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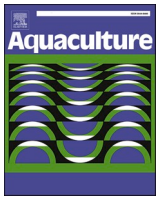

Molecular epidemiology and current management of Infectious Spleen and Kidney Necrosis Virus (ISKNV) infection in Ghanaian cultured tilapia

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ABSTRACT

Infectious Spleen and Kidney Necrosis Virus (ISKNV) is globally gaining more attention, due to its highly significant economic impact on the aquaculture industry. In late 2018, unusually high levels of mortality (60–90%) was reported in some intensive tilapia cage culture systems on the Eastern bank of Lake Volta in Ghana. This resulted in the fold-up of several small and medium scale farms. Preliminary investigations confirmed the involvement of ISKNV, a viral pathogen noted for fatal systemic infections in many fish species. As a follow-up on the outbreak situation, and post-mass vaccination of affected fish farms, the need to investigate further the molecular epidemiology and phylogeography of the virus across Lake Volta became paramount. A multiplexed PCR assay to detect the virus and MinION™ nanopore sequencing of the Major Capsid Protein (MCP) were performed to investigate the presence and genotype of ISKNV in tilapia collected from 30 randomly selected farms at various geographical locations. ISKNV was found to be widely distributed across the lake and detected in 80% of farms with a reported average daily mortality of 40%. Fry and juvenile fish were the most affected, and approximately 50% of fish that tested positive were asymptomatic. These apparently healthy fish are likely contributors of virus transmission across farms. Phylogenetic analysis of the MCP revealed that all 35 isolates from 14 different farms distributed across the lake clustered with ISKNV clade I with 100% homology to isolates from the 2019 outbreak strain. Vaccination and heat-shock treatment; the two main specific interventions currently employed to control the viral pathogen have not achieved much success, and ISKNV remains a threat to the growth of the aquaculture industry in Ghana. The outcome of this study can be useful in improving fish health management and biosecurity policies in the aquaculture industry.

1. Introduction

Infectious Spleen and Kidney Necrosis virus (ISKNV) is a megalocytivirus known to induce a fatal systemic disease in some aquatic animals resulting in mass mortalities and causing significant economic losses in the aquaculture industry (Subramaniam et al., 2016; Subramaniam et al., 2008; Joon et al., 2008; He et al., 2000; Ramírez-Paredes et al., 2021). Symptomatology of the disease in fish usually includes lethargy, ascites, exophthalmia, frayed fin and hemorrhages on body parts (Subramaniam et al., 2016; Thanasaksiri et al., 2018; He et al., 2002; Zhu et al., 2021). The virus is icosahedral and about 150 nm in diameter (He et al., 2002). The virion core contains a single linear dsDNA molecule of approximately 111 kb (Shi et al., 2010; He et al., 2001a).

In recent times the major capsid protein (MCP) gene has been used to classify and assess genetic relationship of unknown megalocytivirus isolates because of its highly conservative DNA sequence (Wang et al., 2009; Fu et al., 2011; Huang et al., 2011; Kurita and Nakajima, 2012). There are three main genotypic clusters of megalocytiviruses: red sea bream iridovirus (RSIV), first isolated in cultured red sea bream (*Pagrus*

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major); infectious spleen and kidney necrosis virus (ISKNV), isolated initially from farmed mandarin fish (*Siniperca chuatsi*); and turbot reddish body iridovirus (TRBIV), initially from farmed turbot (*Scophthalmus maxius*) (Inouye et al., 1991; Shi et al., 2004).

Conventional diagnosis of ISKNV infection is based on histopathological analysis, electron microscopic observation, or immunoassays (Subramaniam et al., 2016; Thanasaksiri et al., 2018; OIE, 2019; Machimbirike et al., 2019). However, several PCR-based methods such as nested PCR, loop-mediated isothermal amplification (LAMP) and qPCR assays have been established for detecting and quantifying ISKNV infection (Subramaniam et al., 2008; Fu et al., 2011; Machimbirike et al., 2019; Rimmer et al., 2012; Lin et al., 2017; Suebsing et al., 2016). The host range of ISKNV is broad, predominantly affecting freshwater and brackish water fish species such as tilapia (*Oreochromis* spp.) (Subramaniam et al., 2016; Suebsing et al., 2016; Dhar and Manna, 2014). The virus was originally identified in a high-mortality outbreak among cultured mandarin fish (*Siniperca chuatsi*) in China and was subsequently detected in other geographies (He et al., 2000; Wang and Wen, 2018; King et al., 2012). The export of asymptomatic infected fish has been linked to the spreading of ISKNV widely to other parts of Asia, Europe and Australia; especially in aquaculture facilities (Subramaniam et al., 2008; Shi et al., 2010; Rimmer et al., 2012; Wang and Wen, 2018; Hick et al., 2016).

Aquaculture of Nile tilapia (*Oreochromis niloticus*) is an emerging industry in Ghana where fish constitutes 50–80% of consumed animal protein. The yearly per capita consumption of tilapia is estimated at 28 kg, significantly higher than most African countries (Sumberg et al., 2016; Santos et al., 2020; FAO, 2023). Because Nile tilapia is the dominant and preferable species for fish farming and consumption, the industry employs thousands of people and is critical to livelihoods and the general economy (Ramírez-Paredes et al., 2021; Béné, 2007). In Ghana, from only 2000 t in 2006, Nile tilapia production soared to over 55,000 t per annum by 2016, with revenues of approximately US$ 200,000,000 (Fish price in Ghana, 2023; Ministry of Fisheries and Aquaculture Development -MOFAD, 2019). More than 90% of this production was derived from high stocking-density floating cage systems on Lake Volta, one of the largest artificial lakes in the world (FAO, 2017; Amenyogbe et al., 2018). In 2018 and 2019 production dropped to about 30,000 t per annum representing 46% economic loss due to disease outbreak (Fisheries Commission, Ministry of Fisheries and Aquaculture Development, 2020). Infection with ISKNV was detected in few farms in 2018 and 2019 and subsequently, this virus was confirmed to be the major cause of the devastating economic loss to the cage farmers and hatchery operators (Ramírez-Paredes et al., 2021). This was the first reported cases of ISKNV in Africa. To curtail the spread of the virus, the Ghanaian Ministry of Fisheries and Aquaculture carried out a mass immunization of fish farms across the lake using the Aquavac irido Vaccine, a commercial iridovirus vaccine from Singapore (MOFAD, 2021). The industry is recovering, but yet to peak to pre-outbreak levels (Ministry of Fisheries and Aquaculture Development, 2021).

Since the first report of ISKNV in farmed tilapia in Ghana by Ramírez-Paredes et al (2019), few reports have emerged on ISKNV genomics and diagnosis using samples from Ghana (Alathari et al., 2023; Delamare-Debouteville et al., 2023) However, no studies have been published on the burden, presentation and scale of the disease in the country. So far, investigation to determine the spread of the virus in tilapia aquaculture has only been restricted to few farms below the dam of the Volta Lake (Alathari et al., 2023), thereby limiting the sampling scope for better understanding of the epidemiological situaton. There has also not been any report on the outcome of the iridovirus vaccine rollout in Ghana. To fill these knowledge gaps, fish samples collected from a broad swathe of farms on the lake were screened for the presence of ISKNV using an in-house multiplex conventional PCR assay. We performed sequencing and phylogenetic analysis to establish strain relatedness and collected important epidemiological data on disease mortalities and controls measures at the farm level using a detailed questionnaire. We present data showing the widespread distribution of the virus on the lake and the need to have better practical control strategies moving forward.

2. Materials and methods

2.1. Bio-specimen collection

Field sampling was carried out between 25 August and 6 November 2021 in 30 tilapia farms including those that had received the iridovirus vaccine at least once from the Fisheries Commission, Ghana. The farms were located along Lake Volta upstream and downstream of the Akosombo dam within Eastern and Volta regions, which is the hub for intensive floating cage culture systems in the country. To ensure good sampling coverage, fish were collected from only farms in active operation, who gave informed consent (Supplementary data 1), and were adequately spaced out across the study area. The farms sampled included small (n = 9), medium (n = 12) and large (n = 9) scales of operations. The scale of operations is categorized based on fish production capacity per annum. Small (10-50mt) /year), Medium (51-100mt /year) and Large (> 100 mt) /year). On average, 10 biological samples of fry, fingerlings, grow-out adult fish, brood-stocks and their eggs were randomly collected at each farm depending on the type of facility (i.e., grow-out and/or hatchery) in operation. For farms with both hatchery and grow-out, it was ensured that good representative samples (10–12 fish and egg material) were collected across the entire production cycle. Symptoms indicative of ISKNV infection were recorded for each fish on a separate form (Table S1). All fish sampled were euthanized with 0.20 mL of clove oil per 500 mL of water for 10 min (Fernandes et al., 2017). Fish tissues (kidney, brain and spleen, eggs/ova) were preserved in RNAlater, and 10% buffered formalin on the field, for subsequent PCR detection of ISKNV in the lab and histopathological analysis. Samples in formalin were shipped to Norwegian Veterinary Institute in Norway where slides were prepared, and digital images generated. Ethical approval was acquired from the University of Ghana Institutional Animal Care and Use Committee (UG-IACUC 007/20–21).

Table 1
List of primers used for PCR assay.

Name of Gene	Function	Gene Location	Primer	Sequence	Tm	Product size (bp)
ATPase	Adenosine triphosphatase	ORF 122R	Forward	CGACGAAATGGGTGGGATGA	60.11	834
			Reverse	CGCCCGCAAGATGATCAAAC	60.25	
TNFR	Tumor necrosis factor Receptor-associated protein	ORF 111L	Forward	ATTGGCGTACAGCAGCTCAA	60.39	987
			Reverse	ATCGCACACGTGTACCTTGA	59.68	
VEGF	Viral Vascular Endothelial Growth Factor	ORF 48R	Forward	ATTGCGTGTCAAGGAGAGCA	59.96	664
			Reverse	AGATGCCAACACCACTCTGTT	59.86	
MCP	Major Capsid Protein	ORF 006L	Forward	AGTCAAGGAACTCGCTGGTG	59.97	295
			Reverse	GTGACCTACTTTGCCCGTGA	59.97	
nMCP	Major Capsid Protein	ORF 006L	Forward	CAATTGAGCACACACGGCTC	60.11	1624
			Reverse	CCGGCACCCTGAACATAGAC	60.46	

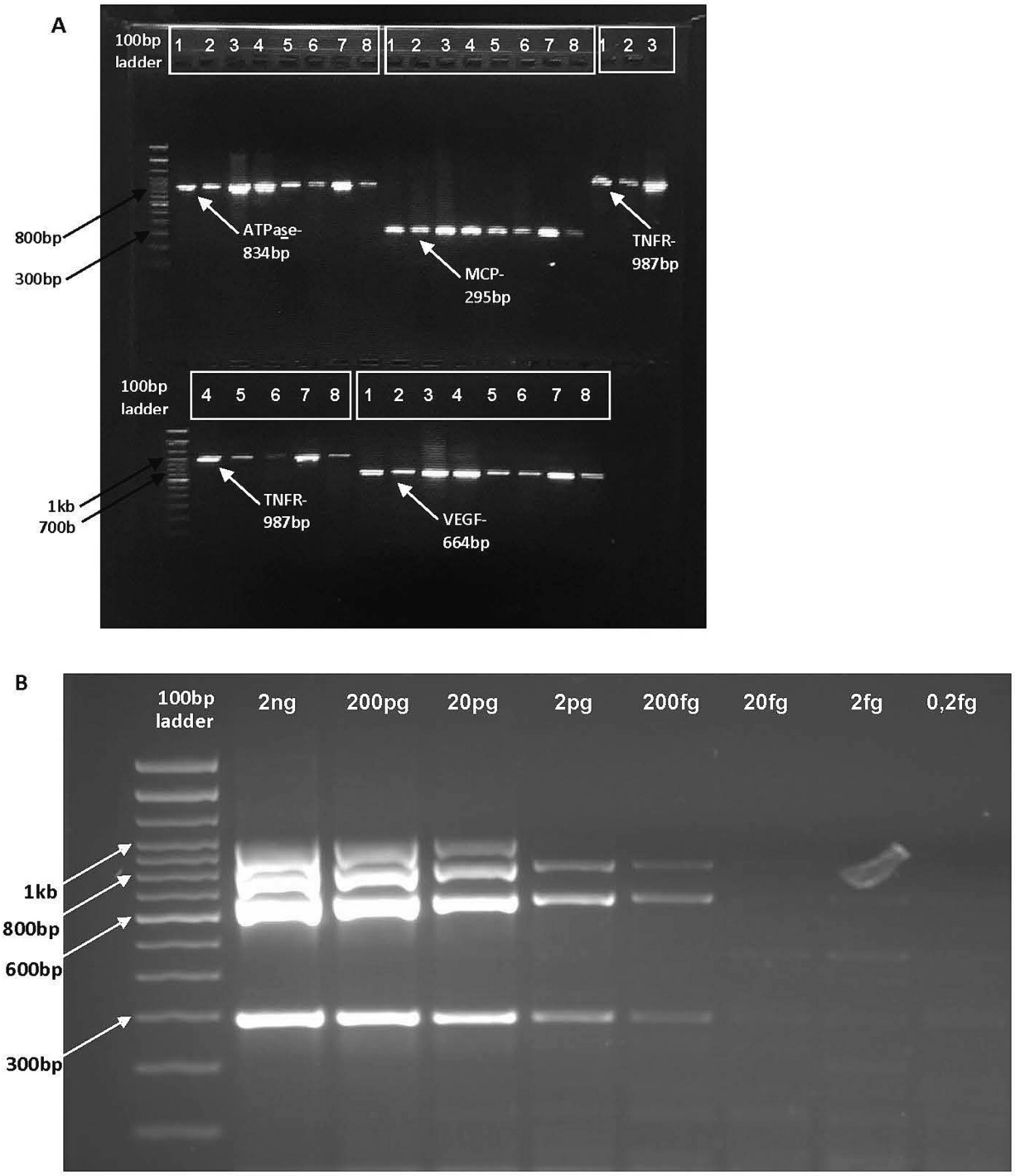

Fig. 1. (A) Gel image showing individual optimization of primers (TNFR (987 bp), ATPase (834 bp), VEGF (664) and MCP (295)) used for the multiple assay (B) Optimized multiplex assay gel image.

2.2. Farm interviews and epidemiological data collection

A structured electronic questionnaire was developed to collect epidemiological information from all farms visited (Supplementary data 1). This obtained data on farm operations, production, disease episodes, mortalities, biosecurity and control measures against ISKNV, as well and other infectious agents. All samples and corresponding questionnaire were linked with a unique farm identification number.

2.3. DNA extraction and PCR amplification

Total DNA was extracted from the RNAlater-preserved tissue samples using the QIAamp DNA Mini Kit (Qiagen), according to the manufacturer's specification. Briefly, tissue (≤25 mg) was removed and washed with sterile 1× PBS (500 μL) twice. Glass microbeads and 100 μL of buffer ATL were added to the tissue and then homogenized by mechanical disruption. The homogenized tissue was incubated with 20 μL of proteinase K at 56 °C overnight for 12 h, following which total nucleic acids were extracted and stored until further use.

Using the extracted DNA as template, detection of ISKNV-like virus

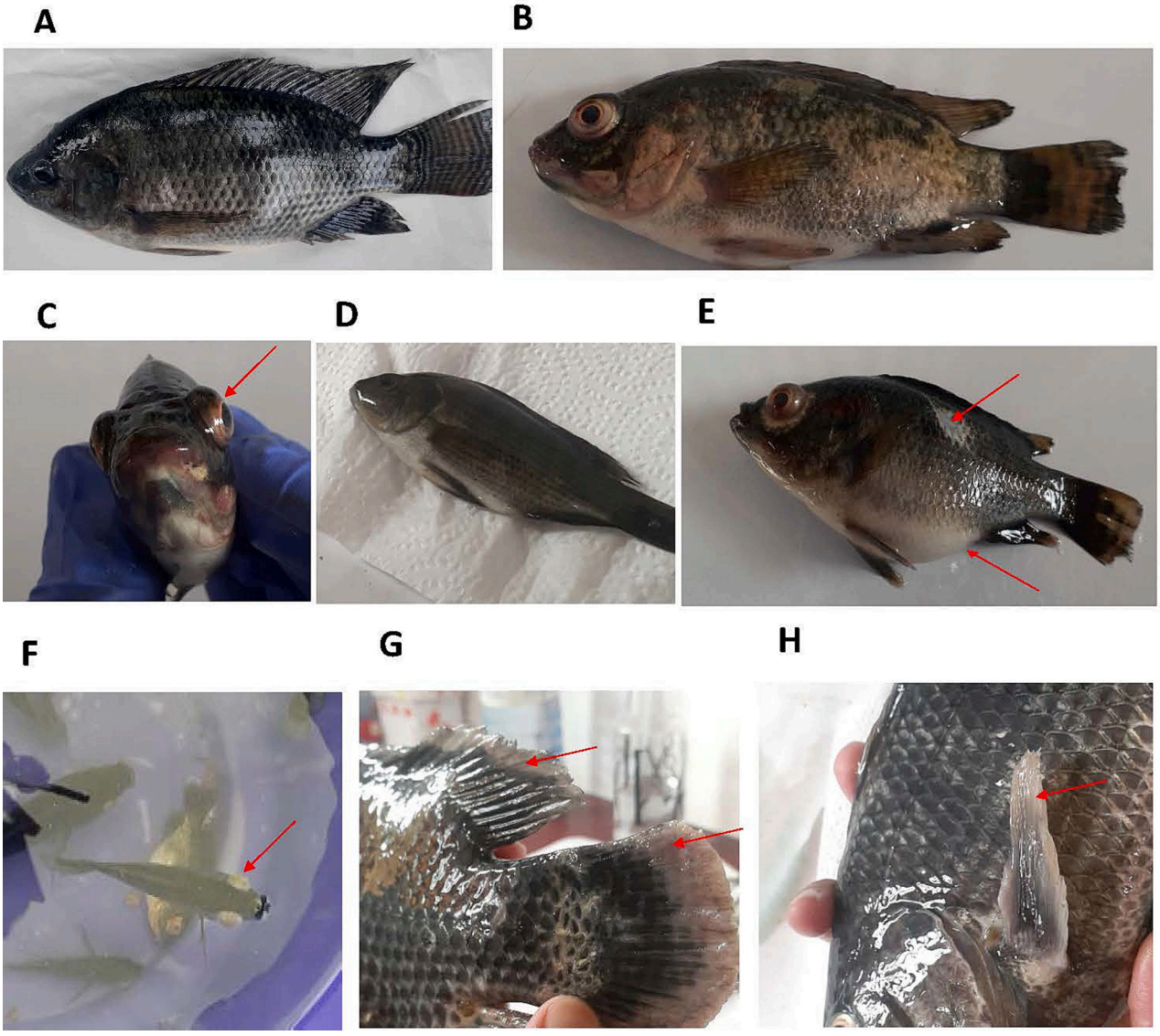

Fig. 2. Some disease symptoms observed in the sampled fish; (A) asymptomatic, (B) discoloration, hemorrhaging and frayed fin, (C) exophthalmia, (D) excess mucus on the skin, (E) ascites & loss of scales, (F). opaque eyes, (G) eroded caudal fin, and (H) eroded pectoral fin.

from the tissue samples was carried out by multiplex PCR amplification. Four putative ISKNV genes were targeted in the multiplex assay and all primers were designed based on the nucleotide sequence of ISKNV (GenBank accession number AF371960) using the NCBI Primer Blast tool (Table 1) (He et al., 2001b; Primer designing tool [Internet], 2023). The 25 μL PCR mixture consisted of 12.5 μL of 2× Go Taq (Hot Start) DNA Polymerase (Invitrogen), 0.5 μL of 0.2 μM forward and reverse primers for each gene (Table 1), 6.5 μL of molecular grade water, and 2 μL of DNA template. The PCR reactions included an initial denaturation of 5 min at 95 °C; followed by 40 cycles of denaturation at 95 °C for 1 min, annealing at 56 °C for 1 min, extension at 72 °C for 1 min; and a final elongation step at 72 °C for 10 min. PCR reactions were subsequently visualized by agarose gel electrophoreses. The assay had previously been optimized to detect ISKNV in 200 fg/μL of fish-derived nucleic acid (Fig. 1). Theoretical specificity of primers used was examined against other genomes by performing basic local alignment searches (Ye et al., 2012). Aquatic bacterial pathogens (*Streptococcus agalactiae, Edwardsiella tarda, Chrysobacterium gambrini, Aeromonas jandaei Aeromonas veronii, Plesiomonas shigelloides*) and extracts from tissues of ISKNV uninfected fish were used as reference samples to confirm the analytical specificity of the multiplex PCR assay (data not shown). To test the sensitivity, DNA from three positive ISKNV samples were diluted serially from a starting concentration of 2 ng in 8 steps 10-fold dilution. Each dilution was used as template in the multiplex PCR as described above. The amplified DNA was analyzed by gel electrophoresis using 1.5% agarose.

2.4. Sequencing and phylogenetic analysis

The complete MCP gene fragment (1358 bp) was amplified for 35 PCR confirmed ISKNV isolates using different set of primers (nMCP) (Table 1). Amplicon sequencing was performed on an Oxford Nanopore™ MinION using a modified protocol based on SARS-CoV-2 whole genome sequencing (Morang'a et al., 2022). Sequencing libraries were prepared using the NEBNext Ultra II End Repair/dA-tailing module (New England Biolabs, UK). Amplicons were barcoded using the EXP-NBD196 kit (Oxford Nanopore Technologies, UK). The barcoded amplicons were then pooled, purified with Ampure XP beads (Beckman Coulter) and quantified using the QubitTM DNA HS Assay Kit (Thermo Fisher ScientificTM, USA). About 75 ng of barcoded libraries were ligated to the AMII sequencing adaptors (Oxford Nanopore Technologies, UK) using the Quick ligation kit (New England Biolabs, UK), then

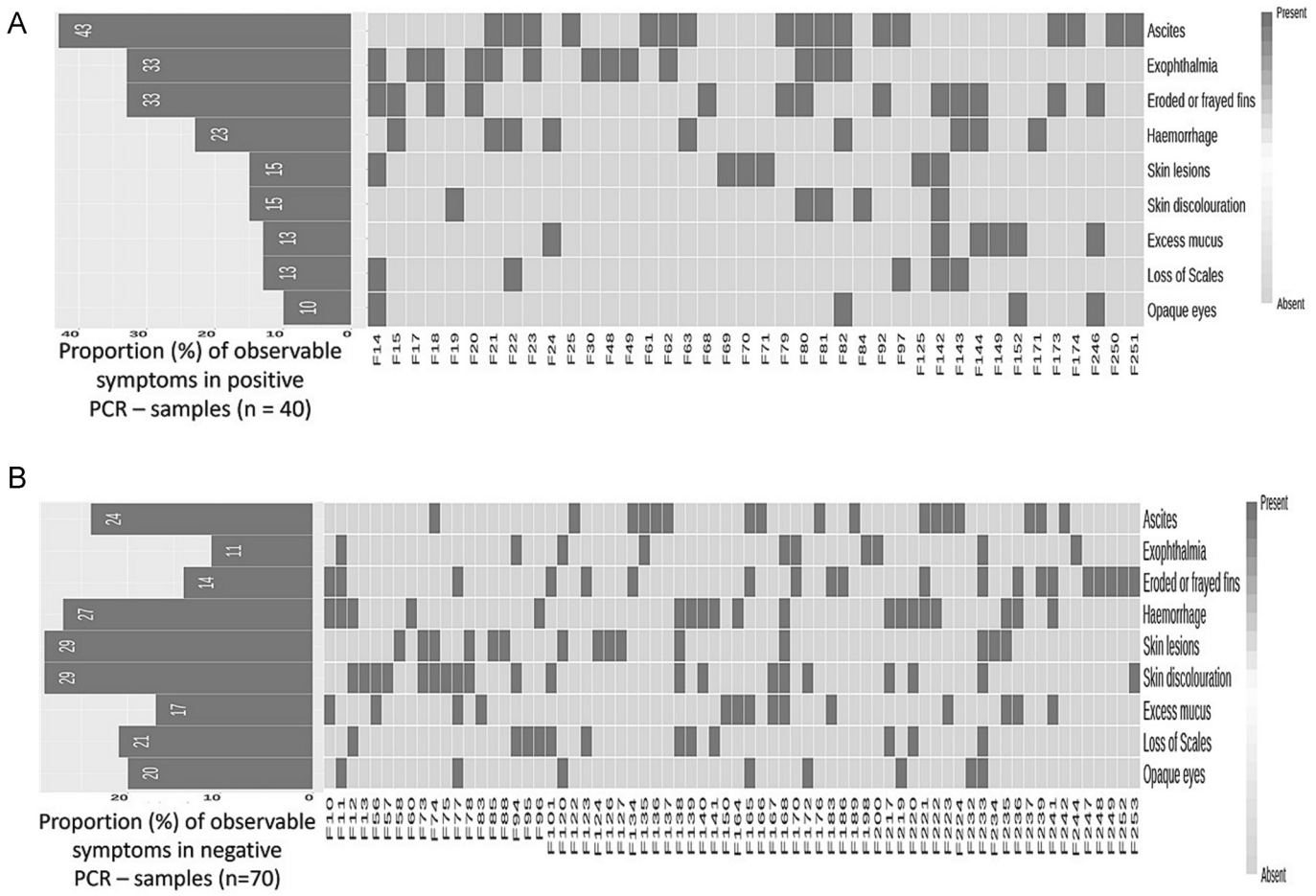

Fig. 3. A. Heat map showing frequency of observed symptoms in ISKNV PCR positive tilapia.
B. Heat map showing frequency of observed symptoms in ISKNV PCR negative tilapia.

finally purified and quantified with Ampure XP beads (Beckman Coulter) and QubitTM DNA HS Assay Kit (Thermo Fisher ScientificTM, USA), respectively. Approximately 20 ng of the purified adaptor-ligated libraries were subsequently loaded on an R9.4.1 flow cell (FLO-MIN106) to be sequenced using a MinION Mk1b device (Oxford Nanopore Technologies, UK). Base-calling and demultiplexing of MinION Fast5 files were achieved using Guppy (Version: v5.0.7) (Wick et al., 2019; GitHub - nanoporetech_rerio_ Research release basecalling models and configurations [Internet], 2023). Alignments and statistics to the reference sequence (AF371960) was done using epi2me-labs/wf-alignment (Version. v0.1.8), followed by a consensus sequence generation using NGSpeciesID (Version - v0.1.2.1) (GitHub - epi2me-labs_wf-alignment [Internet], 2023; NGSpecies ID_DNA barcode and amplicon consensus generation from long-read sequencing data, 2023; GitHub - ksahlin_NGSpeciesID_ Reference-free clustering and consensus forming of long-read amplicon sequencing [Internet], 2023). Sequence alignment and visualization were performed using MAFFT (Version: v7.394) and MEGA 11, respectively (Tamura et al., 2021; Katoh et al., 2002; MAFFT - a multiple sequence alignment program [Internet], 2023).

Nucleotide sequence analysis of ISKNV MCP gene fragments from various isolates and geographical locations were compared with 59 publicly available Megalocytivirus sequences downloaded from NCBI GenBank and covering 13 different countries around the globe. Multiple sequence alignment was performed with CLUSTALW (Thompson et al., 1994). A phylogenetic tree based on the MCP nucleotide sequences was constructed using the Maximum Likelihood method and Tamura-Nei model (Tamura and Nei, 1993). Evolutionary analyses were conducted in MEGA11 (Tamura et al., 2021). Sequences were submitted to GenBank with Accession numbers OP689616–50.

2.5. Statistical analysis

Statistical analysis on the relationship between fish production and interventions adapted by farmers during the outbreak period as well as the relationship between production and ISKNV detection determine by PCR was carried out. Wilcoxon rank sum test with continuity correction was conducted to test the rank differences in fish production between high and low-detection areas. The 'high' and 'low' detection was arbitrary defined as ISKNV positivity rate $> 30\%$ and positivity rate $< 30\%$, respectively. Effect sizes were labelled following Funder's recommendations (Funder and Ozer, 2019). Also, the Kruskal-Wallis test was conducted to examine the differences in fish production according to the types of interventions farmers implemented. Data were checked for completeness and correctness prior to analysis. Two-sided $p < 0.05$ was applied to determine the statistical significance, with a confidence interval of 95%. All analyses were done using R/studio statistical packages (https://www.R-project.org/,Vienna, Austria).

3. Results

3.1. Disease symptoms, histopathology and molecular detection of ISKNV

The common external abnormalities or disease signs observed in sampled fish included lethargy, cloudy/opaque eyes, exophthalmia, loss of eyes/ skin discoloration, loss of scales, skin erosions, distended abdomen, fin rot and hemorrhages on body parts (Fig. 2). Fish which

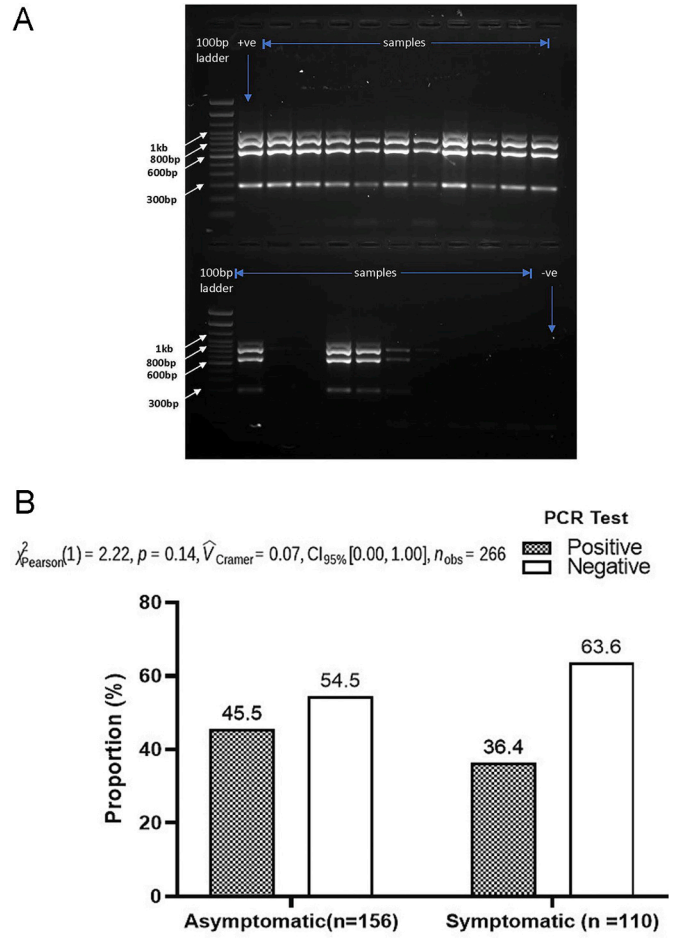

Fig. 4. A. Representative gel image of all samples screened across the Lake Volta for the four putative genes of ISKNV using the multiplex PCR assay. B. ISKNV PCR positivity in symptomatic and asymptomatic tilapia.

tested positive for ISKNV with none of these symptoms above were subsequently referred to as asymptomatic. Of all the fish found infected ($n = 111$), 50.5% ($n = 56$) were asymptomatic. The three most occurring symptom among the ISKNV positive symptomatic fish (49.5%) were distended abdomen due to ascites (43%), bulging eyes (exophthalmia; 33%) and frayed or eroded fins (33%; Fig. 3A). For negative symptomatic fish, skin lesions (29%), skin discolouration (29%) and hemorrhaging (27%) were the most frequent symptoms observed (Fig. 3B). Both symptomatic and asymptomatic fish samples were considered ISKNV positive if they showed bands for all four putative genomic regions representing the TNFR (987 bp), ATPase (834 bp), VEGF (664) and MCP (295) gene fragments (Fig. 4A). Although, the number of ISKNV PCR positives were slightly higher for asymptomatic (45.5%) than symptomatic (36.4%) fish, the difference was not significant (Fig. 4B).

Of the 30 farms screened, ISKNV was detected in 24 farms, indicating 80% prevalence (Fig. 5). Majority of the farms screened were on the Eastern and Western banks within strata 2 of the Volta Lake in the Asuogyaman district, Eastern region. Here, ISKNV was detected in 21 of the 24 farms (87.5%). In the Volta region, 3 out of 7 farms (42.8%) showed positivity. At the time of sampling, most of these farms were closing or demonstrated significantly reduced scales of production. Of the 111 infected fish, ISKNV was predominantly detected in fry and juvenile fish (≤20 g) ($n = 60$, 54%) when compared to adult/grow-out fish (>20 g) ($n = 51$, 46%). In addition, 6.25% (2 out of 32) of eggs screened were positive and only one broodstock out of the 24 sampled was positive for ISKNV. Necrosis as well as typical megalocytic cells observed with ISKNV infected tissue were found in the spleen and kidney of 6 out of 28 samples that tested positive for ISKNV by PCR (Fig. 6). This was not seen in any of the ISKNV negative samples.

3.2. Epidemiological analysis and farm interview responses

Sixty percent of the studied farms ($n = 18$) had been in operation between 1 and 10 years and the remaining 40% ($n = 12$) had been in operation for >10 years. Reported daily pre-outbreak mortality rate ranged from 0.1 to 65% (median = 1%, mean = 6%). Only four farms reported a pre-outbreak mortality above 10%. Twenty-eight farms (93.33%) reported having experienced significant disease outbreak in the past five years with high mortality (22 farms, range 40–95%) and large financial losses (median 70%, range 75–90%). There were also reports of significantly higher mortality in fingerlings than juveniles and grow-outs. Currently, the reported average mortality was ~40%. Approximately 63% of farms were reported to have been outsourcing broodstock and/or fingerlings from a test positive farm on the lake in this study. Fingerlings were purchased from both private and public hatcheries. All the large-scale operators were producing fingerlings in-house to stock their own farms. All farms situated below the dam site tested positive for ISKNV (Fig. 5). Majority of sampled farms (70%, $n = 21$) screened for this study were above the dam site. Only six farms out the 21 farms above the dam site tested negative for the virus. Interestingly, 10 farms reported observable changes in water colour prior to disease events with changes in measured parameters including pH (5.8–10), dissolved oxygen (3–7 mg/L), salinity (~7 ppt) and temperature (23–31 °C).

Farmers adapted several interventions during the outbreak to mitigate the high mortalities. Interventions included vaccination (Iridovirus vaccine), heat-shock treatment, fallowing, in-house fingerling production, reduced stocking density, use of clean nets, sorting out of diseased fish, antibiotics mixed with feed, minimized feeding, starvation, salt treatment and the use of herbs (rosemary, turmeric, garlic, cactus) as immuno-stimulants. Vaccination was conducted on approximately 3 g tilapia only. Heat-shock on the other hand was conducted on much younger fry < 1 g in weight according to the farmers. Vaccination and heat-shock treatment are intended to prime the immune system of the fish to produce helpful immune component that increases the chances of surviving an infection (Dhar and Manna, 2014; Sung, 2014). These were the most reported ISKNV-specific interventions adopted by the farmers. In addition, some few farms attempted to improve the quality of fingerling production by performing in-house crossbreeding of the Akosombo strain (the nationally accredited farmed strain) with wild tilapia species from Lake Volta, as an intervention to the viral disease. Thirteen farms out of the 30 sampled had received the Aquavac iridovirus vaccine once. The vaccine was administered intra-peritoneally to fish weighing 3 g and above after anesthetization with clove oil. Farms that received the commercial vaccine rolled out by the government, reported an improvement in survival rate but still had mortalities rates above 40%. For heat-shock treatment, ISKNV exposed fish were gradually introduced to temperatures 10 °C above the optimal growing temperature (27–30 °C) for 30 mins (Nivelle et al., 2019). This was repeated over a period of 6 days.

Percentage fish production increases from farms using various intervention strategies were also compared. Interventions were categorized into; “heat-shock”, “heat-shock and vaccination”, “neither heat-shock nor vaccination”, and “vaccination” as these were the most frequent interventions used. Generally, farmers practiced other interventions in addition to heat-shock and/or vaccination or practicing other interventions without heat-shock or vaccination, including all the other routine management practices mentioned earlier. As indicated (Fig. 7), significant differences were found among the four categories of interventions; heat-shock, heat-shock and vaccination, neither heat-shock nor vaccination, and vaccination (Chi-square = 36.48, $p < 0.001$, 95% CI: 0.13–1.00). Production seemed highest on farms combining heat-shock treatment and vaccination. Heat-shock treatment

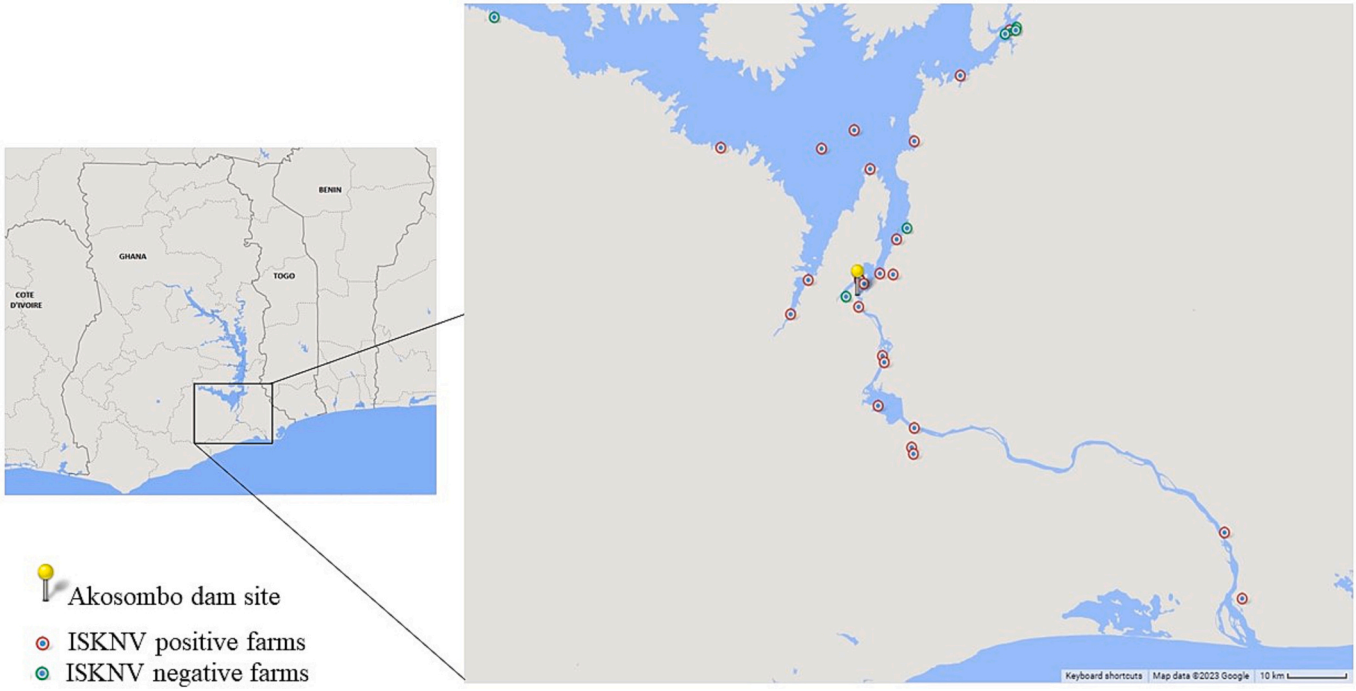

Fig. 5. Map showing the distribution of ISKNV infected farms upstream and downstream of the Akosombo dam. The map was created using the Maptive mapping software (www.maptive.com).

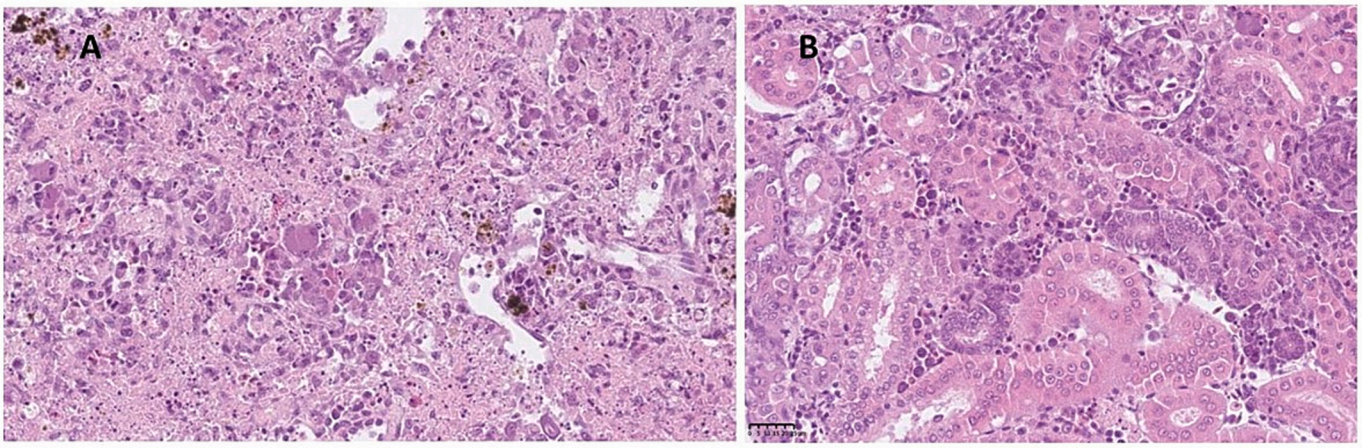

Fig. 6. Histopathology of ISKNV PCR positive tilapia showing necrosis and megalocytes in (A) spleen and (B) kidney. Sections were stained with H&E and Gram-Twort.

without vaccination appeared to be as effective as vaccination, while those implementing other interventions other than heat-shock and/or vaccination demonstrated the lowest average production rates. In order to relate ISKNV positivity to production, the farms were categorized into high (>30% positivity rate), and low (<30% positivity rate) detection areas based on the multiplex PCR test results. Based on Fig. 8, farms implementing only heat-shock as the main intervention recorded the highest ISKNV positivity rates, followed by neither vaccination nor heat-shock group. Those farms within the combined heat-shock and vaccine group as well as the vaccine group only recorded the least ISKNV positivity. Although production, on average, appeared higher in the high than in the low detection areas (Fig. 9), the difference was not statistically significant ($W = 5352$, $p = 0.120$; r (rank biserial) $= -0.21$, 95% CI [−0.27, 0.03]).

3.3. Sequencing and phylogenetic analysis of MCP gene

The PCR assay using the nMCP primer set to amplify the full MCP genomic region yielded expected PCR products (1634 bp) (Fig. S1) from 35 of the confirmed isolates. The optimal tree generated from phylogenetic analysis based on nucleotide sequences is shown in Fig. 10. All positions containing gaps and missing data were eliminated (complete deletion option). There were 1491 positions in the final dataset. The ISKNV reference sequences used for this analysis were spread across 13 countries and from several different hosts (Table S2). Sequence analysis of the amplicons showed that all samples from this study were identical to each other, displaying 100% homology with the outbreak strain OQ513807.1 identified in 2019 from Lake Volta, which has been whole genome sequenced (Alathari et al., 2023). Based on the phylogenetics, all the Ghanaian isolates clustered with ISKNV Clade 1 sequences (Fig. 10). After computation of variants using MegAlign Pro software

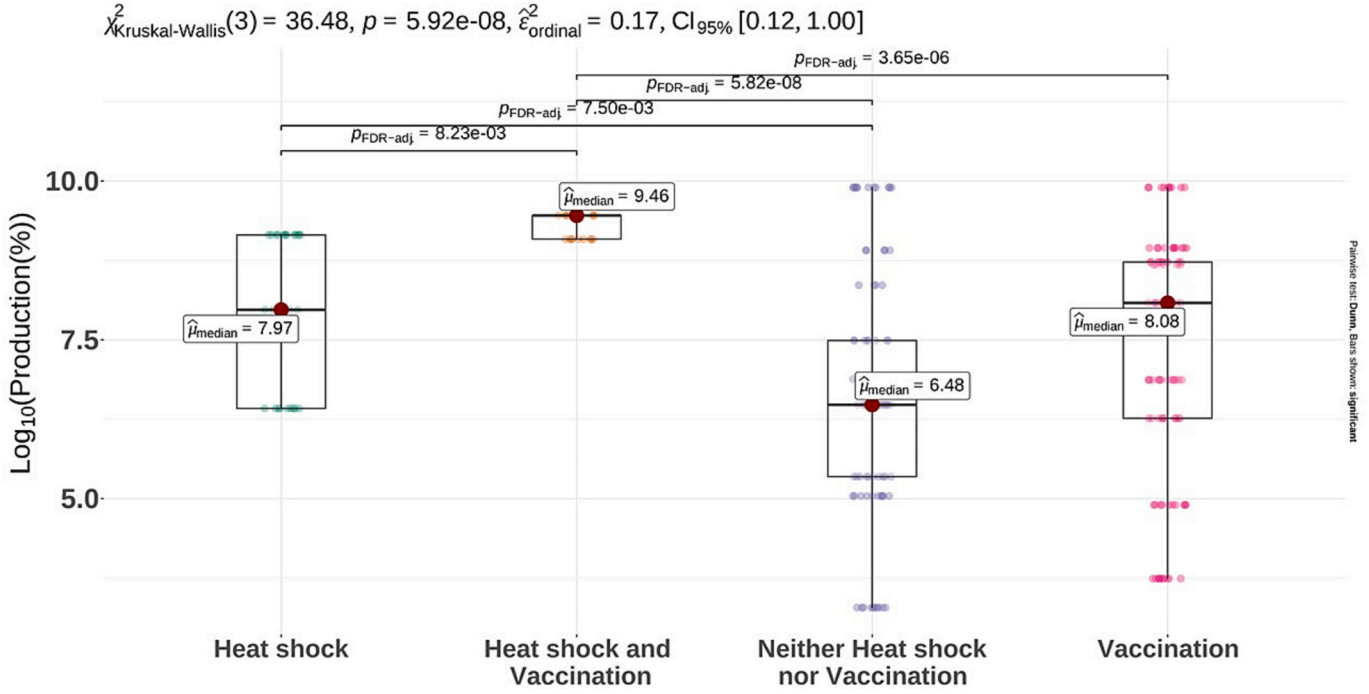

Fig. 7. Percent production over three years (2019–2021) post-ISKNV outbreak as against interventions employed by farmers.

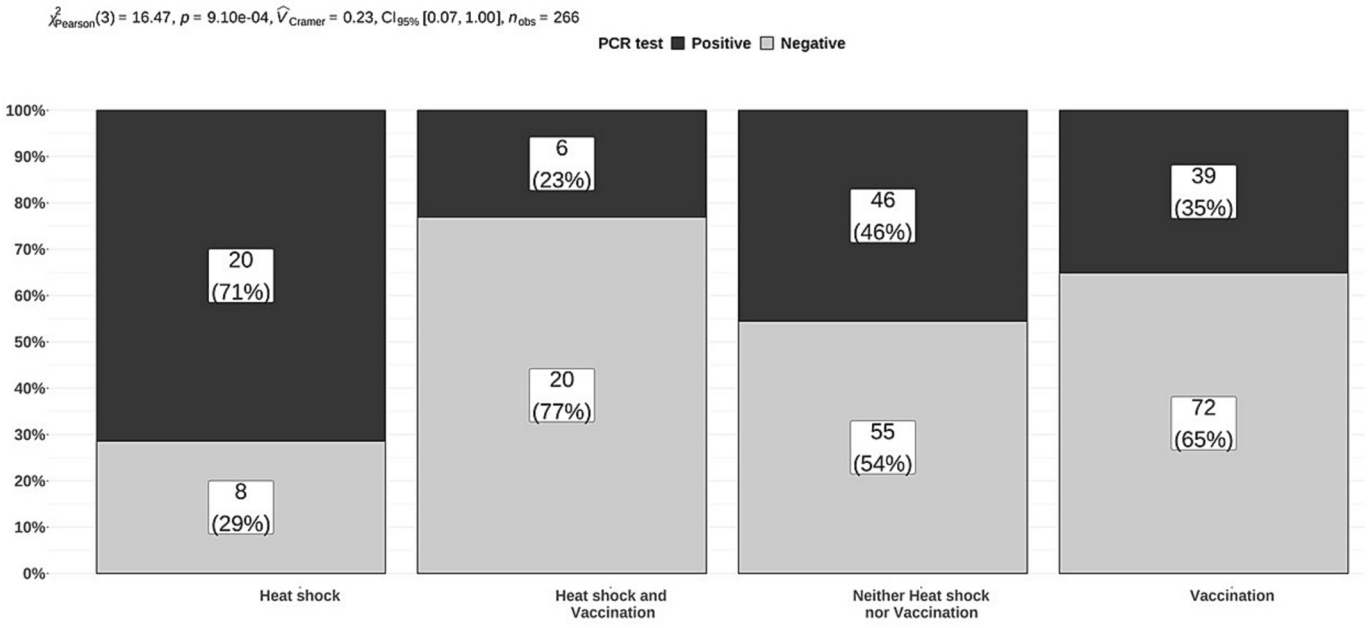

Fig. 8. ISKNV positivity based on various interventions implemented by farmers during the outbreak.

(DNASTAR Lasergene, www.dnastar.com) against the reference sequence AF371960, two Single Nucleotide Polymorphisms (SNPs) were discovered in the MCP genomic region. One SNP (C4328T) was unique for all the Ghanaian sequences including the 2019 Ghana sequence OQ513807, and the divergent sequence on the phylogenetic tree OP689646 from this study. In addition to the unique SNP, a second SNP (T4515G) was discovered in only the divergent strain OP689646 (Fig. 10).

4. Discussion

Megalocytiviruses are well known for their broad host range including both ornamental and food fish, and their ability of more than one genotype infecting the same host species (Fu et al., 2011; Wang et al., 2007). The MCP gene is the standard target for analysis of phylogeny among iridoviruses, due to its relatively conservative nucleotide sequences (Fu et al., 2011; Song et al., 2008). Megalocytivirus show high nucleotide and amino acid sequence identities, and ISKNV have been found to have comparatively lower genetic variation and much more unique biological features (Wang and Wen, 2018). Molecular screening conducted in this study to detect specifically the virus in tilapia, amplified four putative genes in a multiplex PCR assay. The full MCP genomic region was amplified to further confirm the viral pathogen and for the test of phylogeny. The sequence analysis revealed that all isolates

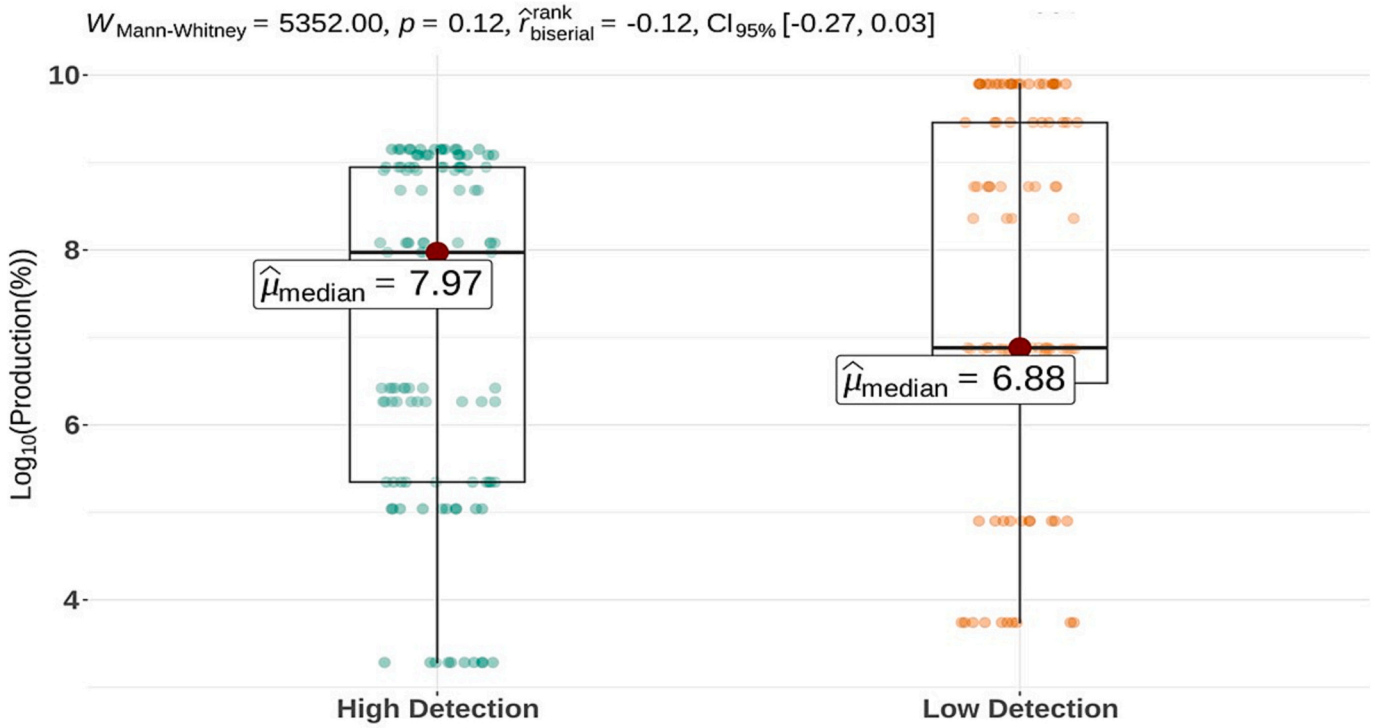

Fig. 9. Percent production over three years (2019–2021) post-ISKNV outbreak as against high (>30% positivity rate) and low (<30% positivity rate) detection areas based on the multiplex PCR test.

based on the MCP clustered with ISKNV clade 1. Expectedly, the sequence identity of the isolates suggests that these isolates may be of the same strain and that no significant variation of sequences had occurred over the three years' period since the virus was first detected in the country (Fig. 10). All the sequences except for the slightly divergent sequence (OP689646.1) exhibited 100% homology with the fully sequenced ISKNV genome isolated from Ghana in 2019 and 99.94% homology with the reference strain AF371960 (Alathari et al., 2023). The presence of the unique SNP (C4328T) in a highly conserved region such as the MCP over the past four years is suggestive of a common ancestry. The new mutation in 2021 (T4515G) and that discovered by Alathari et al. in 2022 (T3934C) based on the MCP is possible evidence of a gradual divergence from the 2018 outbreak strain (Alathari et al., 2023). Because the virus is a DNA virus and the MCP is highly conserved, these mutations may be occurring at a very slow rate. It is likely that the circulating iridovirus causing high mortalities across the farms on Lake Volta descended from the original strain or from new introductions as recently reported by Alathari et al. (Alathari et al., 2023). Given the scale of coverage of this study, we can tentatively say that Ghana is no longer experiencing an ISKNV outbreak but rather an epidemic in farmed tilapia. This is a useful information that can inform vaccine design and deployment strategies.

Our data shows that ISKNV was highly prevalent among all farm types and across operational scales, with an apparent overall farm-level prevalence of 80% (24 out of 30 farms). The high prevalence and wide geographical distribution of the virus within the study area suggests significant horizontal transmission between farms. Common horizontal transmission routes in aquaculture includes live infected fish movements, fomite contact between farms and local spread through water contact (He et al., 2002). One of the farms with significant high positivity in their samples (54.5%) also supplies broodstock and/or fingerlings to 17 other farms. Although all farms below the dam site are at a great risk due to their geographical location and were all evidently ISKNV positive, the transfer of infected fingerlings poses the greatest threat to the spread of the virus across the Lake in both upstream and downstream of the dam. Several farms reported having biosecurity measures in place. However, effective biosecurity measures were found to be largely absent during farms visits, with only 3 farms having biosecurity physical barriers (vehicle and footwear dips, signpost) mounted at the farm gate and other strategic entry points. This may increase the likelihood of inadvertent horizontal transmission between farms. Also, about one half (50.5%) of the sampled fish that tested positive for ISKNV, showed no external symptoms (Fig. 4B), making the transfer of supposedly healthy fish to other farms more likely. While there have not been any publications describing true vertical transmission of ISKNV, it was detected in broodstock (4.17%) and eggs (6.25%), suggesting possible rare occurrence of vertical transmission in the studied population (Joon et al., 2008). With a limited number of operational hatcheries in Ghana, and many farmers still depending on a few hatcheries to source their fingerlings, enforcing biosecurity measures and testing at hatcheries will be critical to prevent potential spread of the pathogen through large scale movement of fingerlings from infected broodstocks.

Despite the high detection by PCR, pathological signs of ISKNV infection were only found in 6 out of the 28 ISKNV positive samples. Histopathological analysis usually reveals inclusion body-bearing cells and numerous enlarged cells in hematopoietic tissues (Kurita and Nakajima, 2012; Kawato et al., 2021). Also, visible vacuolization and lymphocytic infiltration can be observed in some tissue. These pathological changes are not always observed even in infections with high viral load during mass mortality event (Rimmer et al., 2017; Dong et al., 2017), emphasizing the usefulness of more sensitive and specific diagnostic tools for ISKNV detection.

Most ISKNV-negative farms were distant from neighboring farms or located further offshore, which may indicate that these farms are exposed to a lower disease pressure or may easier implement effective biosecurity measures to prevent the introduction of the virus. Farm-level routine disease prevention practices including disinfection (use of salt or other disinfectant), access restriction, staff biosecurity training, heat treatment, reduced feeding, herbal remedies and the use of antibiotics, removal of dead and diseased fish, net cleaning, not sharing equipment

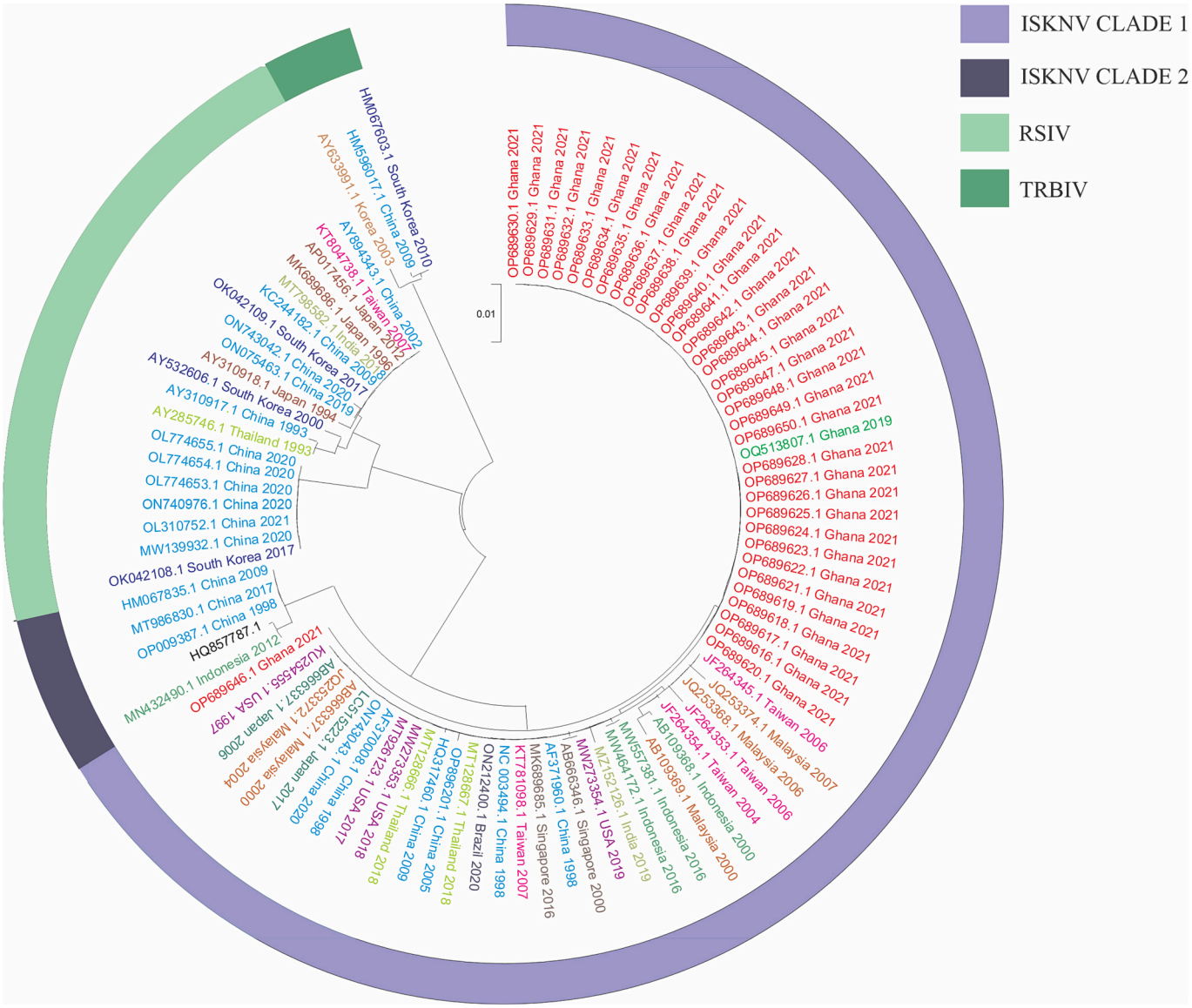

Fig. 10. Phylogenetic tree of 35 sequenced samples based on the MCP genomic region of ISKNV using the Maximum Likelihood method and Tamura-Nei model. This is the tree with the highest log likelihood (−3126.03) after 500 bootstrap replicates. The analysis included 59 reference sequences from GenBank. These sequences were spread across 13 countries and from several different hosts (Table S2). The country of origin and genotypes of the sequences have been colour coded.

and some other biosecurity measures were reported. During the mass vaccination in 2019, 13 farms received the commercial iridovirus vaccine (MOFAD, 2021). Twelve of these farms were found to be still positive for ISKNV with high mortality (40–90%), possibly indicating an ineffective response to the vaccine. Indeed, most farmers could only partially testify to the vaccine efficacy as mortality rates had reduced slightly but remained comparatively high at the time of sampling. Unfortunately, the iridovirus vaccines were rolled out as an emergency response to the outbreak and no efficacy study was done before, during or after its administration. Also, the vaccination was carried out on fry (3 g), and this was not repeated for successive stocks. Therefore, protection against the virus may have not been sustainable on these farms since farmers who did not have in-house hatcheries were still outsourcing from infected farms. Moreover, the possibility of vertical transmission threatens the vaccination programme as fingerlings may already be infected before vaccination.

The production data revealed no differences between farms implementing heat-shock and those that had received the vaccine. From the farm interviews, the heat-shock treatment procedure being practiced by these farmers involved intentionally infecting fish with the virus prepared from fish homogenate prior to the heat-shock. Aquatic organisms in general have been found to synthesize a class of proteins know as heat-shock proteins (Hsps) when subjected to physiological stressors such as temperature extremes, pollutants, parasitism, anoxia and many others (Tine et al., 2010; Eissa et al., 2017). These proteins are implicated in the unspecific protection of stressed cells by mitigating cell damage to enhance tolerance of aquatic organisms to disease (Sung, 2014; Tine et al., 2010). The role of these Hsps is becoming clearer with continuous research at the molecular and cellular levels. It is generally speculated that heat-shock offers better immunity to the fish to withstand the virus, without necessarily clearing the pathogen. This might explain why the relatively high production farms practicing heat-shock still maintained high ISKNV positivity rate. While vaccination is required to provide protection for fish that have not yet been exposed to the virus, heat-shock is recommended by farmers for already infected fish to slowdown or reduce disease symptoms and progression. Although some farmers considered heat-shock treatment an effective pathogen control strategy, a well-structured system for hyperthermia treatment

and an understanding of the mechanism of action is required to evaluate its beneficial role in disease management properly.

5. Conclusion

The findings from this study suggest that ISKNV introduction to Ghanaian tilapia aquaculture was likely through a single source and has reached epidemic status, possibly through the transfer of juvenile fish between farms on the Volta Lake. Although, this study limited analysis of phylogeny to the MCP gene, comparable data was generated using whole ISKNV genomes. Novel measures such as heat-shock, especially when combined with vaccination appeared to have some success against mortality, indicating the possibility of managing the ISKNV disease situation with the appropriate control strategies. Although, the heat-shock treatment may allow the farmers to continue for a short term, this practice cannot completely eliminate the pathogen and the infection will continue to persist, thus preventing the establishment of disease-free farms. It would be prudent to also focus vaccination on the few hatcheries available in the country, to ensure that juvenile fish have a better chance at survival once introduced into the lake. Likewise, vaccine efficacy studies must be carried out to ensure that the vaccines, are yielding the expected response and meet some basic regulatory requirements. The fact that a larger proportion of asymptomatic fish tested positive, and symptoms observed were non-specific for ISKVN makes laboratory diagnostics essential. Introduction and expansion of disease diagnostic laboratories with the prerequisite expertise coupled with regular monitoring of production facilities, keeping of proper production data, reporting of unusual mortality, strict enforcement and adherence to biosecurity measures can provide early warnings and protect the industry from surprise mass mortalities.

CRediT authorship contribution statement

Angela N.A. Ayiku: Methodology, Investigation, Data curation, Writing – original draft, Writing – review & editing. **Abigail A. Adelani:** Methodology, Investigation, Data curation. **Patrick Appenteng:** Methodology, Data curation. **Mary Nkansa:** Methodology. **Joyce M. Ngoi:** Methodology. **Collins M. Morang'a:** Methodology, Data curation. **Francis Dzabeng:** Data curation, Formal analysis. **Richard Paley:** Conceptualization, Supervision, Funding acquisition, Resources. **Kofitsyo S. Cudjoe:** Methodology, Funding acquisition, Resources. **David Verner-Jeffreys:** Conceptualization, Supervision, Funding acquisition, Resources. **Peter K. Quashie:** Methodology, Supervision, Funding acquisition, Resources, Writing - review & editing. **Samuel Duodu:** Conceptualization, Methodology, Investigation, Project administration, Validation, Funding acquisition, Resources, Supervision, Writing – review & editing.

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Declaration of Competing Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Data availability

The hotlinks for all the sequenced isolates uploaded to NCBI will be made available before publishing

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2023.740330.

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