones (nalidixic acid and norfloxacin, 34 mm), followed by ceftriaxone (32 mm), chloramphenicol (24 mm), trimethoprim/sulfamethoxazole (22 mm) and gentamicin (18 mm); while ampicillin, amoxicillin/clavulanic acid, colistin sulphate and erythromycin produced insignificant zones of activities rendering the strain a multidrug resistant (MDR). Moreover, broth micro dilution test explored maximum activities of clove oil and ciprofloxacin (MIC value 0.00195 µg/ml each).

Efficacy of treatment trials in challenged fish

Experimentally infected *C. gariepinus* exhibited diverse clinical signs and mortality records. Positive control group showed abnormal behavior as listlessness, stop feeding, loss of reflexes and inability to swim. Furthermore, it expressed typical clinical picture of *A. sobria* infection including severe erythematic and hemorrhagic patches on different parts of the body surfaces, fins, operculum and around barbells. Internally, dissection of the freshly dead fish revealed friability and congestion of all internal organs, enlarged kidneys and distended gall bladder.

Mortality patterns in experimentally infected fish were first recorded 18 h post exposures and increased gradually. Remarkably, the cumulative mortalities varied among experimentally infected groups, it was 100% in infected non-treated one, however, both prophylactic and therapeutic treatment trails led to ameliorating the general health condition of fish with dimensioning the intensity of clinical signs and mortalities. It was noticed that the lowest cumulative mortality rate (10%) was observed in infected-treated group with 3% clove oil as a prophylactic use indicating the protective effect on *A. sobria* infection in African catfish and its potential to replace antibiotics for controlling the disease, while, 30% fish mortality was recorded in infected-treated group with a prophylactic dose of ciprofloxacin. Interestingly, moderately high mortality rates were apparent in infected-treated groups with clove oil (60%) and ciprofloxacin (40%) as therapeutic treatments.

Re-isolation of aeromonas bacterial cells was recovered from freshly dead and clinically diseased sacrificed fish allover the experiment with lower bacterial loads from internal organs of infected-treated fish. On the contrary, control negative groups showed neither clinical signs nor mortalities and no *A. sobria* was isolated yet.

Serum parameters

Serum analysis revealed various metabolic disorders among experimental groups (Table 1). Obviously, positive control group displayed a significant increase of AST and ALT activities, serum urea, creatinine, total proteins and MDA levels together with decreasing the reduced GSH levels in comparison with respective parameters of the negative control group. Certainly, prophylactic or therapeutic administration of the essential oil succeeded to significantly enhance the conditions of all analyzed parameters. Moreover, serum IgM demonstrated significantly higher values in clove oil treated groups comparing to the positive control one appointed in the *in vivo* design. Hence, a more robust inference delivered from our study is the non-progressive disorders accompanied by no serious changes in serum parameters after exposing fish to clove oil.

Contrary to the eligible impact of clove oil, certain significant variations of blood chemistry in all fish groups received ciprofloxacin comparing with both positive and negative controls emphasized that antibiotic administration is an under recognized stress factor drastically alter fish biochemistry and metabolism, which must be dealt through unified local and global preventive approaches.

In vivo modulation of virulence genes expression

Quantitative RT-PCR analysis explored that the highest mRNA expression levels of *A. sobria* aerolysin and hemolysin genes were ubiquitously detected in

Table 1. Hematological changes in *Clarias gariepinus* within each group of the experiment protocol.

Serum parameter	Experiment groups							
	G1	G2	G3	G4	G5	G6	G7	G8
Total proteins (gm/dl)	6.03± 0.3^{b}	5.4±0.26^{c}	5.6±0.38^{c}	6.8±0.13^{a}	5.7±0.23^{c}	6.9±0.24^{a}	5.3±0.34^{c}	5.9±0.6^{bc}
GSH (ng/ml)	6.21±0.23^{d}	9.59±0.26^{a}	7.56±0.42^{c}	6.98±0.31^{d}	7.24±0.22^{c}	6.56±0.51^{d}	9.7±0.6^{a}	8.2±0.24^{b}
MDA (nmol/l)	34.31±1.34^{b}	22.4±1.3^{e}	26.4±0.96^{d}	36.4±0.8^{a}	29.4±1.1^{c}	35.4±1.26^{ab}	22.2±0.93^{e}	27.1±0.98^{d}
ALT (IU/dl)	75.7±2.33^{b}	48.67±0.67^{e}	55.2±1.57^{d}	80.3±2.5^{a}	60.4±1.8^{c}	78.8±0.95^{ab}	47.23±0.21^{e}	54.6±0.62^{d}
AST (IU/dl)	36.14±0.5^{b}	21.05±0.53^{e}	27.34±1.3^{d}	38.05±1.5^{a}	30.5±1.2^{c}	37.5±1.87^{a}	20.65±0.21^{e}	26.5±1.2^{d}
Creatinine (mg/dl)	1.15±0.05^{c}	0.47±0.03^{f}	0.61±0.02^{e}	1.4±0.05^{b}	0.76±0.08^{d}	1.54±0.02^{a}	0.48±0.04^{f}	0.46±0.08^{f}
Urea (mg/dl)	1.67±0.03^{c}	0.82±0.05^{f}	0.93±0.33^{e}	1.83±0.04^{a}	1.04±0.06^{d}	1.74±0.07^{b}	0.8±0.04^{f}	0.79±0.09^{f}
IgM value (µg/ml)	10.38±0.43^{f}	23.33±0.08^{a}	18.42±0.11^{c}	15.53±0.36^{e}	17.8±0.62^{cd}	16.84±0.8^{de}	24.8±0.1^{a}	20.63±0.08^{b}

G, Group; G1, infected non-treated; G2, non-infected non-treated; G3, G4, infected and prophylactic treated with either clove oil or ciprofloxacin, respectively; G5, G6, infected and therapeutic treated with either clove oil or ciprofloxacin, respectively; G7, G8, non-infected treated with either clove oil or ciprofloxacin, respectively.

Means ± Standard Error in the same row carrying different superscript are significantly different ($P < 0.05$).

Table 2. Relative expression of *A. sobria* virulence genes in challenged *Clarias gariepinus* exposed to various treatments.

Virulence gene	Infected non-treated group	Infected-treated groups*				F value	*P* value
		G3	G4	G5	G6		
Aero	1.04±0.01[a]	0.002±0.0002[e]	0.015±0.003[de]	0.493±0.038[b]	0.129±0.025[c]	446.5	< 0.0001
Hly	1.01±0.03[a]	0.008±0.0005[e]	0.031±0.006[de]	0.785±0.019[b]	0.147±0.005[c]	826.1	< 0.0001

G, Group; G3, G4, infected and prophylactic treated with either clove oil or ciprofloxacin, respectively; G5, G6, infected and therapeutic treated with either clove oil or ciprofloxacin, respectively.

*Values represent the means of fold change in comparison with the transcription level of *A. sobria* strain extracted from infected non-treated group. Means within the same raw carrying different superscripts are significant different at $P < 0.05$ based on Tukey's Honestly Significant Difference test.

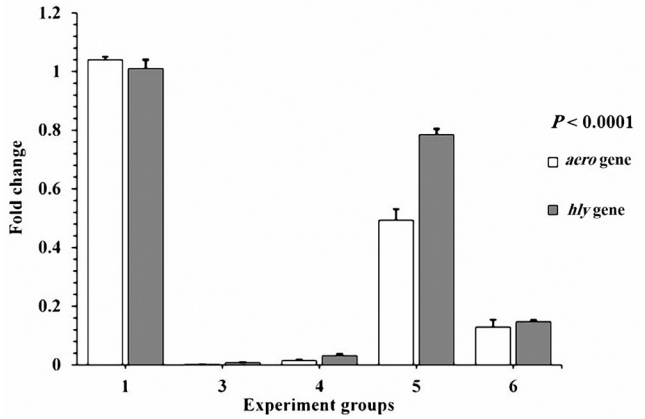

Figure 1. Transcript levels of aerolysin and hemolysin genes of *A. sobria* in tissue specimens of challenged catfish**.** The fold change of infected-treated groups was calculated with the ΔΔCt method using 16S rDNA as a reference gene and was compared to that of infected-non treated one. *P* value refers to significant decrease of the transcript levels of *aero* and *hly* genes in infected and treated groups compared to positive control one.

positive control group; however, the transcript levels were decreased significantly within infected and treated ones ($P < 0.0001$) (Table 2) (Figure 1). Interestingly, the lowest expression levels were detected in group 3 supplied with clove oil as a prophylactic use, where the aerolysin and hemolysin transcripts decreased 0.002 and 0.008-folds post-challenge, respectively pointing to the wide range role of clove oil on the down-regulation of *A. sobria* virulence genes. Moreover, the use of ciprofloxacin as a prophylactic agent (group 4) induced weak expressions of the examined genes. Alternatively, both therapeutic treatments with either clove oil or ciprofloxacin (groups 5, 6, respectively) showed different responses of moderate expressions compared to prophylactic ones.

Discussion

Beyond any doubt, *Aeromonas* species are increasingly recognized among the most critical causes of mass mortalities in Egyptian aquaculture ventures (23). Because motile aeromonas septicemia has been considered a disease with a significant major economic impact on fish industry worldwide, it is etiologically important to control the potentially pathogenic aeromonads that have swiftly attacked certain cultured fish population (24). As a consequence, this work focused on the *in vitro* and *in vivo* antimicrobial properties of clove oil and ciprofloxacin against *A. sobria*.

Throughout this work, the combination of morphological and biochemical schemes easily characterized and identified the Aeromonas strain at the phenotypic level, but genetic studies with the aid of PCR- RFLP analysis of the 16S rRNA gene accurately reflected the genomic complexity of a given species supposing to be *A. sobria*. Similar to previously reported data, Borrell and coauthors reported identical 16S rRNA gene RFLP profile ranging from 33 to 207 bp as genome-specific markers belonging to *A. sobria* indicating that the molecular strategies provide valuable insights into the identification of this strain (6).

The pathogenesis of *A. sobria* is multifactorial and depends upon the secretion of numerous extracellular factors that influence virulence. In the present investigation, supposed *A. sobria* strain demonstrated positive reaction to hemolytic test and Congo red binding assay given preliminary information about its virulence. More obviously, aerolysin and hemolysin virulence genes were identified in the tested strain by PCR. Similar phenotypic and genotypic detection of virulence factors of *A. sobria* were also reported (2, 25). With sufficient background data, this situation emphasizes the high pathogenicity potential of *A. sobria*, which suggests its high capability of causing disease in fish, especially because they were mostly isolated from diseased animals.

In vitro antimicrobial susceptibility testing declared that clove oil and ciprofloxacin exhibited high inhibitory effects against the *A. sobria* strain. The fact that clove oil presented a high antimicrobial activity is supported by numerous investigations ruling out the use of antibiotics and favoring the use of clove oil as an alternative strategy for the treatment of bacterial infections (26, 27).

Herein*, in vivo* virulence study of *A. sobria* displayed generalized septicemia lesions among experimentally challenged fish concerning it a successful invader of aquatic animals. The retrieved clinical signs and tissue alterations in experimentally infected fish were conformant with different reports describing the disease picture caused by the intended microbial agent (1, 28, 29).

In respect to mortality patterns in experimentally infected *C. gariepinus* with *A. sobria*, the commutative mortality was 100% in positive control group which could be attributed to the well documented pathogenicity mechanism induced by the virulence factors analyzed as declared in many previous studies (29, 30). It is noteworthy that the mortality rates were significantly decreased in fish administered clove oil or ciprofloxacin as a prophylactic use reporting cumulative mortalities of 10 and 30%, respectively. A recently documented *in vivo* assay indicated that clove oil used to develop a protective effect on catfish experimentally infected with *A. sobria* could replace antibiotics for controlling diseases

(1). In addition, ciprofloxacin had the priority on therapeutic treatment of experimental *A. sobria* infection in comparable to clove oil as anecdotally reported (31), nonetheless, a growing interest in using plant extracts in animal production has been made an additional or supporting medicine as the unrestricted use of antibiotics in aquaculture even in the prophylactic dose may considered as a pronounced stress factor in the fish having a detrimental effect on human and animal health on a global scale (32).

Clinical chemical analysis is a fundamental tool, precisely reflecting the condition of the organism and changes taking place in it under the influence of internal and external factors. In the present study, serum analysis revealed marked increase in ALT, AST, total serum proteins, MDA, serum urea and creatinine values and decrease in the reduced GSH and IgM levels in infected non-treated fish when compared to respective parameters of the negative control group. In harmony with our findings, hematological parameters changes would occur subsequently in response to the invading pathogens, thus important internal organs such as kidney, spleen, liver and pancreas, which have considerable duty in fish physiology, must be affected acutely by infectious pathogens (33). Possible explanations were announced in previous literatures stating that elevated total serum proteins is an indicative for osmo-regulatory dysfunction, hem-dilution or tissue damage surrounding blood vessels (34); increased the two key transaminases, AST and ALT indicates liver dysfunction (35), elevated serum urea and creatinine levels attributes most probably to degenerative changes of the kidney (36), and increased MDA together with decreased the reduced GSH levels denotes oxidative stress (37).

Very little information is available regarding the effect of essential oils or antibiotics on fish biochemistry and metabolism, however, our investigation attained that either prophylactic or therapeutic administration of clove oil could improve the hematological parameters, enzymatic activities, immunological status and subsequently, general health conditions of challenged fish with *A. sobria*. Some discrepancies were discerned in the data published elsewhere (38), in which blood parameters were elevated immediately after administration of fish with an anesthetic concentration of clove oil then returned back to normal within 24 hours. Conversely, blood chemistry after ciprofloxacin administration showed adverse alterations which provide its stress effect on challenged fish as previously documented (19).

Indeed, qRT-PCR analysis indicated that the lowest expression levels of *A. sobria* aerolysin and hemolysin genes were reported in prophylactic treated groups with superiority of clove oil over ciprofloxacin. However, *in vivo* therapeutic application of both agents induced unexpected response. Noteworthy, although it is a relatively emerging practice, there is still no helpful work carried out on the *in vivo* data determining the potential usefulness of clove oil in down-regulation of *A. sobria* virulence genes. However, an *in vivo* study validating modulation of certain virulence genes in bacterial colonization was previously warranted using other medicinal plants and considered on a remote when compared with our first effort (39).

To our knowledge, this is the first report on the *in vivo* influence of clove oil on the expression profiles of virulence-associated genes of *A. sobria*, which highlights a strong evidence for aqua-culturists recommending the mandate of clove oil as a complementary medicine not only to protect fish from *A. sobria* infection, but also to modulate their virulence genes.

Acknowledgments

The authors express their deep gratitude to Dr Alshimaa A. khalil and Dr Abd elhakim Y, Lecturers of Fish Diseases and Management Department, Faculty of Veterinary Medicine, Zagazig University, Egypt for their cooperation and participation during carrying out the research experimental design.

Authors' contributions

MIA and NKA: contributed equally in the conception and design of the study, acquisition of data, analysis and interpretation of data, writing the paper, revising it critically for important intellectual content and final approval of the version to be published. HAA: application of blood chemistry, analysis and interpretation of data, statistical analysis and final approval of the version to be published.

References

1. Ghaly, F.M., Baraka, D.M., Mahmoud, S.H., Abd EL-Salam, S.S., Awad, S.M. and EL-Makhzangy, A.A., Efficacy of ciprofloxacin and clove extract on bacterial infection of clarias gariepinus. Middle East Journal of Applied Sciences 2015, 5: 1-9.

2. Das, A., Rathore, A., Janani, C., Hemanth, C. and Arvind Balakrishnan, R., Diagnosis of motile Aeromonas Sobria from cat fishes with septicemia by PCR. IOSR Journal of Agriculture and Veterinary Science 2013, 2: 87-91.

3. Schmidt, A.S., Bruun, M.S., Dalsgaard, I., Pedersen, K. and Larsen, J.L., Occurrence of antimicrobial resistance in fish-pathogenic and environmental bacteria associated with four Danish rainbow trout farms. Appl. Environ. Microbiol. 2000, 66: 4908-4915.

4. Citarasu, T., Herbal biomedicines: a new opportunity for aquaculture industry. Aquacult. Int. 2010, 18: 403-414.

5. Noga, E.J., Fish disease: Diagnosis and treatment, 2nd ed., Wiley-Blackwell, Ames, Iowa, 2010.

6. Borrell, N., Acinas, S.G., Figueras, M. and Martínez-Murcia, A.J., Identification of aeromonas clinical isolates by restriction fragment length polymorphism of PCR-Amplified 16S rRNA genes. J. Clin. Microbiol. 1997, 35: 1671-1674.

7. Li, Y., Liu, Y., Zhou, Z., Huang, H., Ren, Y., Zhang, Y., Li, G., Zhou, Z. and Wang, L., Complete genome sequence of Aeromonas veronii strain B565. J. Bacteriol. 2011, 193: 3389-3390.

8. Dorsch, M., Ashbolt, N.J., Cox, P.T. and Goodman, A.E., Rapid identification of Aeromonas species using 16S rDNA targeted oligonucleotide primers: a molecular approach based on screening of environmental isolates. J. Appl. Bacteriol. 1994, 77: 722-726.

9. Castro-Escarpulli, G., Figueras, M.J., Aguilera-Arreola, G., Soler, L., Fernández-Rendón, E., Aparicio, G.O., Guarro, J. and Chacón, M.R., Characterisation of Aeromonas spp. isolated from frozen fish intended for human consumption in Mexico. Int. J. Food Microbiol. 2003, 84: 41-49.

10. Yousr, A.H., Napis, S., Rusul, G.R.A. and Son, R., Detection of aerolysin and hemolysin genes in Aeromonas spp. isolated from environmental and shellfish sources by polymerase chain reaction. ASEAN Food Journal 2007, 14: 115-122.

11. Singh, V., Rathore, G., Kapoor, D., Mishra, B.N. and Lakra,

W.S., Detection of aerolysin gene in Aeromonas hydrophila isolated from fish and pond water. Indian J. Microbiol. 2008, 48: 453-458.
12. Starlipera, C.E., Ketolab, H.G., Noyesc, A.D., Schilla, W.B., Hensond, F.G., Chalupnickib, M.A. and Dittmanb, D.E., An investigation of the bactericidal activity of selected essential oils to Aeromonas spp. Journal of Advanced Research 2015, 6: 89–97.
13. Ortez, H.J., Test methods: Disk diffusion testing. In: Manual of antimicrobial susceptibility testing, Coyle, B.M. (ed.), American Society for Microbiology, DC, Washington, 2005, pp. 39-52.
14. Senthil Kumar, A., Venkatesalu, V., Kannathasan, K. and Chandrasekaran, M., Chemical constituents and antibacterial activity of the leaf essential oil of Feronia limonia. Indian J. Microbiol. 2010, 50: S70–S73.
15. Clinical and Laboratory Standards Institute (CLSI), Methods for antimicrobial dilution and disk susceptibility testing of infrequently isolated or fastidious bacteria. 3rd ed., CLSI guidelines M45, Wayne PA, USA, 2015.
16. Monir, M.S., Ahammed, T., Borty, S.C., Bagum, N., Islam, M.A. and Mahmud, Y., Pathogenesis of Aeromonas species in stinging catfish Shing (Heteropneustes fossilis) of Bangladesh. Trends in Fisheries Research 2015, 4: 7-13.
17. Canadian Council on Animal Care (CCAC), Guide to the Care and Use of Experimental Animals, Cross, B.M. and McWilliam, A.A. (eds.), Vol. 1, 2nd ed., Ottawa, Ontario, Canada, 1993, Available at: www.ccac.ca/en/CCAC_Programs/Guidelines_Policies/GDLINES/Guidelis.htm.
18. Rattanachaikunsopon, P. and Phumkhachorn, P., Protective effect of clove oil-supplemented fish diets on experimental Lactococcus garvieae infection in tilapia. Biosci. Biotechnol. Biochem. 2009, 73: 2085-2089.
19. Rizkalla, E.H., Fahmy, B.G., Zaghloul, H.H., El-Sadek, S.E. and Feisal, R.A., Comparative study of some antibiotics on biochemical changes in plasma of the sharptooth catfish Clarias gariepinus. Egypt. J. Aquat. Biol. Fish. 2003, 7: 381- 402.
20. Cooke, S.J., Suski, C.D., Ostrand, K.G., Tufts, B.L. and Wahl, D.H., Behavioral and physiological assessment of low concentrations of clove oil anaesthetic for handling and transporting largemouth bass (Micropterus salmoides). Aquaculture 2004, 239: 509–529.
21. Weatherby, D. and Ferguson, S., Blood Chemistry and CBC Analysis: Clinical Laboratory Testing from a Functional Perspective, 1st ed., Vis Medicatrix Press, OR, Jacksonville, 2002.
22. Livak, K.J. and Schmittgen, T.D., Analysis of relative gene expression data using real-time quantitative PCR and the 2- ΔΔCt Method. Methods 2001, 25: 402-408.
23. Eissa, I.A.M., Badran, A.H., Azza Abdel-Rhman, M.M. and Somayah Awad, M.M., Studies on cultured black carp (Mylopharyngodon picens) regarding Aeromonas sobria septicemia for the first time in Egypt. SCVMJ 2011, XVI: 13-22.
24. Beaz-Hidalgo, R. and Figueras, M.J., Molecular detection and characterization of furunculosis and other aeromonas fish infections. In: Health and environment in aquaculture, Carvalho, E.D., David, G.S. and Silva, R.J. (eds.), InTech, 2012, pp. 97-132.
25. Das, A., Vinayasree, V., Santhosh, C.R. and Sree Hari, S., Surveillance of Aeromonas sobria and Aeromonas hydrophila from commercial food stuffs and environmental sources. Journal of Experimental Sciences 2012, 3: 36-42.
26. Mansour, A.M.A., Zaki, H.M., Hassan, N.A. and El-Nashar, N.A.M., Phenotyping, virulence characteristics of Aeromonas species and the effects of essential plant oils as antimicrobial agents against pathogenic isolates from different sources. American Journal of Infectious Diseases 2014, 10: 21-35.
27. Eissa, I.A.M., El-lamei, M., Sherif, M., Youssef, F., Zaki, M.S. and Bakry, M., Detection of hemolysin gene and antibiogram of Aeromonas veronii biovar sobria isolated from mass mortalities in cultured Nile Tilapia in El-Sharkia governorate, Egypt. Life Science Journal 2015, 12: 85-89.
28. Janda, J.M. and Abbott, S.L., The genus Aeromonas: taxonomy, pathogenicity and infection. Clin. Microbiol. Rev. 2010, 23: 35-73.
29. Anyanwu, M.U., Chah, K.F. and Shoyinka, V.S., Evaluation of pathogenicity of motile Aeromonas species in African catfish. International Journal of Fisheries and Aquatic Studies 2015, 2: 93-98.
30. Austin, B. and Austin, D.A., Aeromonadaceae representatives (Motile Aeromonads). In: Bacterial fish pathogens: Disease of farmed and wild fish, 5th ed., Springer Science+Business Media Dordrecht. 2012.
31. Ashiru, A.W., Uaboi-Egbeni, P.O., Oguntowo, J.E. and Idika, C.N., Isolation and antibiotic profile of Aeromonas species from tilapia fish (Tilapia nilotica) and catfish (Clarias betrachus). Pakistan Journal of Nutrition 2011, 10: 982-986.
32. Cabello, F.C., Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. Environ. Microbiol. 2006, 8: 1137–1144.
33. Austin, B. and Austin, D.A., Bacterial Fish Pathogens: Diseases of farmed and wild fish. Ellis Harwood Ltd, Chichester, England, 1987, pp.364.
34. Wood, C.M., Turner, J.D. and Graham, M.S., Why do fish die after severe exercise? Journal of Fish Biology 1983, 22: 189-201.
35. Palanivelu, V., Vijayavel, K., Balasubramanian, S.E. and Balasubramanian, M.P., Influence of insecticidal derivative (cartap hydrochloride) from the marine polychaete on certain enzyme systems of the fresh water fish Oreochromis mossambicus. Journal of Environmental Biology 2005, 26: 191-195.
36. Holloway, H.L. and Shoemaker, C.A., Humoral creatinine in walleye (Stizostedion vitreum). Great Basin Naturalist 1993, 53: 199-202.
37. Dröge, W., Free radicals in the physiological control of cell function. Physiol. Rev. 2002, 82: 47-95.
38. Velisek, J., Svobodova, Z., Piackova, V., Groch, L. and Nepejchalova, L., Effects of clove oil anaesthesia on common carp (Cyprinus carpio L.). Veterinary Medicine – Czech 2005, 50: 269-275.
39. Upadhyaya, I., Upadhyay, A., Kollanoor-Johny, A., Darre, M.J. and Venkitanarayanan, K., Effect of Plant Derived Antimicrobials on Salmonella Enteritidis Adhesion to and Invasion of Primary Chicken Oviduct Epithelial Cells in vitro and Virulence Gene Expression. International Journal of Molecular Sciences 2013, 14: 10608-10625.