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Aeromonas Spp in Sardinian Snail Farms

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Abstract

Snails, also known as Gastropod molluscs of the order Stilommatophoridae, belonging to the family Helicidae, are farmed in Sardinian agriculture. Nonetheless, this practice is currently causing numerous problems and critical issues. Setting up snail farming facilities demands high organisational standards to optimise production, as well as continuous in-depth technical training for dedicated personnel. The aim of this paper was to acquire knowledge on the epidemiology and ecology of Aeromonas spp in snail farms and to assess the pathogenicity of the different strains, isolated through molecular characterisation of virulence genes. In order to do this, snails belonging to the genus and species Helix aspersa (N.22) and irrigation water (N.22) from various farms in Sardinia were sampled and microbiological culture investigations for Aeromonas spp. were carried out. The microorganisms isolated were subjected to culture and molecular investigations for the characterisation of virulence genes. From this study, it was possible to highlight the presence of 15 positive samples for Aeromonas spp. In addition, it was discovered that the origin of the contamination was often associated with unexpected factors, which were not under the control of those responsible for the plant. As a result, this study revealed the need for further preventive actions in two key aspects;

1. Improve the production in the surveillance of the farms;

2. Increase the assessment of associated risks caused by virulent strains that may compromise product quality and consumer safety.

Keywords: Aeromonas spp; virulence genes; snail farming; Helix aspersa Muller; watering

Introduction

The genus Aeromonas consists of Gram-negative, rod-shaped, facultatively anaerobic, non-spore-forming, catalase- and oxidase-positive bacteria found in soil and aquatic environments. Most motile strains produce a single polar flagellum, while peritrichous or lateral flagella may form on solid media in some species. They grow at temperature ranges between 22 °C and 35 °C, and in some species, growth may also occur at 0-45 °C. There are more than 30 genetically diverse species with complex taxonomy. Current literature indicates that A. hydrophila, A. veronii bv sobria and A. caviae are responsible for the majority of human infections and clinical isolations [1]. Aeromonas hydrophila is a pathogenic microorganism, while the pathogenicity of Aeromonas caviae, and Aeromonas sobria is still being studied. Aeromonas sobria is a micro-organism that overlaps and complicates other infection states, and is capable of developing gastrointestinal pathology in humans [2]. The first case of Aeromonas spp disease caused by snails dates back to 1994 and occurred in France [3]. In addition, several other cases of infections with septicaemia, meningitis, wound infections, peritonitis, hepatobiliary infections and necrotising fasciitis were reported [4]. Cytotoxic heat-labile enterotoxin (Act) is the main virulence factor of A. hydrophila and is responsible for haemolytic, cytotoxic and enterotoxic activities. Indeed, Haemolysis involves the formation of pores in the target cell membrane and the entry of water from the external medium, resulting in cell swelling and subsequent lysis [5]. The toxin interacts with erythrocyte membranes, inserts itself into the lipid bilayer and creates pores in the range of 1.14 to 2.8 nm. The cholesterol present on cell membranes acts as a receptor for Act, enabling its activation with subsequent oligomerisation and pore formation. The toxin's activity also includes tissue damage and elevated fluid secretion in intestinal epithelial cells, resulting from the induction of proinflammatory response in the target cells [6]. The Cytotonic heat-labile enterotoxin (Alt) and Cytotonic heat-stable enterotoxin (Ast), do not produce degeneration of the epithelium and act similarly to cholera toxin by raising levels of adenylated cAMP and prostaglandins in intestinal epithelial cells. This induces an efflux of chloride ions, which leads to osmotic leakage of water into the intestinal lumen causing diarrhoea. On the other hand, Cytotonic enterotoxins produced by Aeromonas spp. show variable reactivity to cholera antitoxin: the thermolabile enterotoxin alt (56°C for 10 min) does not show cross-reactivity with cholera antitoxin, whereas the thermostable enterotoxin Ast (56°C for 20 min) reacts with cholera antitoxin [7].

Aeromonas spp possesses several haemolysins (AerA, Ahh1 and Asa1) that cause α- and β-haemolysis. Aerolysin is a β- haemolysin and is one of the best-characterised virulence factors. The most frequent haemolysin in Aeromonas strains is a thermolabile haemolysin (encoded by Ahh1) that exhibits increased haemolytic activity when it coexists with Aerolysin. A Most Aeromonas strains also produce a variety of extracellular enzymes, which can contribute to overall virulence, such as proteases, lipases, collagenases, nucleases, amylases, chitinases and elastases [8]. Aeromonas hydrophila, caviae and sobria are among the 'Motile Aeromonad species' frequently isolated from fresh water, treated or purified sewage, seawater, and water intended for human consumption. Isolation is also reported from seafood and meat products that may represent vehicles for the indirect transmission of infectious diseases in humans, particularly in immunocompromised individuals. Also, the presence in snails of *Aeromonas* spp. represents mainly a risk of cross-contamination.

The presence of Aeromonas spp was detected in various environmental matrices, in particular, high isolation rates were found in snail samples and from different water sources, indicating these samples as likely reservoirs and sources of infection for humans. Low isolation rates were found in faeces from transient hosts such as poultry, cattle and humans [9]. Aeromonas spp has been isolated in several plant species (cabbage, carrot, cucumber, aubergine, lettuce, onion, tomato, potato and spinach) with Aeromonas caviae as the most frequent species followed by A. hydrophila [10-12]. Indeed, the surface of vegetables can be contaminated by different microorganisms depending on the microbial population in their original environment, their condition, processing method, storage time and conditions [13]. The environment of a snail plant should ensure the absence of chemical, microbiological and physical contaminants that pose a risk to product quality due to contamination of meat. Furthermore, snails accumulate different

types of contaminants in their tissues [14] and therefore for the construction of the facility, soil and irrigation water must be analyzed to check for the presence of any environmental contaminants that may affect the quality of the meat and be incompatible within the snails' life. The diet is important for the quality of the meat and is characterized by the intake of quality, fresh and selected vegetables represented mainly by: chard, radicchio, kohlrabi, kale, sunflower, Savoy cabbage, and rape. Additional food intake could also be provided by fruits such as sunflower calatis and watermelon, or carrot tubers. The presence of leguminous plants in the seed bed, in addition to food crops, contributes to the maintenance of soil fertility and visibly improves the leaf apparatus of other cultivated species (Brassicaceae).

The Italian and regional territories are still in the midst of a growing phase for snail production, which saw significant growth in the 1980s and 1990s. Snails are traditionally consumed in Sardinia at a rate that is nearly eight times higher than the national average.

Although snail farming was acknowledged as "primary agricultural production" in 1984, it has evolved over time to encompass the supply chain idea, which extends from harvest to slaughter, distribution, and trading of raw materials to derived goods. Since it is now categorized as an agro-zootechnics activity, it must abide by the rules established for general farming operations and the complete supply chain. At the same time, European regulations fall short of defining specific rules for official controls on products of animal origin products intended for human consumption and for the definition of specific diagnostic requirements, providing no guarantee of the "snail" food and its derived products.

A farm starts its operations by introducing "broodstock" snails into the previously prepared and delineated regions. This is then followed by the stages that represent the biological cycle and the final collection. The soil is the snail's natural habitat, and it is essential to provide the mollusc with the best conditions for growth and to take care of its physiological requirements. The soil used for rearing the snails must be comparable to what it would encounter in nature and must be confirmed to be free of pollutants, which are a significant source of meat contamination.

Indeed, one of the crucial factors for the soil is water. Determining the right daily amount is essential as too much salt in the water can cause fermentations that could kill subjects while not enough water causes subjects to die from dehydration. To feed the snails, another essential element of the farm, fresh vegetables must be added to the other vegetables that are fed by mowing. This requires careful management of the farm's vegetation.

The stages of snail cultivation are:

1) "broodstocks" snails are released into the fences. This is typically done in March and April. High mortality may occur if the introduction of the snails happen in the late summer season.

2) Snail regeneration and acclimatization

3) Reproduction, laying and growth of the first generation

4) Harvesting of adults: occurs when there is the "edging" formation

5) Gutting: elimination of intestinal contents.

Drying occurs when the mollusc retracts into the shell, forming the operculum (NOTE BIBLIOGRAFICHE)(15,16). Thus, the aim of this work was to acquire knowledge on the epidemiology and ecology of Aeromonas spp in snail breeding in Sardinia and to assess the pathogenicity of the different strains isolated from the various environmental matrices. This was done through the molecular characterization of virulence genes, also in order to understand the origin of the contamination for the setting up of preventive actions to improve meat quality.

Methodology

Sampling

Microbiological Investigations

Microbiological investigations for the detection and enumeration of Aeromonas spp. were conducted on 22 samples of snail meat. From the primary sample of 500g, 50g of meat was taken and 25g was subjected to microbiological investigation according to [15]. Briefly, this method

involved the preparation of the initial sample suspension using alkaline peptone water (APA) in a P/V ratio of 1:10 (25g + 225 ml) in sterile food bags subsequently subjected to stomacher for sample homogenization. Scaled dilutions were then set up in sterile saline by transferring 1 ml of each dilution onto three Petri dishes containing Aeromonas agar medium. The inoculum was spread evenly over the surface of the culture medium using a sterile spatula. Once the inoculum was absorbed, the plates were incubated at 37° C ±1 for 24 hours. Then, 5 colonies attributable to Aeromonas spp were selected and transferred to Klighler Iron Agar (Microbiol Diagnostici, Uta, Cagliari, Italy) and subsequently incubated at 28°C ±1 for 24h. Colonies were then exposed to the oxidase test. On each farm, Aeromonas spp. was tested in the water used for irrigation and animal husbandry purposes. By means of polycarbonate filter membranes with a porosity of 0.45 µm placed in a filtration ramp, 100 ml of water was filtered and the membrane was placed in Aeromonas agar plates incubated at 37°±1 for 24h. The 5 colonies were attributable to Aeromonas spp were selected and transferred to Klighler Iron Agar and subsequently incubated at 28°±1 for 24h. Colonies were again exposed to the oxidase test.

The colonies that resulted positive for the oxidase test were identified using the API20NE system (Biomerieux). A bacterial suspension of 1 McFarland was inoculated into the API 20 NE (Biomerieux) miniature gallery, humidifying the medium and incubating it at 37°C±1 for 24 h. Subsequently, reading of the tunnel through APIweb and subsequent species identification took place.

Molecular Investigations

The bio molecular investigation was performed, by multiplex PCR, on 44 bacterial strains isolated from snail meat and irrigation water samples, for species identification and characterization of the main virulence genes. Genomic DNA extraction was performed from a pure culture using InstaGeneTM Matrix extraction kits (Bio-Rad Laboratories). An isolated colony was dissolved in 200 µl of InstaGeneTM Matrix and placed for 10 minutes at 99 °C. After centrifugation, the supernatant containing the extracted nucleic acid was removed. For all multiplex PCR methods, the reference strain of Aeromonas hydrophila ATCC 7966 was used as a positive control. A negative control (molecular grade water) was included at each run. The samples were subjected to molecular investigation in multiplex PCR for the genes encoding Aeromonas spp and Aeromonas hydrophila. Primers amplifying a fragment of the 16S rRNA gene, conserved for the genus Aeromonas, were used to confirm the presence of Aeromonas spp [16] while target sequences of the DNA gyrase subunit B (gyrB), a housekeeping gene, were used to identify the species Aeromonas hydrophila [17,18]. Strains that tested positive in the initial screening were processed for the characterization of specific virulence factors. These included cytotoxic aerolysin-related enterotoxin (Act), thermolabile cytosolic enterotoxin (Alt), thermostable cytosolic enterotoxin (Ast), haemolysin (HlyA), aerolysin (AerA), elastase (Ela) and lipase (Lip) [19]. The genes were amplified by PCR using oligonucleotide-specific primers (Table 1) under the conditions of three multiplex PCR reactions (Table 2) with defined thermal cycles (Table 3). All primers were synthesized by Sigma Aldrich and the visualization of the amplicons was carried out on 1% agarose gels (Sigma Aldrich, Saint Louis, Missouri, U.S.A.).

Statistical analysis

The significance of the results differences between the observed quantitative variables Aeromonas spp and Aeromonas hidrophila in Helix aspersa Muller in Snail farms were carried out applying the Chi-square test with 1 degree of freedom for the 95% confidence interval analysis. The results were processed using Microsoft Excel XP 2010 (http://in-silico.net/tools/statistics/fisherexacttest). The value of $p< 0.05$ was considered significant for all comparisons of results.

Table 1: PCR Primers for Aeromonas genus-specific and virulence associated

Target gene	Primer Sequenza 5' – 3'	Amplicon size (bp)	Concentration (µM)	Reference
A16S F	GGG AGT GCC TTC GGG AAT CAG A	356 bp	0.24	Wang et al, 2003
A16S R	TCA CCG CAA CAT TCT GAT TTG		0.24	
A-hyd F	AGT CTG CCG CCA GTG GC	144 bp	0.48	S.Persson et al,2015
A-hyd R	CRC CCA TCG CCT GTT CG		0.48	
astF	ATG CAC GCA CGT ACC GCC AT	260 bp	0.12	Kingombe et al,2010
astR	ATC CGG TCG TCG CTC TTG GT		0.12	
lipF	ATC TTC TCC GAC TGG TTC GG	382 bp	0.8	K.SenandM.Rodgers, 2004
lipR	CCG TGC CAG GAC TGG GTC TT		0.8	
elaF	ACA CGG TCA AGG AGA TCA AC	513 bp	0.2	K.SenandM.Rodgers, 2004
elaR	CGC TGG TGT TGG CCA GCA GG		0.2	
	GAG AAG GTG ACC ACC AAG AACA	232 bp	0.8	Kingombe et al,2010
actF				
actR	AAC TGA CAT CGG CCT TGA ACT C		0.8	
altF	GCA CGG CGT GAC TTC GGT GA	576 bp	0.8	Kingombe et al,2010
altR	ACC GCG GTC TTG CAG TTG GG		0.8	
aerF	AAC CGA ACT CTC CAT	301 bp	1.2	Pabbs et al, 2009
aerR	CGC CTT GTC CTT GTA		1.2	
	GGC CGG TGG CCC GAA GAT ACGGG	597 bp	0.12	Wong et al, 1998
hlyAF				
hlyAR	GGC GGC GCC GGA CGA GAC GGG		0.12	

Table 2: Mix Reaction Multiplex PCR 1,2,3

Multiplex 1		Multiplex 2		Multiplex 3	
dNTPs	0,5 µl	dNTPs	0,5 µl	dNTPs	0,5 µl
A16S-F	0,5 µl	alt-F	2 µl	hlyA-F	0,3 µl
A16S-R	0,5 µl	alt-R	2 µl	hlyA- R	0,3 µl
A-hyd -F	0,5 µl	lip-F	2 µl	ela-F	0,5 µl
A-hyd -R	0,5 µl	lip-R	2 µl	ela-R	0,5 µl
		ast-F	0,3 µl	aer-F	3 µl
		Ast-R	0,3 µl	aer-R	3 µl
				act-F	2 µl
				act-R	2 µl
H2O PCR	14,2 µl		9,2 µl		6,2 µl
DNA template	2 µl		2 µl		2 µl
Taq Polymerase	0,2 µl		0,2 µl		0,2 µl
MgCl2	2 µl		2 µl		2 µl
Buffer	2,5 µl		2,5 µl		2,5 µl

Volume totale	25 µl		25 µl		25 µl

Table 3: Multiplex 1,2,3 PCR: thermic profiles

Step	Temperature	Time (Min)	Cycle
Initial denaturisation	95°C	0.208333333	1
Denaturisation	95°C	0.020833333	30
Coupling	60°C	0.020833333	
Extension	72°C	0.041666667	
Final Extension	72°C	0.291666667	1

Discussion and Results

Overall, 61% of the meat and water samples tested for detection and enumeration of Aeromonas spp (n=19, n= 8 meat, n=11 water, A.sobria and caviae) and Aeromonas hydrophila (n=8) only in the meat were positive with an average concentration of 4.8 and 4.7 logs respectively (not significant statistical difference was observed), while the results of the other samples were below the detection limit of the method (<10 CFU/g). In the meat samples, the presence of virulence genes in Aeromonas hydrophila isolates was constant for the Alt, Lip, Ela genes (n. 8/8 samples), while the Ast (n.3/8 samples), Hly (n.6/8 samples) and Act genes were present in one sample. Aer gene was not detected (Table 4).

Table 4: Detection enterotoxin gene in isolates of Aeromonas hidrophila and spp in the meat samples

		Alt	Lip	Ast	Hly	Ela	Aer	Act
1C	A. h	+	+	-	+	+	-	-
2C	A. spp	-	-	-	-	-	-	+
3C	A. spp	-	+	-	+	-	-	+
4C	A. h	+	+	+	+	+	-	-
5C	A. h	+	+	-	-	+	-	+
6C	A. h	+	+	-	+	+	-	-
7C	A. spp	-	+	-	-	+	-	-
8C	A. h	+	+	-	+	+	-	-
9C	A.spp	-	+	-	+	-	-	+
10C2	A. spp	-	-	-	-	-	-	+
10C1	A. h	+	+	+	-	+	-	-
13C	A. spp	-	+	-	-	+	-	-
11C	A. h	+	+	-	+	+	-	-
12C	A. h	+	+	+	+	+	-	-
14C	A. spp	-	+	-	-	+	-	-
17C	A. spp	-	+	-	+	-	-	+

The presence of cytotoxic enterotoxins such as alt and ast may present an important aspect in the virulence expression of the strains tested, as they are similarly to cholera toxin by raising the levels of adenylated cAMP and prostaglandins in intestinal epithelial cells [17,18]. In samples with Aeromonas spp isolates, the virulence genes alt, ast, aer were never detected, the lip gene in six samples, act gene five samples, the Ela and Hly genes in three samples were

present of the isolates. Moreover, 46% of the water samples tested were only positive for Aeromonas spp (n=10) with 1 sample showing a concentration of 2.2 log, 4 samples showing a concentration of 2.7 log, 3 samples showing a concentration of 3.5 log and only 1 sample showing a concentration of 4.2 log. The rest of the samples were below the detection limit of the method (<1 CFU/ml). The species isolated from the water systems at the same frequency were Aeromonas sobria and caviae.

All the microorganisms isolated showed the presence of a single but relevant virulence gene (act). Cytotoxic enterotoxin (act) is, in fact, the main virulence factor of A. hydrophila and Aeromonas spp and is responsible for the haemolytic, cytotoxic and enterotoxic activities. Haemolytic activity occurs with a haemolysin that through the formation of pores from 1.14 to 2.8 nm in the erythrocyte membrane allows the entry of water from the external medium resulting in cell lysis [5,6]. In the samples examined, most of the Aeromonas strains showed virulence genes and thus a potential expression of extracellular enzymes, such as protease, lipase, collagenase, nuclease, amylase, chitinase and elastase. Analysis of the presence of virulence genes in the meat of the snails and in the irrigation water of the snail plants examined reveals a mismatch in the presence of virulence genes between the different strains isolated. This suggests that the origin of Aeromonas contamination in snails does not originate from these waters but rather from other reservoirs or sources of contamination such as supplementary feeding sources represented by vegetable mowings of various origins and sources. Indeed, studies have found that Aeromonas spp. in water used for agricultural irrigation may pose a risk as a source of contamination of bacteria in the food chain and thus in snail plants [20,21]. This can contaminate the surface of vegetables and together with the lack of proper hygiene at various stages of the chain can contribute to the spread of Aeromonas spp. [13]. Several studies have reported the presence of Aeromonas spp. in different vegetable species (cabbage, carrot, cucumber, aubergine, lettuce, onion, tomato, potato and spinach) with Aeromonas caviae being the most prevalent species followed by A. hydrophila [9-12]. In addition to the pathogenetic mechanisms of Aeromonas spp in humans with virulence factors contributing to biofilm formation, cell adherence, invasion and cytotoxicity, the literature confirms that Aeronomas can severely damage the production of snail farms [3,5].

Conclusion

This study revealed that Aeromonas spp was frequently detected in Snail farms in Sardinia, in particular within isolated snail meat and in water used for zootechnical purposes in different seasons. The study underlines a critical control point for minimising the risk of Aeromonas ssp contamination by adopting appropriate management and microbiological monitoring of supplementary food sources from outside environments, which are often irrigated with insufficiently controlled water.

Contributions

The authors contributed equally

Conflict of Interest

The authors declare no potential conflict of interest

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