Research Article

Oral Liposome Vaccines Containing *Cyprinid herpesvirus* 3 (CyHv3) and *Aeromonas hydrophila* Antigen for the Immunization of Common Carp *Cyprinus carpio*

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Abstract | This study aims to develop two oral vaccines for immunization against Koi Herpesvirus (KHV) and Aeromonas hydrophila antigen in common carp, Cyprinus carpio, using formalin-inactivated versions of both virus and bacteria (mean body weight of 108.2±1.2g). Vaccines that have been prepared and loaded into Nano liposome blank that have been formulated according to the thin film method (Bangham). The experimental was designed into four fish groups for vaccinination. The Formulated vaccines (viral and bacterial) was vaccinated once daily for fish along five days, dosed: 0.63g/fish/day and 1×109cfu/fish/day, respectively. The first two groups of fish were challenged by mingling them with KHV-infected fish at a ratio of two infected fish to ten vaccinated fish under simulated acclemtized normal condition. In contrast, fish from the second group were challenged with live *Aeromonas hydrophila* at a dose of (3×10^8cfu/fish and 3×10^7cfu/fish) for 22 days then had been vaccinated single dose. Preliminary findings reveal that fish groups treated with KHV liposomes pharmaceutic vaccine exhibit Relation present survival of 45% protection against KHV infection in bad quality water. Similarly, in fish groups raised in high-quality water, Relation present survival 62.5 % protection was test screening in fish groups. In contrast, fish groups treated with Aeromonas bacterium liposomes had Relation present survival values of 67.50% and 78.65%, respectively, against bacterial infection. This result enables us to initiate the subsequent phase of our research, wherein we employ tissue culture of fin cells from common carp fish to propagate the Koi herpes virus. The objective is to develop a vaccine with a dose containing the antigenic standard of 1×102.5 TCID50/dose, aiming to achieve a heightened level of immunity compared to the outcomes observed in the initial phase. Additionally, we aim to combine the two vaccine agents, namely the virus and bacterial components, into a single product. Conclusion the study successfully developed and tested two liposomal vaccines for common carp, one against *Koi Herpesvirus* (KHV) and another against *Aeromonas hydrophila* bacteria. The vaccines were prepared using formalin-inactivated virus and bacteria and encapsulated in liposomes formulated using the thin film method. KHV vaccine: 45% protection against KHV infection in fish raised in bad quality water. 62.5% protection in fish raised in high-quality water. Whereas Aeromonas hydrophila vaccine: 67.50% protection against bacterial infection, 78.65% protection in another group of fish.

Keywords | *Aeromonas hydrophila*, Common carp, *Cyprinus carpio* Liposome, Virosome

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INTRODUCTION

Koi Herpesvirus (KHV) is a new Herpesvirus that exclusively infects carp (*Cyprinus carpio*). The end of October 2018 affected hundreds of tons of farmed common carp (*Cyprinus carpio*) throughout the Euphrates and Tigris Iraqi rivers. Fish were discovered dead and moribund along rivers (Toffan *et al*., 2020). It has resulted in a significant number of fatalities and massive economic damage (Ababneh *et al*., 2020). Aeromonas hydrophila is a significant pathogen that has also been identified as a secondary invader in a variety of infectious diseases (Ali and Faruk, 2018) We isolated this bacterium from 70% of KHV samples that have been sent to CVL (unpublished data). There is an urgent need for a vaccine to prevent KHV and Aeromonas hydrophila infection; the traditional way has been injection vaccination (Ellis, 1988). A live vaccination was created utilizing attenuated KHV for intramuscular injection (Ronen *et al*., 2003). However, vaccination by injection route is time and labor-intensive and also severe stress to fish by handling also there is a risk that the live vaccine might mutate to highly virulent virus Oral immunization is a more practical strategy, as fish in the posterior intestine takes up both crude lipids and proteins (Miyazaki and Fujiwara, 1988). Liposomes are vesicles of phospholipid that spontaneously form concentric bilayers when exposed to aqueous solutions; they become confined between the biodegradable bilayers and slowly release their contents as they are degraded within biological systems. Therefore, liposomes may effectively function as immuno-adjuvants (van Rooijen and van Nieuwmegen, 1980). The use of vaccines encapsulated in liposomes in fish has not been thoroughly investigated. According to a number of studies, vaccines encapsulated in liposomes substantially enhance antibody protection, immune response, and disease protection in a variety of fish and other animals (Yasumoto *et al*., 2006a, b; Irie *et al*., 2003) indicating that liposomes could be utilized profitably for fish vaccination (Yasumoto *et al*., 2006a).

This designated experiment was aimed and projected to developed a liposome vaccine containing the antigen of the cyprinid herpesvirus 3 (CyHv3) viruses (virosome CyHv3) and a liposome vaccine loaded antigen Aeromonas hydrophila both were evaluated the immunization and development of immunity to common carp Cyprinus carpio by oral administration.

MATERIAL AND METHODS

KHV antigen preparation

Our Baghdad laboratory CVL received 10 infected fish from a private farm on the Tigris River near Tarmiyh. Tissue samples (kidney, spleen, and gills) were taken from fish exhibiting severe KHV infection, including death. The Portion of the tissue samples was taken for virological testing by PCR, while the other part was crushed and centrifuged at 5000 g for 15 minutes at 4°C to remove any depression and tissue particles and filter at 450nm. Clarified suspension 1/1 diluted by sterile growth MEM with 10% fetal calf serum kept at -70°C for future use.

The cleared suspension was inactivated with formalin at a final concentration of 0.3 % (v/v) for 24 hr, at 24° C. The viral antigen was adjusted to a protein concentration of 0.63g/fish/day, and the protein concentration was measured with a Bio-Rad protein assay kit.

Aeromonas hydrophila antigen preparation

The virulent isolate of *Aeromonas hydrophila* strain isolated from a skin lesion of a received fish that exhibits significant hemolysis on sheep blood agar plates. Aeromonas strain cultured in tryptic soy broth at 24°C for 48 hours. The cultures were killed with formalin at a final concentration of 0.5% (v/v) for 24hrs. At 24°C, the bacteria were collected by centrifugation at 3000g for 15 min at 4°C, and the pellet was re-suspended in saline solution. The bacteria were adjusted to 1×10^9cfu/fish/day.

Liposome synthesis

The empty liposome was prepared according to (Bangham thin-film methods) (Bangham, 1993) and standardized. The mixture of phosphatidylcholine 0.5 g and 0.5 g of cholesterol w/w were dissolved for liquefaction in mixed solvent chloroform 10 ml and methanol 5 ml v/v, the mixture was dispersed for 30 minutes, 1500 rpm by the vortex. The pro liposome base on the homogenized mixture was vacuumed for evaporation by reducing pressure conducted with the setting, and intermittent settling in water bath 38°C achieved liquefaction temperature.

The stock solution of each antigen 2 ml was mixed with empty liposome 1g the mixture was vortex for 30 minutes 1500rpm. the ensure and facilitated entire antigens (virus, bacteria) into the liposome, stock of liposome was kept in the refrigerator overnight at 8°C by diffusion loading manner, the cold liposome stock solution was incubated in the water bath for 10 minutes prior vortexing for 30 minutes.

The non-incorporated antigen suspension was removed, and the KHV liposome vaccine was adjusted to a protein concentration of 0.63g/fish/day, while the Aeromonas liposome vaccine was adjusted to $1*10^9$cfu/fish/day. In order to reduce the size of bacteria and facilitate their entry into the liposome, it is necessary to subject them to sonication for duration of 30 minutes. This ensures the successful incorporation of the antigen into the liposome.

So, the resultant suspension was transferred to a new tube and repeatedly sonicated with a probe-type sonicator. The two liposome vaccine stores at 4 °C and were dispersed before feeding fish.

Micrograph: liposome size and lamellar

Light micrograph: A liposome smear was generated and investigated using phase-contrast microscopy. The microscope was equipped with an optical filter and set to oil immersion magnification. The creation and distribution of liposomes were seen in the optical field (Oliveira *et al*., 2005).

Electron micrograph: Take 1% of the liposome samples and place them in the Eppendorf tube at a temperature of 4°C till they are examined. The materials were analyzed using scanning and transmitted techniques. The techniques, procedures, and micrograph were conducted in the Department of Pharmaceuticals and Toxicology, College of Veterinary Medicine, University of Baghdad-Iraq. The imaging scan type used was transmission scattering method (Szebeni *et al*., 1985).

Liposome size: The liposome's dimensions were determined using programmed image analysis and a metric scale on a scanning electron micrograph. The average size ratio was then estimated (Yokoyama *et al*., 2008).

Osmolarity tolerance challenge: A solution of liposome KHV and liposome Aeromonas was prepared in different salt concentrations 0, 0.05, 0.15, 0.25, 0.35, 0.45, 0.55, 0.65, 0.75, 0.85, 0.95, and 1% of NaCl. The osmolarity tolerance challenge included suspending 0.1g of liposome in a 5 ml solution of normal saline 0.85% as a stock solution. Then, 0.2ml of the suspension was added to each tube and stirred gently. The liposome count was determined using a spectrophotometer at a wavelength of 476 at both the initial time and after one hour. The spectrophotometer was calibrated using a standard curve (Cicuta *et al*., 2007).

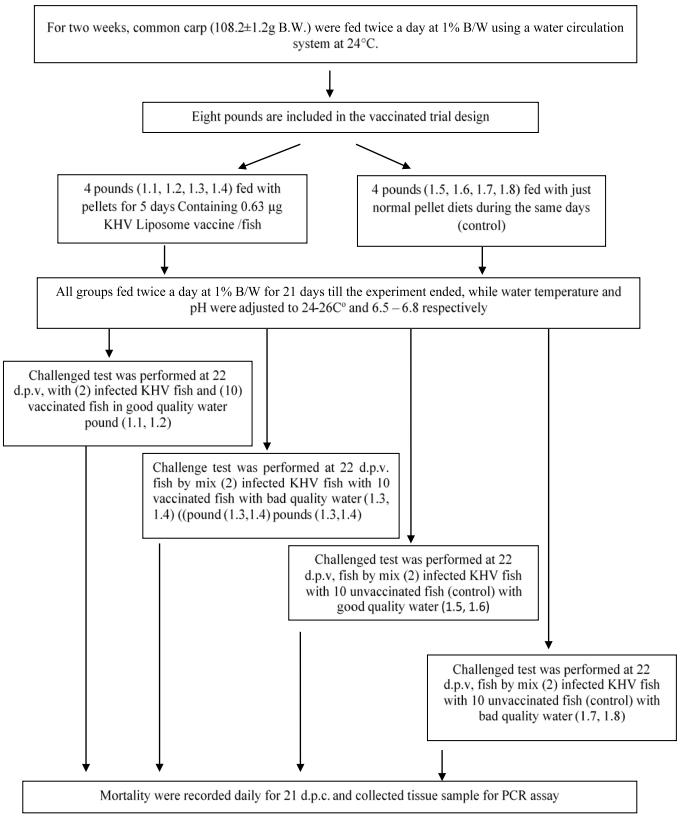
pH tolerance challenge pH challenge of liposome KHV and liposome Aeromonas was done by prepared solution in a tube containing different pH labeled as 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 0.9%, Liposome 1 g was suspended into 10 ml of distal water and added 0.2 ml of the suspension to each tube to determine the liposome survival in different pH (Yokoyama *et al*., 2008).

Experimental design and protocol

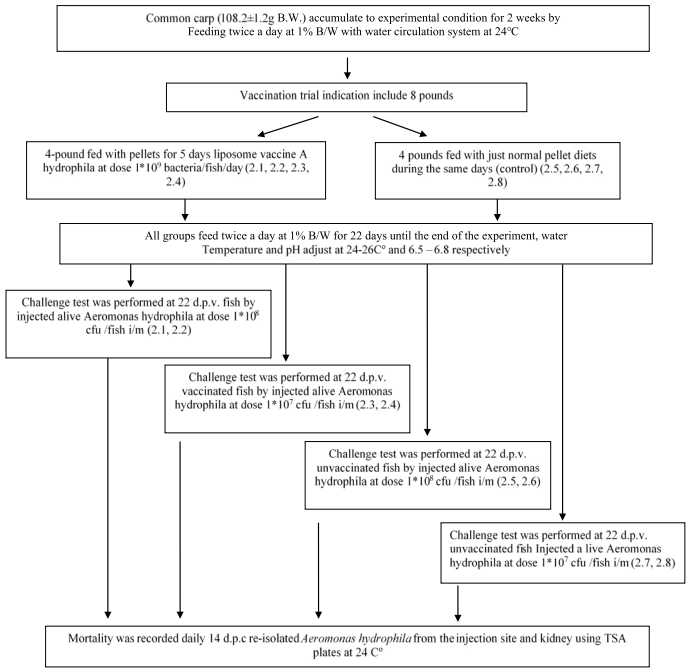
One hundred and sixty Common carp fish (108.2±1.2g mean body weight) were purchased from the Essaouire government hatchery, so each ten fish was maintained in a glass pound with dimensions of 80*45*28 cm (L*W*D).

Challenge test

Challenge of vaccinated and unvaccinated fish in all experimental groups by exposure to natural infection (virulent viral and bacterial) and the level and moment of exposure should be the same in all groups of fish. Observation of signs of disease is recorded and supported by laboratory tests by detected and identified the agent of the diseases.



Scheme 1: Experimental design.



Scheme 2: Experimental design.

The protection against KHV and *Aeromonas hydrophila* after vaccination was calculated as the Relation Present Survival RPS= (1-immunized groups mortality/ control

group mortality) × 100 (Amend, 1981).

Results and Discussion

The electron microscope showed the yield liposome KHV and *A. hydrophila* formation was standardized the working units liposome via identified forming multilamellar-multivesicle liposome and the geometrical scales was shown in Table 1 the size of KHV and A hydrophila liposome was achieved 89.46±8.92nm and 91.10±3.85nm, lamellar. Whereas the loading capability of liposome was attended as a percentage of entrapped antigen 3.13±0.21 % and 4.25±0.17% respectively as the apparent amount and the efficiency of entrapment was 75.83±1.46% and 84.29±3.07%, respectively as productivity of actual load of antigen.

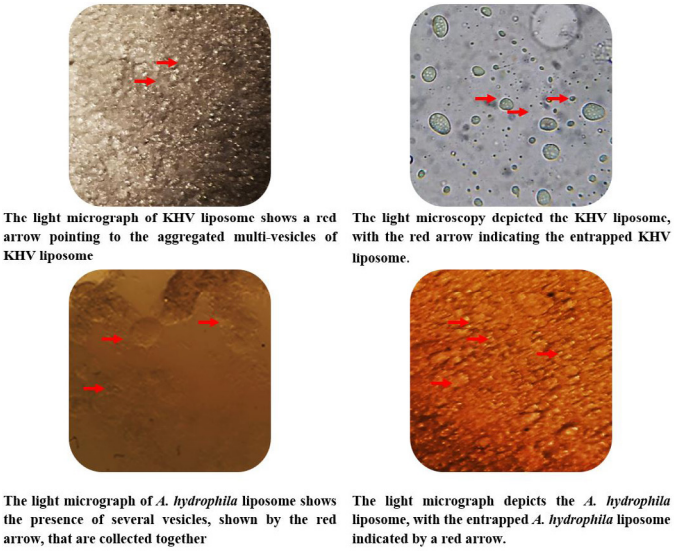
Table 1: Size, lamellar and entrapment efficiency of encapsulated KHV and *A. hydrophila*.

Efficiency entrapment liposome %	Lamellar number	Size of liposome nm	Type of vaccine
75.83 ± 1.46	3.13 ± 0.21	89.46 ± 8.92	KHV liposome
84.29 ± 3.07	4.25 ± 0.17	91.10 ± 3.85	*A. hydrophila* liposome

Transmission electron microscopy revealed a strong association between Phosphatidylcholine and cholesterol, resulting in the formation of closed vesicles that have enhanced thermodynamic stability. The liposomal composition consists of lipids that closely resemble the phospholipids found in cell membranes. These lipids are fully biodegradable within the cell membrane. The inclusion of cholesterol in the liposome affects its rigidity and fluidity, leading to increased stability and reduced susceptibility to degradation (Marasini *et al*., 2017). In general, many parameters may influence the entrapment effectiveness of the KHV and *A. hydrophila* liposome vaccine in multi-vesicular liposomes. These factors include solubility, permeability, and osmolarity tolerance of the liposome vaccine containing the first solution. These impact results collectively indicated to osmolarity was affected encapsulation efficiency of Liposome vaccine in the multi-vesicular liposomes and that the maximum encapsulation efficiency.

The size of multi-lamellar liposomes was reduced by subjecting them to overtaxing at lower temperatures during the production process (Schaeffer and Krohn, 1982). Enhance the permeability of molecules across various biological tissue membranes (Anderson *et al*., 1999).

The light micrograph shows the lamellar layers of multi-lamellar multi-vesicles liposomes containing KHV or A. hydrophila, indicated by a red arrow. The light micrograph depicts a red arrow pointing to the liposome vaccination containing the entrapped antigene, whereas a yellow arrow indicates the empty liposome. The light micrograph of KHV or *A. hydrophila* liposome shows a red arrow pointing to the aggregated multivesicular antigen vaccination.



The light micrograph of KHV liposome shows a red arrow pointing to the aggregated multi-vesicles of KHV liposome

The light microscopy depicted the KHV liposome, with the red arrow indicating the entrapped KHV liposome.

The light micrograph of *A. hydrophila* liposome shows the presence of several vesicles, shown by the red arrow, that are collected together

The light micrograph depicts the *A. hydrophila* liposome, with the entrapped *A. hydrophila* liposome indicated by a red arrow.

Figure 1: The light micrograph of liposome KHV and *A. hydrophila* show the lamellar of liposome with clusters of liposomes of multivesicles form and in otherwise denoted to the general profile of loading incidence and empty form. Further, the signs of inclusive aggregation of liposome KHV and *A. hydrophila* behavior trend to signify a crowed grab form.

Osmolarity tolerance challenges

The osmo-tolerance of liposome KHV and *A. hydrophila* showed no significant change within the range of (0.4-0.85) when compared to other points of osmotic change. Additionally, there was fluctuation seen between the exposure durations and NaCl concentrations, as shown by the shaded region Figure 2.

KHV liposome *A. hydrophila* was disseminated in phosphate buffers with a pH of 7.2±1.02. The buffer allowed the combined lipids to swelling efficiently, resulting in the formation of well-defined liposomes. The swelling of the material is influenced by the pH and concentration of the liquid media. The particular combination of lipid ratios in this composition exhibited enhanced swelling characteristics within this pH range (Ghazi and Al-Bayati 2020).

pH tolerance challenge pH

The pH tolerance of *A. hydrophila* and KHV, liposomes are a small spherical vesicle made up of a lipid bilayer. The pH tolerance of liposome KHV and *A. hydrophila* did not exhibit any significant variations within the pH range of 4 to 8, as compared to other pH values. Additionally, there was no notable variance seen between the duration

of exposure to the pH-changing environment and the resulting pH alterations, Figure 3. The liposomal vaccines exhibited pH tolerance within the range of pH 4 to pH 8. The liposome serves as a carrier to administer oral vaccination and maintain a favorable response to stomach secretion, so achieving an oral mode of administration (Kesisoglou *et al*., 2005).

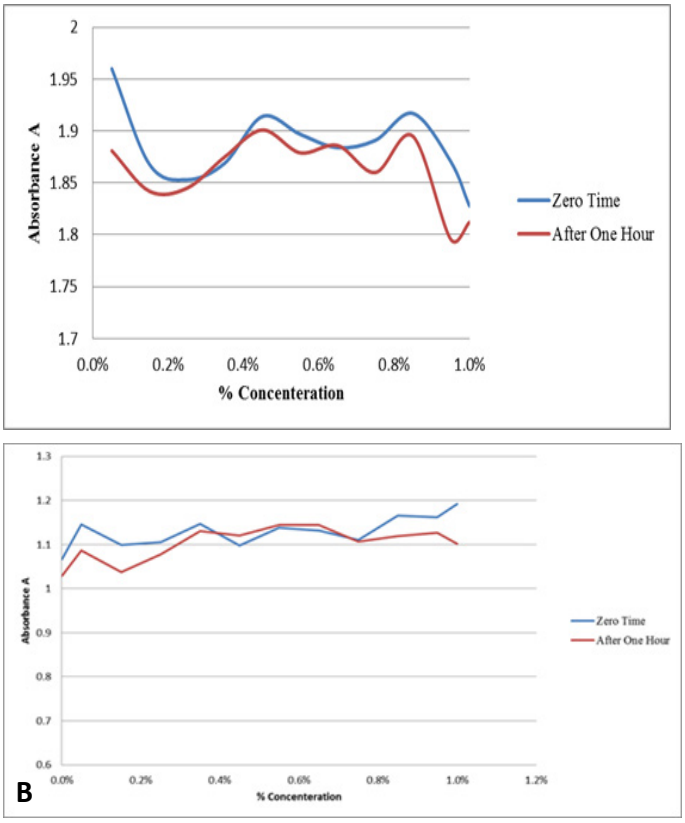
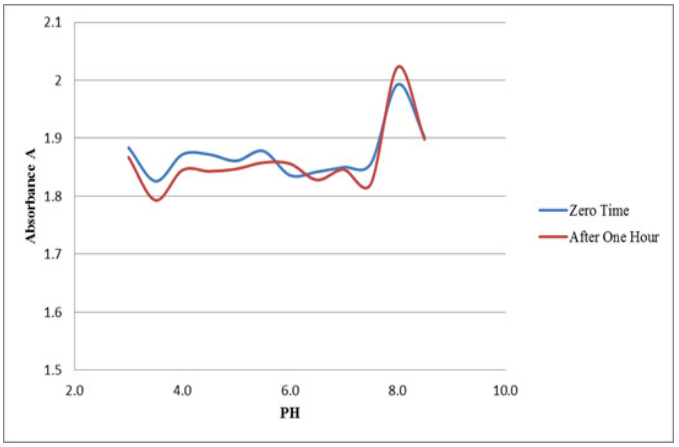


Figure 2: The Osmo-tolerance in NaCl challenge solution of (A) liposome KHV and (B) *A. hydrophila* at zero time and after one hour.

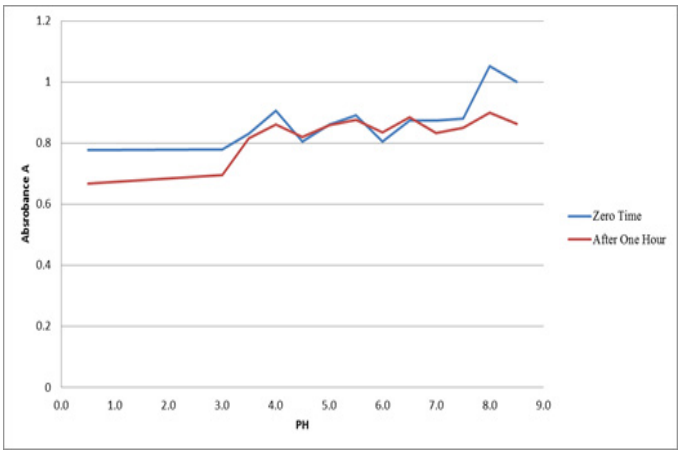
Challenge test

In vaccinated fish with liposome, KHV with bad water quality pound (1.3, 1.4) shown that ten out of twenty fish died within 21 days post challenge with accumulative mortality of 50%. In contrast, unvaccinated fish with bad water quality pound (1.7, 1.8) had shown mortality 90%. The percent survival RPS was 45%.

Vaccinated fish with good water quality that changes water every 2 days pound (1.1, 1.2) post challenged six out of twenty fish died within 21 days post challenge, the accumulative mortality was 30% while in unvaccinated fish with good water quality pound (1.5, 1.6) sixteen out of twenty fish died, the accumulative mortality was 82.5% so RPS was 62.5%, as indicated in Figures 4. All fish died and survivors in vaccinated and unvaccinated were KHV positive at gill and kidney by PCR assay except 4 vaccinated fish.



(A) The pH tolerance of liposome KHV in an acid-base challenge solution was measured at both zero hours and after one hour.



(B) The pH tolerance of liposomes in an acid-base challenge solution was measured at both zero hours and after one hour

Figure 3: The pH tolerance of (A) liposome KHV and (B) *A. hydrophila* in an Acid-Base challenge solution.

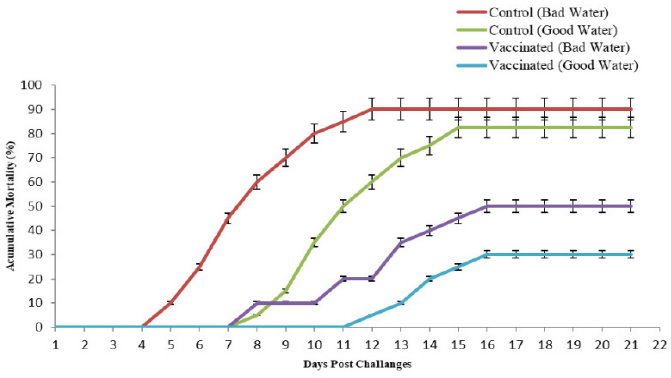


Figure 4: Mortality in groups (immunized fish in good quality water by oral vaccination Containing 0.63 µg KHV Liposome vaccine /fish/day for five days (1.1, 1.2*) and unvaccinated fish groups in good quality water (1.5, 1.6) and Mortality in groups (immunized fish in bad quality water by oral vaccination Containing 0.63 µg KHV Liposome vaccine/fish/day for five days(1.3, 1.4*) and unvaccinated fish groups in bad quality water (1.7, 1.8). *Significantly difference from control (p≤0.05).

The vaccinated fish group with liposome Aeromonas bacteria that challenged with alive bacteria at dose 3×10^8 cfu/fish pond (2.1, 2.2) showed six out of twenty fish died within 14 days post challenge so the accumulative mortality was 30% while unvaccinated fish with same challenged dose (2.5, 2.6) eighteen out of twenty fish died the accumulation mortality was 90% that recorded RPS was 67.5%.

When challenged fishes with Aeromonas bacteria at dose 3×10^7cfu/fish, (2.3, 2.4) three out of twenty fish died within 14 days post challenge recording an accumulative mortality of 15%, while unvaccinated with the same challenged dose (2.7, 2.8) showed fourteen out of twenty fish died recording 72.5% accumulation mortality and RPS was 78.65%, as depicted in Figure 5.

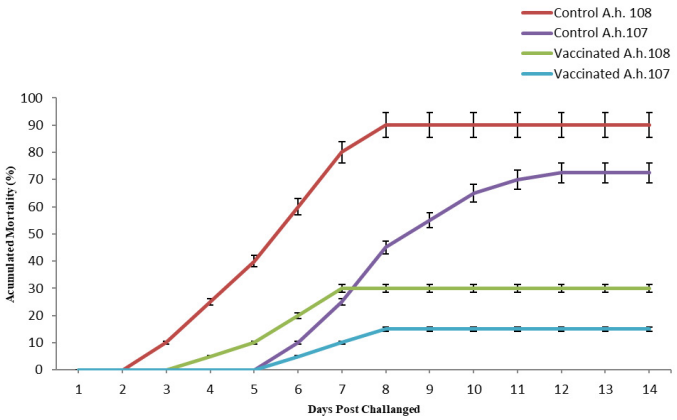


Figure 5: Mortality in vaccination fish for 5 days *Aeromonas hydrophila* liposome vaccine at dose $1*10^9$ bacteria/fish/day pound (2.1, 2.2) and unvaccinated fish pound (2.5, 2.6) was challenged by intramuscular injection with *A. hydrophila* at $3*10^8$ CFU/fish, Mortality in vaccination fish for 5 days Aeromonas hydrophila liposome vaccine at dose $1*10^9$ bacteria/fish/day pound(2.3,2.4) and unvaccinated fish pound (2.7, 2.8) was challenged by intramuscular injection with *A. hydrophila* at 3×10^7 CFU /fish. *Significant difference between vaccinated and control at level ($p\leq0.05$).

All fish that died post challenged in vaccinated and unvaccinated were re-isolated Aeromonas bacteria from kidney and injection site in triptych soy agar plate at 24 °C.

According to the findings, the vaccine created from an antigen encased in a liposome is efficient in immunizing fish when administered through the oral method. This is mainly a result of the fact that fish have a posterior gut that is capable of absorbing crude lipids (Miyazaki and Fujiwara, 1988), Multilamellar liposomes consist of several bilayered structures and vary in diameter from a few to hundreds of nanometers. They are particularly well-suited for encapsulating hydrophobic antigens (Marasini *et al.*, 2017). The amount of lipid bilayers in liposomes also affects the encapsulation efficiency and the release of vaccines. When liposomes are taken up or processed in the cell, the lamellarity of the liposomes influences their intracellular destiny (Laouini *et al.*, 2012). The vesicles exhibited a dense arrangement when seen using the transmission electron microscope, suggesting the presence of amphipathic lipids such as phosphatidylcholine and cholesterol (Jaafer *et al.*, 2021).

The liposome vaccine developed in this investigation, including KHV antigen and Aeromonas bacteria, was intended to be taken up in the posterior intestine via the oral route the oral vaccination is regarded optimal since it may stimulate both mucosal and systemic immune responses (Quentel and Vigneulle, 1997). Additionally, it serves as a handy pathway for administration. Nevertheless, the primary issue with oral vaccinations is from the degradation of antigens due to stomach acidity and proteolytic enzymes in the intestinal lumen.

Consequently, substantial dosages are necessary to attain adequate immune responses. Liposomes are a very efficient vehicle for transporting antigens, and they are taken up by antigen-presenting cells (APCs), such as macrophages in the stimulation of immunological response (Watarai *et al.*, 1998). Oral administration of liposomes containing antigen effectively induced immune responses in carp (Irie *et al.*, 2003). However, an attempt to develop oral vaccines has failed (Marasini *et al.*, 2017).

The current work demonstrated that vaccination with liposomes containing KHV and *A. hydrophila* effectively stimulated the production of antibodies specific to both antigens, potentially leading to excellent protection against both the virus and bacterium. Three mechanisms have been identified in fish: humoral antibody response, cell mediated immune response, and non-specific defensive mechanisms (Bernoth *et al.*, 1997). Furthermore, studies have shown that the administration of liposomes may effectively stimulate the development of cell-mediated immunity (Ninomiya *et al.*, 2002).

Miyazaki and Fujiwara (1988) showed that fish take in crude lipids and proteins in the posterior intestine. Since this pathway is widespread in juveniles, antigen-containing lipid particles are utilized to transfer antigens to the posterior intestine and enhance their absorption in the area. This would make such particles suitable for the induction of immunity (Yasumoto *et al.*, 2006a). Experiments using KHV cohabitation with sick fish were used to cause infection (Ronen *et al.*, 2003). Orally vaccinated fish were immunized and protected from experimental infection, resulting in high RPS. This indicated that the efficacy of immunization with the liposome vaccine is reproducible, especially in fish cohabitation in a good environment. However, following these methods, it is unknown exactly

how many viruses invade fish. Challenge tests with one infected fish and five vaccinated fish revealed that orally vaccinated fish were immunized and protected from experimental infection. The challenge test and PCR findings support the efficiency of oral vaccination with KHV liposome vaccine in carp and the importance of a healthy environment (high water quality) to prevent fish from sickness. Additionally, the attenuated KHV vaccine induces immunity in common carp (Ronen *et al*., 2003). On the other hand, the live attenuated KHV may potentially evolve into the virulent form, but the liposome KHV vaccination would never have that danger.

According to another group of researchers, carp may be immunized by oral administration of a liposome vaccine that contains inactivated *Aeromonas hydrophila* antigens (Yasumoto *et al*., 2006b). However, the liposomes they used in their research had a different molar ratio of phospholipids than the liposomes we used in our investigation. Furthermore, the amount of antigen used in the current study was much lower. Additionally, researchers (Siriyappagouder *et al*., 2014; Nayak *et al*., 2004) have shown that common carp given a biofilm vaccination containing *Aeromonas hydrophila* antigens established protection against bacterial infection.

The findings of this study, which was carried out for the first time in Iraq, indicate that the two vaccines prepared by the liposome method are useful and feasible for immunizing fish against infection with Koi herpes virus and bacterial infection with *Aeromonas hydrophila* and encourage the start of work in the second stage of the project's by This result enables us to initiate the subsequent phase of our research, where in we employ tissue culture of fin cells from common carp fish to propagate the Koi herpes virus. The objective is to develop a vaccine with a dose containing the antigenic standard of $1\times10^{2.5}$ $TCID_{50}$/dose, aiming to achieve a heightened level of immunity compared to the outcomes observed in the initial phase. Additionally, we aim to combine the two vaccine agents, namely the virus and bacterial components, into a single product.

CONCLUSIONS AND RECOMMENDATIONS

Conclusion this challenge created liposomal vaccines against both *Koi herpesvirus* (KHV) and *Aeromonas hydrophila* bacteria. Early results show: KHV vaccine: Protected fish by 45-62.5%, depending on water quality. Aeromonas vaccine: Protected fish by 67.5-78.65%. Next recommended steps: Strengthen the KHV vaccine for potentially even better protection. However, the current study strongly recommended that we should employ tissue culture of fin cells from common carp fish to propagate the Koi herpes virus. The objective is to develop a vaccine with a dose containing the antigenic standard of $1\times10^{2.5}$ $TCID_{50}$/dose, aiming to achieve a heightened level of immunity compared to the outcomes observed in the initial phase. Additionally, we aim to combine the two vaccine agents, namely the virus and bacterial components, into a single product. Combine both vaccines into one for broader defense. Overall, these liposomal vaccines offer promising protection for carp, with further improvements on the horizon.

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NOVELTY STATEMENT

The results of this first research conducted in Iraq demonstrate the efficacy and practicality of using liposome-based vaccinations to immunize fish against the Koi herpes virus and bacterial illness caused by Aeromonas hydrophila

AUTHOR'S CONTRIBUTION

The writers of this study collaborated as a cohesive team, providing mutual assistance in conducting experiments, analyzing data, and reporting the findings.

Conflict of interest

The authors have declared no conflict of interest.

REFERENCES

Ababneh, Mustafa, Hananeh W, Alzghoul M (2020). Mass mortality associated with koi herpesvirus in common carp in Iraq. Heliyon, 6(8). https://doi.org/10.1016/j.heliyon.2020.e04827

Ali M, Faruk M (2018). Fish parasite: Infectious diseases associated with fish parasite.

Amend DF (1981). Potency testing of fish vaccines. Fish biologics: Serodiagnostics and vaccines, 49: 447-454.

Anderson KE, Stevenson BR, Rogers JA (1999). Folic acid-PEO-labeled liposomes to improve gastrointestinal absorption of encapsulated agents. J. Contr. Release, 60: 189-198. https://doi.org/10.1016/S0168-3659(99)00072-3

Bangham AD (1993). Liposomes: The Babraham connection. Chem. Phys. Lipids, 64(1): 275-285. https://doi.org/10.1016/0009-3084(93)90071-A

Bernoth, Eva-Maria, Anthony EE, Paul JM, Gilles O, Peter S (1997). Furunculosis: Multidisciplinary fish disease research (Elsevier). https://doi.org/10.1016/B978-012093040-1/50003-8

Cicuta P, Sarah LK, Sarah LV (2007). Diffusion of liquid domains in lipid bilayer membranes. J Phys. Chem. B, 111(13): 3328-3331. https://doi.org/10.1021/jp0702088

Ellis AE (1988). Current aspects of fish vaccination. Dis. Aquat. Org., 4: 159-164. https://doi.org/10.3354/dao004159

Ghazi AM, Al-Bayati MASA (2020). Anti-proliferative of the phytosome propolis, phytosome lycopene and synergistic effect on the benign prostatic hyperplasia cells *in-vitro*. Plant Arch., 20: 6579-6589.

Irie T, Watarai S, Kodama H (2003). Humoral immune response of carp (*Cyprinus carpio*) induced by oral immunization with liposome-entrapped antigen. Dev. Comp. Immunol., 27: 413-421. https://doi.org/10.1016/S0145-305X(02)00137-4

Jaafer NS, Balqees AHA, Al-Bayati MA (2021). Comparison between Newcastle disease vaccine and liposomal entrapped Newcastle disease vaccine in chicks. J. Genet. Environ. Resour. Conserv., 9: 157-167.

Kesisoglou F, Zhou SY, Niemiec S, Lee JW, Zimmermann EM, Fleisher D (2005). Liposomal formulations of inflammatory bowel disease drugs: local versus systemic drug delivery in a rat model. Pharm. Res., 22: 1320-1330. https://doi.org/10.1007/s11095-005-5376-3

Laouini, A, Chiraz J-M, Imen B, Souad S, Catherine C, Hatem F (2012). Preparation, characterization and applications of liposomes: State of the art. J. Colloid Sci. Biotechnol., 1: 147-168. https://doi.org/10.1166/jcsb.2012.1020

Marasini N, Ghaffar KA, Skwarczynski M, Toth I (2017). Chapter twelve liposomes as a vaccine delivery system. In: (eds. M. Skwarczynski and I. Toth), micro and nanotechnology in vaccine development. William Andrew Publishing. pp. 221-239. https://doi.org/10.1016/B978-0-323-39981-4.00012-9

Miyazaki T, Fujiwara K (1988). Histological studies on yolk utilization and digestive function in larvae and juvenile of red sea bream and black sea bream. Bull. Fac. Bioresour. Mie Univ., 1: 15-27.

Nayak DK, Asha A, Shankar KM, Mohan CV (2004). Evaluation of biofilm of *Aeromonas hydrophila* for oral vaccination of *Clarias batrachus*. A carnivore model. Fish Shellf. Immunol., 16: 613-619. https://doi.org/10.1016/j.fsi.2003.09.012

Ninomiya A, Ogasawara K, Kajino K, Takada A, Kida H (2002). Intranasal administration of a synthetic peptide vaccine encapsulated in liposome together with an anti-CD40 antibody induces protective immunity against influenza A virus in mice. Vaccine, 20: 3123-3129. https://doi.org/10.1016/S0264-410X(02)00261-X

Oliveira, MF, Stefan WK, Ariel G, Aaron JK, Bohle DS, Hempelmann E, Menezes D, Vannier-Santos MA, Oliveira PL, Ferreira ST (2005). Structural and morphological characterization of hemozoin produced by *Schistosoma mansoni* and *Rhodnius prolixus*. FEBS Lett., 579: 6010-6016. https://doi.org/10.1016/j.febslet.2005.09.035

Quentel C, Vigneulle M (1997). Antigen uptake and immune responses after oral vaccination. Dev. Biol. Stand., 90: 69-78.

Ronen A, Perelberg A, Abramowitz J, Hutoran M, Tinman S, Bejerano I, Steinitz M, Kotler M (2003). Efficient vaccine against the virus causing a lethal disease in cultured *Cyprinus carpio*. Vaccine, 21(32): 4677-4684. https://doi.org/10.1016/S0264-410X(03)00523-1

Schaeffer HE, Krohn DL (1982). Liposomes in topical drug delivery. Invest. Ophthalmol. Visual Sci., 22(2): 220-227.

Siriyappagouder, Prabhugouda, Shankar KM, Kumar BTN, Patil R, Byadgi OV (2014). Evaluation of biofilm of Aeromonas hydrophila for oral vaccination of *Channa striatus*. Fish Shellfish Immunol., 41: 581-585. https://doi.org/10.1016/j.fsi.2014.09.021

Szebeni J, Di Iorio EE, Hauser H, Winterhalter KH (1985). Encapsulation of hemoglobin in phospholipid liposomes: Characterization and stability. Biochemistry, 24: 2827-2832. https://doi.org/10.1021/bi00333a003

Toffan A, Marsella A, Abbadi M, Abass S, Al-Adhadh B, Wood G, Stone DM (2020). First detection of koi herpesvirus and carp oedema virus in Iraq associated with a mass mortality in common carp (*Cyprinus carpio*). Transb. Emerg. Dis., 67: 523-528. https://doi.org/10.1111/tbed.13428

van Rooijen N, Nieuwmegen R (1980). Endotoxin enhanced adjuvant effect of liposomes, particularly when antigen and endotoxin are incorporated within the same liposome. Immunol. Commun., 9: 747-757. https://doi.org/10.3109/08820138009109684

Watarai S, Han M, Tana, Kodama H (1998). Antibody response in the intestinal tract of mice orally immunized with antigen associated with liposomes. J. Vet. Med. Sci., 60: 1047-1050. https://doi.org/10.1292/jvms.60.1047

Yasumoto S, Kuzuya H, Yasuda M, Yoshimura T, Miyazaki T (2006a). Oral immunization of common carp with a liposome vaccine fusing koi herpesvirus antigen. Fish Pathol. (Japan), 41: 141-145. https://doi.org/10.3147/jsfp.41.141

Yasumoto S, Yoshimura T, Miyazaki T (2006b). Oral immunization of common carp with a liposome vaccine containing aeromonas hydrophila antigen. Fish Pathol., 41: 45-49. https://doi.org/10.3147/jsfp.41.45

Yokoyama, T, Masuda H, Suzuki M, Ehara K, Nogi K, Fuji M, Fukui T, Suzuki H, Tatami J, Hayashi K, Toda K (2008). Chapter 1 basic properties and measuring methods of nanoparticles. In: (eds. M. Hosokawa, K. Nogi, M. Naito and T. Yokoyama). Nanoparticle Technology Handbook (Elsevier: Amsterdam). https://doi.org/10.1016/B978-044453122-3.50004-0