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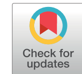
Virulence pattern of circulating aeromonads isolated from farmed Nile tilapia in Tanzania and novel antibiotic free attenuation of *Aeromonas hydrophila* strain TZR7-2018



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ABSTRACT

Aeromonads are gram-negative, rod-shaped, facultative anaerobes bacteria known to cause motile aeromonads septicemia diseases (MAS) in warm freshwater farmed fish. Outbreaks are associated with pathogenicity of aeromonads in fish which is partly contributed by virulence characteristics of the etiological agent. The objective of this study was to assess the virulence characteristics of the previously isolated and identified aeromonads, and attenuate potential *Aeromonas hydrophila* strain TZR7-2018 to serve as local vaccine candidate. Six virulence genes and other virulence characteristics were molecularly and phenotypically assessed both using in-vitro and in-vivo approaches. Attenuation of *A. hydrophila* parent strain TZR7-2018^{+} was performed by passaging through thermal continuous sub-culturing 40 times in Tryptic soy agar (TSA). Bacterin was prepared by formalin inactivation from the same parent strain. Humoral responses were assayed using quantitative serological agglutination test (qSAT) while protective efficacy was measured through relative percent survival (RPS). A total 240 Nile tilapia fingerlings with an average weight of 8.1 ± 0.4 g were used in all in-vivo studies. The presence of aerolysin (*aer*), cytotoxic enterotoxin (*act*), elastase (*ahy*), haemolysin (*hly*), serine (*ser*) and polar flagella (*fla*) genes were determined using PCR. Out of 201 isolates, 75.1 % (151/201) of the aeromonads possessed virulence genes (120 = *A. hydrophila* and 31 = *Aeromonas veronii)*. The virulence gene pattern of *aer/hly/fla* was the most prominent with the prevalence of 12.6 %. The attenuated strain TZR7-2018^{-} showed reduced: colon size, multiplication rate, cell size and loss in; haemolysis, motility and capsule. Humoral responses increased gradually and reached maximum at day 28 in both attenuated and bacterin formulation given through intraperitoneal (IP) injection and immersion (IM). A RPS of 82.3 %, 71.4 % and 85.1 %, were recorded to the attenuated vaccine given through IP and IM and bacterin provided through IP respectively.

Therefore the attenuated strain TZR7-2018^{-} obtained through thermal continuous subculture technique and the bacterin proved to be efficacious and can serve as vaccine candidate.

1. Introduction

Aeromonads are gram-negative, rod-shaped, facultative anaerobes bacteria found largely in the aquatic environment(Oliveira et al., 2012; Ruhil et al., 2015; Rasmussen-Ivey et al., 2016; Tomás, 2012; Sen and Rodgers, 2004; Li et al., 2011a, 2011b). Several species of genus *Aeromonas* are known to cause diseases in warm freshwater farmed fish named as hemorrhagic septicemia, ulcerative syndrome, haemorrhagic enteritis, red body disease, and dropsy (Abdelhamed et al., 2017; Igbinosa et al., 2012; Mzula et al., 2019a). *Aeromonas hydrophila*, *Aeromonas veronii*, *Aeromonas sobria* and *Aeromonas caviae* are said to be the main secondary pathogens, however, recent studies have reported certain strains of *A. hydrophila* to be primary pathogen of human and farmed fish causing high mortalities in fish farms (Bravo et al., 2003; Esteve et al., 1993; Li et al., 2011a, 2011b; Pridgeon and Klesius, 2011). *A. hydrophila* ST251 clonal group marked to be the very virulent strain serving as a primary pathogen and caused an outbreak in channel catfish farms in USA (Pang et al., 2015).

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Table 1
Primers for virulent factors.

Gene	Primer	Sequence (5′-3′)	Size(bp)	References
Hemolysin	AHH1F	GCCGAGCGCCCAGAAGGTGAGTT	130	(Wang et al., 2003)
	AHH1R	GAGCGGCTGGATGCGGTTGT		
Elastase	*ahyB*-F	ACACGGTCAAGGAGATCAAC	540	(Sen, 2005)
	ahyB-R	ATCTTCTCCGACTGGTTCGG		
Flagella	*fla*-F	TCCAACCGTYTGACCTC	608	(Sen and Rodgers, 2004)
	fla-R	GMYTGGTTGCGRATGGT		
Aerolysin	*aer*-F	CCTATGGCCTGAGCGAGAAG	431	(Howard et al., 1987)
	aer-R	CCAGTTCCAGTCCCACCACT		
Enterotoxin	AHCF1	GAGAAGGTGACCACCAAGAACA	232	(Kingombe et al., 1999)
	AHCF2	AACTGACATCGGCCTTGAACTC		
Serine	Ser F	ACGGAGTGCGTTCTTCCTACTCCAG	211	(Nam and Joh, 2007)
	Ser R	CCGTTCATCACACCGTTGTAGTCG		

The pathogenicity of aeromonads in fish as well as in humans is attributed to several virulence factors working in a multifactorial manner making the phenomenon complex (Galindo et al., 2006; Li et al., 2011a,2011b; Sha et al., 2009). These virulence factors include but not limited to Outer membrane proteins (OMPs), lipopolysaccharides (LPS), adhesive structures and extracellular factors such as siderophore, enterotoxins, aerolysins, haemolysins proteases and lactamases (Al-Fatlawy et al., 2013; Janda and Abbott, 2010).Their virulence genes have been broadly saved as a determinant of pathogenicity of *Aeromonas* species (Kingombe et al., 1999; Li et al., 2011a, 2011b).There is a great variation of virulent gene occurrence, possession and distribution in aeromonads isolates between and within genus and species. The differences that exist may be also linked to differences in geographical location (Ghenghesh et al., 2014). Therefore assessment of occurrence, possession, and distribution of virulence genes and their phenotypic characteristics based on geographical location is important for improved control and prevention strategies of disease occurrence.

Vaccination of fish is one of the effective disease control strategies. An autogenous vaccine made from a particular pathogen is a good option provided that there are accurate typing and evidence-based definitions of the epidemiological unit for their use (Sheng, 2018). The use of local autogenous vaccines to control fish diseases is the most viable approach, especially in situations where licensed commercial vaccine is not available, vaccination is a matter of agency due to emerging diseases and when providing a solution for a disease described as minor or secondary. Furthermore these vaccines helps during fighting against vaccination failure and when strive to reduce cost of production (Fish Site, 2009).

Knowledge is required to facilitate the development of these vaccines and it includes identification of the pathogen and its major characteristics such as strains and serotypes, their virulence, their antigenicity, and the nature of essential immunogens (Committee on Issues and Priorities for New Vaccine Development, 1986). Therefore choice of a suitable isolate is important for effective vaccine development (Swain et al., 2010). Live attenuated vaccines candidate against *A. hydrophila* have been developed and they include aeroA mutant and a novobiocin selection vaccine candidates. Heat-shock has been used to attenuate bacteria for their virulence (Allen, 1923; Selby et al., 2017). However, the attenuation of *A. hydrophila* using heat-shock (thermal) continuous subculture has never reported. The objective of this study was to assess the virulence characteristics and potential pathogenicity of the previously isolated and identified aeromonads by Mzula et al. (2019a, 2019b) and attenuate potential *A. hydrophila* strain TZR7-2018 to serve as local vaccine candidate.

2. Materials and methods

2.1. Study site and sampling procedure

A cross-sectional study was carried out between February 2017 and October 2018. A total of 816 whole fish samples were aseptically collected from 32 ponds in Ruvuma, Mbeya, Iringa and Kilimanjaro regions (8 in each region). The sample size, sampling procedures, packaging and transportation to the laboratory has been described in previous Mzula et al. (2019b) work.

2.2. Isolation and molecular identification

Two hundred and one (201) isolates tested for virulence in this study were previously isolated and molecularly identified and confirmed to be *A. hydrophila* (n = 133) and *A. veronii* (n = 68) by Mzula et al.(2019b).

2.3. Virulence gene characterization

The presence of the following genes encoding virulence factors was determined in the isolates by PCR (T1000™ thermocycler, BIORAD): Aerolysin (*aer*), cytotoxic enterotoxin (*act*), elastase (*ahy*), haemolysin (*hly*), serine (*ser*) and polar flagella (*fla*). Specific primers for the virulent genes have been given in Table 1.

The PCRs were performed under similar conditions: the first step was denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing for 30 s and an extension step at 72 °C for 30 s. After the end of the cycles, one final extension step at 72 °C for 10 min was added. Among the genes, the difference consisted of the annealing temperature (60.6 °C for elastase and 55.5 °C for aerolysin). Parameters for the amplification of haemolysin gene included an initial denaturation at 95 °C for 5 min, followed by 50 cycles of denaturation at 95 °C for 30 s, annealing of the primers at 59 °C for 30 s, and primer extension at 72 °C for 30 s. A final extension at 72 °C for 7 min was used. PCR amplification for cytotoxic enterotoxin gene was done following the temperature program: 1 cycle of denaturation for 10 min at 95 °C; 35 cycles of melting at 95 °C for 15 s, annealing at 66 °C for 30 s, and elongation at 72 °C for30 s; and a final extension round at 72 °C for 10 min. Cycling conditions for flagella (flaA/flab) consisted of an initial single cycle at 95 °C for 5 min, followed by 35 cycles of melting for 25 s at 95 °C, annealing for 30 s at 55 °C, elongation for 1 min at 72 °C and a final single cycle at 72 °C for 5 min. The cycling requirement used for serine protease gene was adopted from the work conducted by Nam and Joh (2007). The products of PCR were as well analyzed on agarose gel 1.5 % and visualized in a gel doc machine.

2.4. Phenotypic biotyping of virulence factors

Six virulence factors characteristics were assayed phenotypically as described by Al-Fatlawy et al.(2013); Aljanaby and Alfaham (2017) and Osman et al. (2018). Briefly, isolates were tested for haemolytic activity by streaking on 7% horse blood agar culture media. Lipase activity was determined by Tween 20 agar and a colour change of the colonies on the media was characterized using $CuSO_4.5H_2O$ Solution. Protease

hydrolysis was tested by streaking on 2% agar-agar containing 10 % (w/v) skimmed milk. Gelatinase was assayed by inoculating the colonies in tubes of medium containing 1.2 g of gelatin in 100 ml of Nutrient broth. A motility test was done in sulphide, indole motility (SIM) medium by stabbing a sterile needle containing a well-isolated colony 1 cm to the bottom of the tube containing the medium. All incubations were done at 37 °C for 24 h. Capsule possession was demonstrated through staining the slide with India ink and counterstained with crystal violet.

2.5. In-vivo virulence study with selected virulence factors frequencies of A. hydrophila in Nile tilapia fingerlings

The virulence study was carried out at Sokoine University of Agriculture (SUA). It involved 120 Nile tilapia fingerlings having an average weight of 8.1 ± 0.4 g sourced from SUA. The fingerlings were randomly distributed in four groups with two replication tanks, each tank with 15 fingerlings. After five days of acclimatization, fingerlings were inoculated with *A. hydrophila* in a combination having aerolysin and haemolysin (B), aerolysin, haemolysin, elastase and enterotoxin (C) and aerolysin, haemolysin, enterotoxin, elastase, flagella and serine (D) virulence genes through intraperitoneal route. The inoculum contained bacterial concentration of 1.6×10^8 CFU/mL established by Oliveira et al. (2012) and the injection dose was 0.2 mL/fish. The same dose of normal saline was given to a control group (A).

The tanks were aerated and physical chemical parameters; pH, temperature and dissolved oxygen were monitored. All fingerlings were fed three times in a day. Water samples were collected from the tanks before inoculation took place for sterility checking. Mortality was recorded and one way ANOVA was used to assess variation of the treatments. Culture of dead fish was conducted to recover the bacterium.

3. Attenuation of *A. hydrophila* strain TZR7-2018

Based on the virulence gene possession, phenotypic virulence characteristics, and in-vivo virulence study, the *A. hydrophila* strain TZR7-2018; having all the six assessed virulent genes, capsulated and showed to cause high mortality in the in-vivo virulence experiment was selected for vaccine candidate development. Attenuation of *A. hydrophila* strain TZR7-2018 here referred to as parent strain TZR7-2018$^+$ was performed by inoculating the isolate in the Tryptic soy broth (TSB) and incubated at 28 °C for 24 h. Then the culture was distributed in 1.5 mL eppendorf tubes containing sterile normal saline and preheated using water bath at a relatively higher than the incubated temperature before inoculation on a Tryptic soy Agar (TSA). Subsequent subculture in TSA was performed proceeded by preheating the passages in the water bath at increasing temperature. The continuous sub-culturing was done and reached a maximum of 40 passages at a maximum temperature of 45 °C. During subsequent sub-culturing the bacterium was observed for loss of capsule, motility, haemolytic activity, cell morphology and bacterial growth rate as compared to the parent strain.

4. Preparation of bacterin of *A. hydrophila* strain TZR7-2018$^+$

Bacterial isolate of the parent strain TZR7-2018$^+$ was inoculated into the Tryptic Soy Broth and incubated at 28 °C for 24 h. Then the bacterium was inactivated by addition of 40 % (W/V) formalin to the broth culture at a final concentration of 0.5 % (V/V) and left at room temperature for 48 h. The suspension was centrifuged at 4000 *g* for 10 min to collect the inactivated cells and then the cells were washed twice in a 0.3 % formalized PBS solution and resuspended at a concentration of McFarland standard tube No3 (10^8 cells / ml). The preparation was checked for sterility by inoculating in Tryptic soy agar (Kamelia et al., 2009).

5. Vaccination of Nile tilapia fingerlings with the attenuated *A. hydrophila* strain TZR7-2018$^-$

The experimental setup (number of fish, weight, and source of fish) is similar to the in vivo virulence study above with slight modification. Briefly, the fishes were randomly grouped into four groups, each having 30 fingerlings. Each group constituted two replication tanks with15 fingerlings in each. Group 1 (G1) got the attenuated *A. hydrophila* TZR7-2018$^-$ through the intrapretoneal (IP) route at the dose of 1.6×10^8 CFU /mL) at the injection volume of 0.1 mL. Group 2 (G2) fish were immersed in a attenuated *A. hydrophila* TZR7-2018$^-$ diluted vaccine in a separate vaccine tank at a ratio of 1 vol of vaccine to 10 volumes of tank water at the same dose of 1.6×10^8CFU / mL for 30 min) (Kamelia et al., 2009). Group 3 (G3) were given *A. hydrophila* TZR7-2018$^+$ bacterin mixed with Fraund's complete adjuvant at the same dose of 1.6 × 108 CFU / mL at the total volume of 0.1 mL via IP route. Group 4 (G4) remained unvaccinated and served as a control group. A booster dose of bacterin was given to group 3 in day 14 of the observation period which took 28 days before the challenge trial.

6. Immunogenicity and efficacy of *A. hydrophila* TZR7-2018

Guideline on the design of the studies to evaluate the immunogenicity, efficacy and safety of fish vaccines (EMA/CVMP/IWP/314550/2010) were adhered. Briefly, in determining the humoral response, antibody titres against *A. hydrophila* TZR7-2018$^-$ was measured at the intervals of 7, 21, 14 and 28 days post vaccination (dpv) while day zero saved as a baseline. A maximum of 1 mL blood samples from fish was drawn using a syringe through caudal vein and collected in eppendorf tubes and stored at 4 °C. Sera were separated by centrifuging the clotted blood at 6000 rpm for 10 min. Each serum was heat-inactivated on water bath at 55 °C for 30 min. Two-fold serial dilutions of the serum (25 μL) was titrated against equal volumes of the heat-inactivated TZR7-2018$^-$ bacterial suspension (10^9 CFU ml^{-1}). The titre was recorded after 24 h as the highest dilution indicating a clear agglutination and then it was expressed as $\log_2$ values (Kalita et al. 2006).

Fish were challenged with a parent virulent *A. hydrophila* TZR7-2018$^+$; 28 days post vaccination (dpv) at a dose of 10^9 CFU/mL (established LD 50) by IP injection and immersion. The challenge process was conducted through Intraperitonial injection (IP). Mortalities were recorded for 15 days post challenge and internal organs were collected from dead fish and cultured for checking the presence or absence of *A. hydrophila*.

The finding of the protective efficacy experiment was presented as relative per cent of survival (RPS) that was calculated according to the formula described previously by Jeong et al.(2016) and (Zhang et al., 2014).

$$\text{RPS} = 1\text{-}\left(\frac{\%\text{Mortality in vaccinated}}{\%\text{Mortality in control}}\right)*100$$

7. Data analysis

Statistical analysis was performed using Graph pad Prism 5 software. Variation between treatments groups were assessed using one way ANOVA. Antibodies titer between treatments and control group were analysed using Newman-Keuls Multiple comparison test at level of $p < 0.05$. Data were presented in tables, graphs and figures.

8. Ethics statement

Sampling of fish and all dissections has been carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, the European and the National Institutes of

Table 2
Outcome (in %) of phenotypic biotyping of selected virulence factors of Aeromonads genospecies.

Virulence| factor	Observation	Outcome(n = 201)
Hemolysin	Presence of colourless zone surrounding the colonies (β-haemolysis)	147 (73.1)
Lipase	Turbid zone around colonies with a blue colour change	148(73.6)
Protease	Presence of transparent zone around the colonies	151 (75.1)
Gelatinase	Absence of liquefaction upon refrigeration	149 (74.1)
Motility	Red turbid area extending away from the line of inoculation	131(65.2)
Capsule	Unstained clear holo surrounding individual bacilli	76(37.8)

Health – Office of Laboratory Animal Welfare policies and laws and the Tanzania Animal Welfare Act of 2008 was complied. This study also complied with the ARRIVE guidelines.

9. Results

9.1. Phenotypic characterization of selected virulence factors of aeromonads isolates

The different phenotypic approaches were used to investigate virulence factors: haemolysis, lipase activity, protease hydrolysis, gelatin liquefaction, capsule possession, and motility. Protease hydrolysis and capsule possession were observed in 151 isolates (75.1 %) and 76 isolates (37.8 %) respectively (Table 2).

9.2. Virulence gene detection of aeromonads obtained from fish

Out of 201 isolates previously identified to be aeromonads by Mzula et al. (2019b), 50 isolates (24.9 %) did not possess any of the assessed virulent genes. Of the 6 assessed virulence genes Hemolysin (*hly*), flagella and Aerolysin (*aer*) were observed to occur in most of the isolates of aeromonads with the occurrences of 97 %, 87 % and 83 % (Table 3). Only 4 isolates showed to have no Hemolysin gene. The detection of these virulent genes resulted in the amplification of their respective fragment sizes (Fig. 1). It has been observed that 151(75.1 %) of the aeromonads isolates had at least 1 virulent gene where 120 isolates were *A. hydrophila* and 31 isolates were *A. veronii*. The number of isolates of the two genospecies with the given virulence factors has been shown in Table 4. Of the 151 isolates, 25.2 % had a combination of two genes while 37.7 % had a combination of three genes and more. The distribution or possession of virulence genes in aeromonads isolates has been shown in Table 5.

9.3. Combination patterns of virulent genes of isolated aeromonads

Generally, there was a varied combination of virulence genes in most of the isolates obtained from samples collected from the four geographical regions of Tanzania namely; Ruvuma, Mbeya, Iringa, and Kilimanjaro. Sixty-three percent of the isolates had at least two virulent genes while on the other hand, only 2 isolates had all the six virulent genes assessed. Thirteen different combinations were revealed with the virulence gene pattern of *aer/hly/fla* and *aer/ser/hly* being the most prominent with the prevalence of 12.6 % and 10.6 % respectively (Table 6).

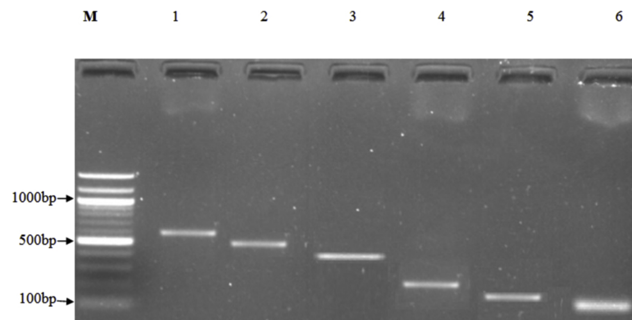


Fig. 1. PCR amplification products of the six assessed virulence genes: Flagella (608bp), Elastase (540bp), Aerolysin (431bp), Enterotoxin (232 bp), Serine (211bp) and Hemolysin (130 bp); respectively. Lane M is DNA size marker (100 bp DNA ladder).

Table 4
A summary of virulence factors occurrence between genospecies.

Virulent factors	Genospecies No.(%) positive		
	A. hydrophila (n = 120)	*A. veronii* (n = 31)	Total (n = 151)
Hemolysin *(hly)*	120 (100)	27 (87.1)	147 (97.4)
Aerolysin *(aer)*	99 (82.5)	26 (83.8)	125 (82.9)
Enterotoxin *(act)*	68 (56.7)	13 (41.9)	81 (53.6)
Elastase *(ahy)*	55 (45.8)	27 (87.1)	82 (54.3)
Serine *(ser)*	52 (43.3)	29 (93.5)	81 (53.6)
Flagella *(fla)*	118 (98.3)	13 (41.9)	131(86.8)

9.4. In-vivo virulence study of selected A. hydrophila in Nile tilapia fingerlings

Mortality of fish was highly observed in all three experimental groups while only 6.7 % and 3.3 % of fish died in control group at day one and day two respectively. Generally high mortality was recorded in day two compared to day one. The mortality increased based on the number of virulence genes the *A. hydrophila* isolate possessed. However no significant difference in mortality was observed between the

Table 3
Occurrence of virulence factors of Aeromonads genospecies in study areas as determined by PCR method.

	Ruvuma (n = 17)		Mbeya (n = 47)		Iringa (n = 50)		Kilimanjaro (n = 37)		Total % (n = 151)
V/genes	# isolates	%	# isolates	%	#isolates	%	# isolates	%	
Hemolysin	16	94	47	100	48	96	36	97	97
Aerolysin	14	82	38	81	43	86	30	81	83
Enterotoxin	7	41	25	53	38	76	11	30	54
Elastase	8	47	27	57	24	48	23	62	55
Serine	10	59	31	65	28	56	12	32	54
Flagella	11	65	39	83	44	88	37	100	87

Table 5
Genospecies virulence factors possession by isolates in study areas.

V/ genes possession	Ruvuma (n = 28)		Mbeya (n = 56)		Iringa (n = 68)		Kilimanjaro(n = 49)	
	Frequency	%	Frequency	%	Frequency	%	Frequency	%
0	11	39.29	9	16.07	18	26.47	12	24.49
1	9	32.14	6	10.71	17	25.00	24	48.98
2	2	7.14	11	19.64	17	25.00	8	16.33
3	2	7.14	25	44.64	11	16.18	4	8.16
4	2	7.14	1	1.79	3	4.41	1	2.04
> 4	2	7.14	4	7.14	2	2.94	0	0.00
Total	28	100.00	56	100.00	68	100.00	49	100.00

Table 6
Generalized combination pattern of virulence factors of the two isolated aeromonads genospecies.

Name of the gene	No of isolates detected n = 151	Percentage (%)
hly	18	11.9
act	3	2.0
fla	8	5.3
aer	10	6.6
Ser	9	6.0
ahy	8	5.3
hly / act	5	3.3
hly / fla	11	7.3
hly / aer	12	7.9
act/ fla	6	4.1
act/ aer	4	2.6
aer /hly/fla	19	12.6
hly/act/ fla	7	4.6
aer/ser/hly	16	10.6
hly/act/fla/aer	4	2.6
hly/ser/aer/act	2	1.3
ahy/aer/act/fla	1	0.7
ahy/aer/fla/act/hly	6	4.0
ser/aer/fla/hly/act/ahy	2	1.3
Total	151	100

NOTE: *hly* = Haemolysin gene; *act* = Cytotoxic enterotoxin gene; *fla* = Flagella gene; *ahy* = elastase gene; *ser* = Serine gene and *aer* = Aerolysin gene.

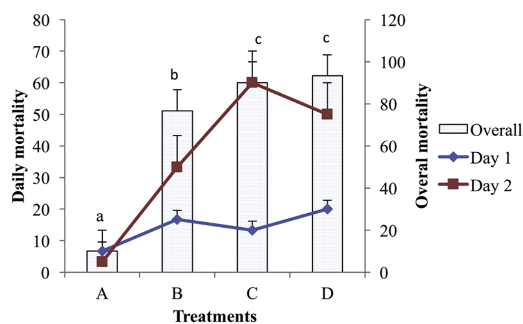


Fig. 2. The in-vivo virulence study outcome in Nile tilapia fingerlings injected with *A. hydrophila* isolates possessing different combination of virulence genes. Treatment B (two virulence genes), treatment C (four virulence genes), treatment D (six virulence genes) and treatment A (control, no any *A. hydrophila* injected).

treatment groups administered with the isolate possessed 4 combination of virulence genes and six virulence genes (Fig. 2).

9.5. Attenuation of A. hydrophila strain TZR7-2018

The *A. hydrophila* TZR7-2018^{-} was assessed for motility, haemolysis, cell size, colon appearance and capsule possession. The isolate was shown to lose the capsule at the 30th passage, no motility was observed at the 30th passage. No haemolysis, was seen to the isolate at the 25th passage and colonies appeared to be small in size as compared to the parent strain TZR7-2018^{+}. No difference in cell morphology was observed however, the cells of TZR7-2018^{-} were appeared to be smaller than the *A. hydrophila* parent strain TZR7-2018^{+} (Table 7, Fig. 3). Bacterial load was observed to increase with time of incubation being high in parent *A. hydrophila* TZR7-2018^{+} than TZR7-2018^{-} (Fig. 4) indicating high multiplication rate in the former than the latter.

9.6. Immunogenicity and efficacy of the A. hydrophila TZR7-2018^{-}

The Geometric mean titre (GMT) increased with increase in time during the observation period in all the treatment groups and the maximum titre (GMT $\log_2$ 6.4) was observed in group1 administered with attenuated *A. hydrophila* TZR7-2018^{-} through IP route. Lower antibodies titres were observed in fingerlings in the experimental group given attenuated *A. hydrophila* TZR7-2018^{-} via immersion throughout the period of observation (Fig. 5A). The overall results showed no significance difference in antibodies levels between the treatment groups ($p > 0.05$), however, marked difference were recorded between all the treatment groups and the control unvaccinated group ($p < 0.05$, Fig. 5B).

The mortality and relative percent survival (RPS) findings indicated high cumulative mortality in a control unvaccinated group during the entire 15 days period of observation post challenge. Bacterin showed high protective efficacy having RPS of 85.1 % (Fig. 6) while the attenuated *A. hydrophila* TZR7-2018^{-} given via immersion showed a lower relative percent survival (71.4 %). However, no significant difference in protection (RPS) was observed between the three treatment groups ($p > 0.05$). *A. hydrophila* were recovered and confirmed by PCR in all died fish post challenge. The died fish in the control unvaccinated group showed scattered skin haemorrhages and exophthalmia (figure not shown).

10. Discussion

Detection of virulence factors through their phenotypic activity and or presence of their genes in clinically sick fish or apparently healthy fish have become a crucial and common measure of virulence and pathogenicity of several species of the genus *Aeromonas* (Hoel et al., 2017; Khajanchi et al., 2010; Oliveira et al., 2012; Silva et al., 2017).

In this study, we assessed six virulence genes in the 201 *Aeromonas* isolates obtained in the previous study by Mzula et al. (2019a, 2019b) and conducted in vivo virulence study in selected isolates based on virulence gene combinations. It has been observed from this study that 151(75.1 %) of the aeromonads isolates had at least one virulence gene constituting 120 isolates for *A. hydrophila* and 31 isolates for *A. veronii.* However, 63 % of these aeromonads isolates had at least two virulence genes. These figures closely fall to those reported by Oliveira et al. (2012).

Li et al. (2011a, 2011b) and Oliveira et al. (2012) showed that the phenotypic characteristics of virulence factors and the presence of their genes in different combinations correlate very well with animal pathogenicity. They also stated that more mortality in fish is observed

Table 7
Changes observed in *A. hydrophila* TZR7-2018$^-$ following attenuation in comparison with parent strain TZR7-2018$^+$.

Factor/Passages	5	10	15	20	25	30	35	40
Haemolysis								
TZR7-2018$^+$	+	+	+	+	+	+	+	+
TZR7-2018$^-$	+	+	+	+	−	−	−	−
Colony appearance								
TZR7-2018$^+$	Large	Large	Large	Large	Large	Large	Large	Large
TZR7-2018$^-$	Large	Large	Large	Small	Small	Small	Small	Small
Motility								
TZR7-2018$^+$	+	+	+	+	+	+	+	+
TZR7-2018$^-$	+	+	+	+	+	−	−	−
Capsule								
TZR7-2018$^+$	+	+	+	+	+	+	+	+
TZR7-2018$^-$	+	+	+	+	+	−	−	−
Cell size								
TZR7-2018$^+$	Large	Large	Large	Large	Large	Large	Large	Large
TZR7-2018$^-$	Large	Large	Large	Large	Large	Small	Small	Small

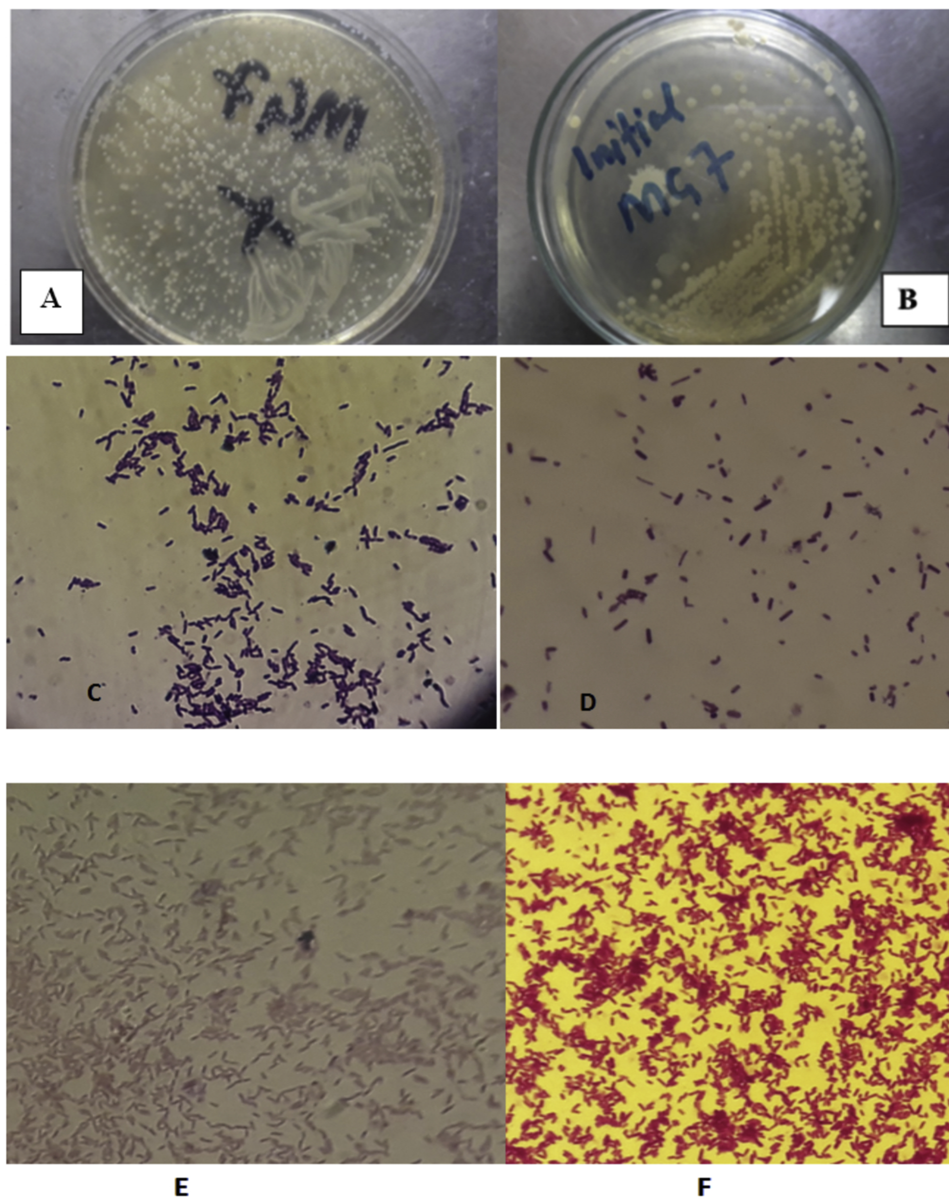


Fig. 3. Changes of different characteristics in a passaged *A. hydrophila* TZR7-2018$^-$ in comparison to parent *A. hydrophila* TZR7-2018$^+$. Fig. 2A and B show colony size in TSA, being smaller in TZR7-2018$^-$ (A). Fig. 2C and D is Indian ink staining showing presence of capsule in parent *A. hydrophila* TZR7-2018+ (C) and absent in *A. hydrophila* TZR7-2018- (D). Fig. 2E and F indicates small cell size in TZR7-2018$^-$ (F) than TZR7-2018+ (E).

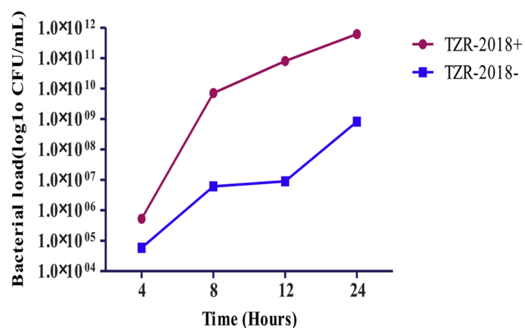


Fig. 4. Bacterial load at different incubation time between parent *A hydrophila* TZR7-2018^{+} and TZR7-2018^{-}.

with aeromonads isolates with more virulence factors.

Similar observation has been recorded in this study where high mortality was observed in *A. hydrophila* with a combination of four and six genes. In addition, the mortality peaked up and reached 95 % at day 2 of the observation time. A similar trend was also recorded by Shayo et al. (2012) during their in-vivo virulence study for *Aeromonas* spp and *Pseudomonas* spp. This indicates how potential these isolates are in establishing diseases in farmed fish provided host susceptibility and suitable environmental conditions are met for them to do so (Hoel et al., 2017; Silva et al., 2017). This is because opportunistic pathogens and potentially pathogenic *Aeromonas* may not necessarily lead to disease due to host responses and the infectious bacterial dose.

Nonetheless, the absence of these six virulent genes in 24.9 % of the isolates does not exclusively eliminate them from being potential pathogens of fish. This is because different species and isolates may possess extra different pathogenicity instruments (Silva et al., 2017) and this is justified by Oliveira et al. (2012), who observed mortalities in the experimental group of fish injected with *Aeromonas* strains having no virulence gene they assessed.

Hemolysin gene, aerolysin gene, and flagella gene were the most prevalent virulence genes of the assessed virulence factor regardless of geographical origin, demonstrating that the circulating aeromonads in the four study regions are closely related in terms of putative virulence. While Oliveira et al. (2012) observed a high prevalence of aerolysin gene in aeromonads, this study is reporting a relatively high prevalence of haemolysin gene from the investigated aeromonads isolates, a similar finding was also reported by Hoel et al. (2017) in their study conducted in fresh retail Sushi foods.

Attenuation of the selected *A. hydrophila* strain TZR7-2018 to serve as a local vaccine candidate was effective through thermal continuous sub-culturing technique. The process led to the loss of some virulence factors such as motility, haemolysis and capsule. Reduced multiplication rate, reduced colon size and changes in cell size were also observed in the attenuated strain TZR7-2018^{-}. These effects were also demonstrated by Pridgeon et al. (2012) when used a novobiocin selection. However, while Jiang et al. (2016) and Pridgeon et al. (2012) reported these attenuation outcomes with antibiotic selection after 20 passages, the current study observed the attenuation effects after more than 20 passages and at different passage in point. This might be probably because of the new approach of attenuation employed in this study.

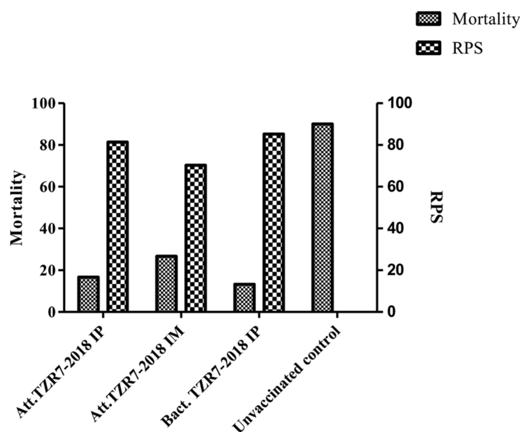
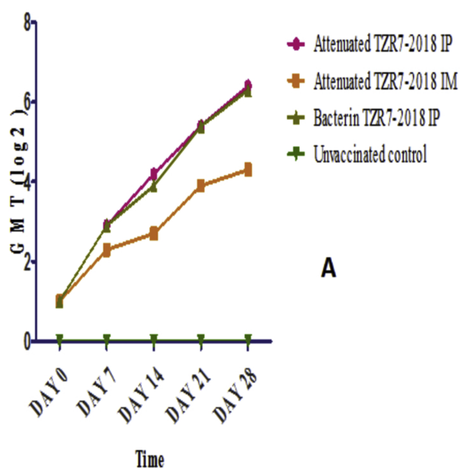


Fig. 6. Mortality and relative percent survival (RPS) of the attenuated and bacterin of *A. hydrophila* TZR7-2018^{-} administered through IP and IM.

In assessing the performance of the attenuated vaccine candidate in Nile tilapia fingerlings, antibodies levels were shown to reach maximum titres at day 28 of the observation period. However there was gradual elevation in antibody titres as from day 7 to day 28 in all three treatment groups, indicating maintenance of potential immunogenicity of the passaged TZR7-208^{-} strain.

Despite the marked difference in humoral response between treatment groups and the unvaccinated control group, no significant variation was observed among the three treatments. However, low immune response was observed from the attenuated vaccine given through immersion.

The in-vitro attenuation outcome and humoral response results of the two vaccine formulation (attenuated and bacterin) of strain TZR7-2018 given through IP and IM routes were revealed through protective efficacy in-vivo study. Bacterin provided through injection showed a



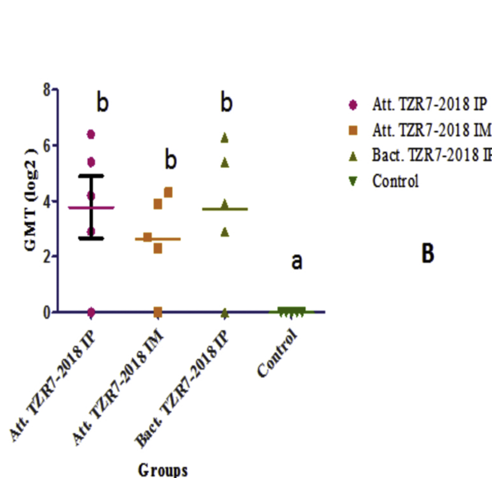


Fig. 5. Antibodies levels in GMT of attenuated and killed *A. hydrophila* TZR7-2018.The Fig. 5A and B indicate trend antibodies increase during observation period and overall performance of each treatment respectively.

high protection level followed by attenuated vaccine given through IP. Contrally to the findings of this study where immersion recorded a lower RPS of 71.4 % compared to IP (82.3 %), Kamelia et al. (2009) reported high humoral response to the vaccine given through immersion than oral and injection. This indicates that optimization of immersion route for the developed vaccine candidate can give better results. However, according to Varvarigos (1999) the immersion vaccine trial of in this study is successful and the outcome is economically acceptable. Many researchers have explained the variability of vaccine efficacy when administered through immersion (Nakanishi and Ototake, 1997). This route largely depend on the fish species, exposure time and vaccine concentration. In addition as it mimic natural infection through skin, gills and oral cavity, the maximum dose that induces optimal immune protection may sometime not be attained. Nonetheless, the use of immersion if successful is a stress free and an economically viable method in terms of cost and labour (Munang'andu et al., 2015).

The use of antibiotic resistant selection as the method of attenuation has been a common procedure in development of *A. hydrophila* vaccine candidate. Rifampicin and novobiocin has been used to *Flavobacterium columnare, Edwardsiella ictaluri* and *Streptococcus iniae* in reducing virulence (Jiang et al., 2016; Pridgeon et al., 2012). However, the application of thermo-continuous sub-culturing technique which has shown to be effective in this study would be helpful as this will reduce the risk of spillover of resistant strain of bacteria in the aquatic environment. As it was stated by Jiang et al. (2016) and Pridgeon et al. (2012), the mechanism of attenuation of *A. hydrophila* with antibiotic selection is not well known, likewise, the attenuation of same using thermo-continuous-sub-culturing technique is not understood. This is because passaging of bacterial isolates with the two approaches does not necessarily end up in partial or complete attenuation.

11. Conclusions

The *aer/hly/fla* and *aer/ser/hly* combination pattern were more frequent in the isolated aeromonads and haemolysin, aerolysin and flagella genes were relatively at high prevalence in all the four studied regions, suggesting a close relatedness in terms of putative virulence.

A selected *A. hydrophila* strain TZR7-2018 has been successfully attenuated through thermo-continuous subculture technique. It proved to be efficacious when given through both IP and IM, with its bacterin given through IP being more efficacious and therefore can serve as vaccine candidate. To the best of my knowledge, this is the first time the thermo-continuous sub-culturing technique has been used in Africa or elsewhere in developing vaccine candidate for controlling aeromonads diseases in fish. The assessment of the changes occurred to the attenuated TZR-2018^{-}strain at genomic level in comparison to the parent TZR-2018^{+}strain is also required to add up knowledge of this inducible attenuation. Optimization of the immersion route of administration with both homologous and heterologous virulent strain of *A. hydrophila* is also recommended. In addition, further work is required to carry out, safety, shelf life and a reverse to virulence study for this vaccine candidate.

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CRediT authorship contribution statement

Alexanda Mzula: Conceptualization, Methodology, Software, Data curation, Writing - original draft. **Philemon N. Wambura:** Supervision, Writing - review & editing. **Robinson H. Mdegela:** Supervision. **Gabriel M. Shirima:** Validation, Supervision.

Declaration of Competing Interest

The authors declare no conflict of interest exist.

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