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A Microwave-Irradiated *Streptococcus agalactiae* Vaccine Provides Partial Protection against Experimental Challenge in Nile Tilapia, *Oreochromis niloticus*

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Abstract

Microwave irradiation, as opposed to formalin exposure, has not routinely been used in the preparation of killed vaccines despite the advantages of decreased chemical toxicity, ability to kill cells quickly, ease of completion requiring only a standard microwave, and potential increased protein conservation during irradiation. We evaluated the potential of microwave irradiation versus formalin fixation of bacteria to improve *Streptococcus agalactiae* vaccine efficacy in 5 gr fish by intraperitoneal (IP) injection and bath immersion (BI). There was no significant difference in the cumulative percent mortality (CPM) post-challenge between fish administered microwave-killed cells (MKC) or formalin killed cells (FKC) within the BI ($p < 0.2026$) or IP ($p < 0.1372$) trials. The CPM in fish sham-vaccinated with tryptic soy broth (TSB) was significantly higher than both the FKC and MKC CPM in the IP trial and the FKC CPM ($p < 0.0019$) in the BI trial. Serum obtained from fish prior to vaccination exhibited minimal anti-*S. agalactiae* antibody activity. Thirty days after vaccination and just prior to challenge, the optical density (OD) levels of the FKC and MKC groups in the IP trials were significantly higher ($p < 0.0001$) than that of the TSB group. None of the groups in the BI trial exhibited significantly different OD levels post vaccination. Fourteen days after the challenge, the OD levels of all groups in both trials increased significantly above their pre-challenge levels. Both the FKC and MKC IP groups ($p < 0.0001$) and only the FKC BI group ($p < 0.0351$) had significantly increased OD level above that of the corresponding post-challenge TSB

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group. These results indicate that the FKC vaccine provides marginally greater protection and increased antibody concentrations than the MKC vaccine by BI and the MKC vaccine may become a non-chemical alternative to FKC in vaccination.

Keywords

Vaccine, *Streptococcus agalactiae*, Microwave

1. Introduction

The group B streptococcal fish pathogen, *Streptococcus agalactiae*, affects numerous fish species and has caused significant mortalities [1]. Affected species include economically and environmentally important fish species, including menhaden, *Brevoortia* sp. [2], striped bass, *Morone saxatilis* [3], tilapia, *Oreochromis* sp. [4] [5], and mullet, *Liza klunzingeri* [5]. Nile tilapia (*O. niloticus*) is highly susceptible to *S. agalactiae* infection and has experienced disease outbreaks worldwide [5]-[8], and an effective vaccine could help decrease related fish losses.

Some authors have developed *S. agalactiae* vaccines for tilapia with promising results. Eldar *et al.* [4] developed killed and modified-killed vaccines against *S. difficile*, later characterized as *S. agalactiae* [9] [10]. Large 150 to 180 g hybrid tilapia (*O. aureus* × *O. niloticus*) was given an intraperitoneal (IP) injection and boostered with whole cell or acellular preparations, resulting in relative percent survival (RPS) values of 100 and 92, respectively, after challenge. Evans *et al.* [11] assessed a vaccine composed of concentrated extracellular products (ECPs) and formalin-killed *S. agalactiae* whole cells created from a Kuwait isolate. The 30 g Nile tilapia (*O. niloticus*) IP injected with this vaccine was significantly protected against *S. agalactiae* challenge, resulting in RPS values of 70 and 80. However, small 5 g tilapia vaccinated IP and by bath immersion (BI) had RPS values of 25 and 34, respectively. Pretto-Giordano *et al.* [12] prepared a formalin-killed vaccine using a Brazil *S. agalactiae* isolate. Nile tilapia weighing 20 g was IP injected with the vaccine, and the authors determined an RPS of 83.6 after challenge. Despite these significant advancements, there is limited information on the successful IP and BI *S. agalactiae* vaccination of fry and fingerling fish. According to the Evans *et al.* [11], because vaccine exhibited the significant efficacy in larger fish after IP injection but showed limited efficacy after BI vaccination, it is possible that this type of vaccine could be successful if the preparation is modified.

Microwave irradiation is well-known to have pathogen killing ability, and a few studies have used microwave irradiation in the preparation of vaccines [13] [14]. Formalin exposure is routinely used to prepare killed vaccines. Microwave fixation of cell cultures introduces ease and speed of handling in addition to a reduction of costs associated with formalin purchase, handling equipment (preparation in a fume hood), disposal, and potential harmful exposure effects to humans. Patterson and Bulard [15] and Husson-van Vliet [16] outlined the advantages of fixing or killing cells using microwave irradiation versus formalin: decreased toxicity because no chemicals were required, ability to fix or kill cells quickly, ease of completion because only a standard microwave was required, and increased protein conservation during irradiation versus significant cell and protein loss with formalin fixation. The last advantage could potentially make microwave-irradiated bacteria generally more efficacious as a vaccine component than the more commonly-used formalin-killed bacteria, and this study was performed to examine this potential. Furthermore, this study focused on whether microwave vaccine preparation could help improve immersion vaccination efficacy.

2. Materials and Methods

Nile tilapia *O. niloticus*, with a mean weight of 5.6 ± 1.5 g were housed at the USDA/ARS Aquatic Animal Health Laboratory in Chestertown, Maryland. The fish were kept in 19 L glass aquaria supplied with flow through dechlorinated tap water and were maintained on a 12 h:12 h light:dark period. The fish were fed daily to satiation with Aquamax Grower (Brentwood, Missouri, USA[1]). Daily water temperature averaged 30.32 ± 0.85˚C, mean daily dissolved oxygen was 5.62 ± 0.81 mg/L, mean pH was 7.02 ± 0.18, and mean total ammonia concentration was 0.42 ± 0.37 mg/L. Five fish were sampled before the study using the microbiologic tech-

[1]Mention of commercial products in this publication does not imply recommendation by the U.S. Department of Agriculture.

niques described here to determine *S. agalactiae*-free status.

The vaccines were prepared using similar methods as previously described [11]. The polysaccharide-encapsulated *S. agalactiae* (01-KU-MU-3B; [5] [11]) was grown in tryptic soy broth (TSB, Difco Laboratories, Sparks, Maryland, USA) at 27˚C for 72 - 125 h. For the formalin-killed vaccine preparation (FKC), the resulting cultures were treated with 3% neutral buffered formalin for 24 h and then centrifuged to separate the cell pellet and culture fluid. The vaccine ECP fraction of the vaccine was prepared by concentrating the cell-free culture fluid containing ECP on a 3 kDa Amicon column (S3Y3) using a Millipore Proflux M12 (Millipore, Billerica, Massachusetts, USA), and sterilized using a 0.22 µm 1 l microbiological filter (Corning, Corning, New York, USA). For the microwave-killed vaccine preparation, cells were centrifuged, the pellet resuspended in TSB, and the cells microwave irradiated on a rotating microwave plate for 2 min on high (Kenmore Microwave Oven 565.60382; 120 V, 60 Hz, 1100 W; Sears, Hoffman Estates, Illinois, USA). The formalin-killed and microwave-killed cells were then diluted to the desired concentration in TSB, and mixed with the FKC ECP fraction for a concentration of 1×10^9 cells/mL for vaccination. A 50 µL sample was obtained, plated on 5% de-fibrinated sheep blood agar (SBA; Remel, Lexena, Kansas, USA), incubated at 30˚C for 24 h, and analyzed for growth to determine that the cells were not viable.

Triplicate groups of 20 fish (**Table 1**) each were injected intraperitoneally (IP) with 0.1 mL of the formalin-killed cell *S. agalactiae* vaccine (FKC) or microwave-killed cell *S. agalactiae* vaccine (MKC). An additional triplicate groups of 20 fish each were injected IP with 0.1 mL sterile TSB to serve as a control group (TSB). Triplicate groups of 20 fish each were vaccinated by a 5 min bath immersion in diluted (1:250) formalin-killed cell *S. agalactiae* vaccine (FKC) or microwave-killed cell *S. agalactiae* vaccine (MKC). Control fish were immersed in TSB using the same procedure. After vaccination or sham-vaccination, all fish were placed in groups of vaccinated or control fish in separate aquaria and maintained as previously described.

Fish from the vaccine and TSB control groups were challenged IP with 1.2×10^8 colony-forming units (cfu) *S. agalactiae*/fish (**Table 1**). During each challenge period, fish were monitored daily for clinical signs of disease and mortality. Moribund and dead fish were removed twice daily, and bacterial samples were aseptically obtained from the nares, brain, head kidney, and intestine of 10% of moribund and dead fish to confirm the presence of *S. agalactiae*. Samples were cultured at 30˚C for 24 h on SBA, and isolate identity was confirmed as *S. agalactiae* using the BIOLOG MicroLog Microbial Identification System (BIOLOG, Hayward, California, USA) according to the manufacturer instructions. Positive cultures were beta-haemolytic, oxidase-negative, catalase-negative, and Gram-positive cocci [5].

Tilapia inoculated with *S. agalactiae* vaccine or TSB were bled from the caudal vein prior to vaccination, 30 d post vaccination and 14 d post challenge (**Table 2**) and the sampled blood was held at 4˚C for 1 h. Serum was separated with centrifugation at 400 × g for 6 min and then stored at −70˚C until use. All serum was tested for anti-*S. agalactiae* antibodies by indirect ELISA based on the methods of Pasnik *et al*. [17]. Plates were washed

Table 1. Vaccination group, vaccination route, dilution or dose of KU-MU-3B *S. agalactiae* vaccine, number of mortalities, cumulative percent mortalities (CPM), and relative percent survival (RPS)[1].

Vaccination group	Vaccination route	Vaccination dilution or dose[2]	Number of mortalities	CPM	RPS	*p* value[3]
TSB	BI	1:250 5 min	42/60	70	-	-
FKC	BI	1:250 5 min	25/60	42	40	0.0019
MKC	BI	1:250 5 min	32/60	53	24	0.0615
TSB	IP	4×10^9	41/60	68	-	-
FKC	IP	4×10^9	11/60	18	74	0.0001
MKC	IP	4×10^9	18/60	30	56	0.0001

[1]Nile tilapia (*O. niloticus*) were vaccinated with TSB, formalin-killed *S. agalactiae* vaccine (FKC), or microwave-killed *S. agalactiae* vaccine (MKC) by immersion (BI) or intraperitoneal (IP) injection. Fish were challenged with 1.2×10^8 cfu/fish *S. agalactiae* 30 d post-vaccination, and the survival of the challenged fish was monitored for 14 d post-challenge. [2]For IP vaccination, doses are represented by colony-forming units (cfu)/mL. [3]*p* value when comparing experimental group to corresponding vaccination route control group with the proclifetest procedure. Significant differences were accepted at $p < 0.05$.

Table 2. Specific anti-*Streptococcus agalactiae* antibody concentrations (ELISA optical density [OD]) in Nile tilapia with tryptic soy broth (TSB), formalin-killed cell *S. agalactiae* vaccine (FKC), or microwave-killed cell *S. agalactiae* vaccine (MKC)[1].

Vaccination group	Vaccination route	Pre-vaccination OD (mean ± S.E.)	Pre-challenge OD (mean ± S.E.)	Post-challenge OD (mean ± S.E.)
TSB	BI	0.011 ± 0.005^{a}	0.019 ± 0.004^{a}	0.056 ± 0.018^{a*}
FKC	BI	0.017 ± 0.010^{a}	0.022 ± 0.009^{a}	0.091 ± 0.042^{b*}
MKC	BI	0.012 ± 0.004^{a}	0.017 ± 0.011^{a}	0.061 ± 0.024^{a*}
TSB	IP	0.011 ± 0.011^{a}	0.018 ± 0.006^{a}	0.047 ± 0.025^{a*}
FKC	IP	0.014 ± 0.006^{a}	0.128 ± 0.024^{b*}	0.192 ± 0.044^{b*}
MKC	IP	0.019 ± 0.004^{a}	0.112 ± 0.032^{b*}	0.158 ± 0.062^{b*}

[1]Nile tilapia (*O. niloticus*) were vaccinated with TSB, formalin-killed *S. agalactiae* vaccine (FKC), or microwave-killed *S. agalactiae* vaccine (MKC) by bath immersion (BI) or intraperitoneal (IP) injection. Fish were challenged with *S. agalactiae* 30 d post-vaccination, and survival of the challenged fish was monitored for 14 d post-challenge. Significant differences between immersion or injection groups within pre-vaccination, pre-challenge (30 d post vaccination), or post-challenge samples are noted by different alphanumeric letters (column), and significant differences between the pre-vaccination and the corresponding pre-challenge and post-challenge samples are noted by an asterisk (row).

between steps as previously described. The test used sonicated whole *S. agalactiae* cells (500 µg protein/mL), serum samples diluted 1:100 in PBS-T, mouse anti-tilapia IgM heavy chain specific monoclonal antibody, and peroxidase-conjugated goat anti-mouse IgG (Pierce Biotechnology, Rockford, Illinois, USA), and 100 µL of One-Step Ultra TMB-ELISA (Pierce). The final reaction was stopped with H_2SO_4, and the ELISA optical density (OD) was read with a Bio-Tek Automated Microplate Reader (Bio-Tek Instruments, Winooski, Vermont, USA).

All statistical analyses were performed using the SAS program (SAS Institute, Cary, North Carolina, USA). Mortality data were compared by the proclifetest with Wilcoxon comparison. ELISA results (mean ± S.E.) were compared by one-way analysis of variance (ANOVA) and Duncan's multiple range test. Significant differences between groups at the same time points and within groups at different time points were accepted at $p < 0.05$.

3. Results

No clinical signs or mortalities were noted among immunized fish before they were challenged. After challenge, fish from each group exhibited clinical signs, including lethargy, confinement to the bottom of the tank, and slow or no feeding response. Mortalities occurred between 2 and 13 d post-challenge in all the groups, and the mean cumulative percent mortality (CPM) (**Table 1**) was 70 (TSB), 42 (FKC), and 53 (MKC) in the BI trial. The BI trial TSB CPM was significantly higher than the FKC group, but the TSB mortality level was not significantly different than the BI trial MKC ($p < 0.0615$). In addition, no significant differences were noted between the FKC and MKC group CPM levels within the BI ($p < 0.2026$) or IP ($p < 0.1372$) trials. A significant difference in CPM was noted between the TSB and the corresponding experimental groups within the IP trials. All sampled organs from dead fish were culture-positive for *S. agalactiae*. BIOLOG identification confirmed that bacteria isolated from all sampled mortalities was *S. agalactiae* (probability = 99%; similarity = 0.84).

Serum obtained from fish prior to vaccination exhibited minimal anti-*S. agalactiae* antibody activity (**Table 2**). Thirty days after vaccination and just prior to challenge, the OD levels of the FKC and MKC groups in the IP trials were significantly higher ($p < 0.0001$) than that of the TSB group. At this time point, none of the groups in the BI trial exhibited significantly different OD levels. Fourteen days after the challenge, the OD levels of all groups in both trials increased significantly above their pre-challenge levels. Both the FKC and MKC IP groups ($p < 0.0001$) and only the FKC BI group ($p < 0.0351$) had significantly increased OD level above that of the corresponding post-challenge TSB group.

4. Discussion

Microwave irradiation has been used to create anti-parasite vaccines, significantly protecting rats against *Strongyloides ratti* [13] and mice against *Trichinella spiralis* [18]. Craciun *et al*. [14] noted increased specific anti-

body titers in bovines inoculated with a microwave-irradiated *Fusobacterium necrophorum* whole cell culture. In this study, an *S. agalactiae* vaccine containing microwave-killed *S. agalactiae* cells (MKC) elicited production of anti-*S. agalactiae* antibodies and exhibited protective effects when IP injected into 5 g fish. The level of antibody production and challenge protection was not significantly different than that conferred by the FKC *S. agalactiae* vaccine, though the RPS for the IP injected MKC was still lower (56) than that of the IP injected FKC (74). Both of these RPS levels were considerably higher than the RPS (25) exhibited in the 5 g IP group from Evans *et al*. [11]. RPS levels for the BI MKC was lower (24) than the RPS (34) exhibited in the 5 g BI group from Evans *et al*. (2004), however the RPS of BI FKC (40) in this study was only slightly higher (35) of than that of the previous study. This may be a result of different study of water temperatures, challenge dose, or dilutions making direct comparison difficult. When studying immersion vaccination, the FKC vaccine stimulated specific antibodies and conferred protection comparable to that of FKC immersion-vaccinated 5 g fish in Evans *et al*. [11]. The concentrated ECP in the FKC vaccine preparation may have imparted specific immunity. Pasnik *et al*. [17] demonstrated a correlation between protection and antibody production to *S. agalactiae* ECP and the importance of the 55 kDa ECP antigen for vaccine efficacy. An ECP vaccine alone, administered intramuscularly was shown to protect hybrid striped bass against *S. iniae* infection 12 weeks post immunization [1]. It is likely that ECP is the key component of vaccines against *Streptococcus* sp. by the stimulation of acquired immune responses.

Overall, the MKC IP vaccine successfully exhibited specific anti-*S. agalactiae* antibody stimulation when assayed with whole cell antigens and showed that the immunogenic effects were protective against challenge. Fixation has long been recognized to create differences in both the chemical and antigenic properties of proteins through a conformational change in the tertiary structure of the proteins. This has a profound significance when considering antibody-antigen interactions particularly when cross-linking fixatives such as formalin are used. Microwave fixation coagulates proteins to preserve antibody-antigen interactions and retrieval. Nasri *et al*. [19] demonstrated a significant increase in the amount of outer membrane proteins (OMP) following microwave irradiation. They attributed this increase to an overexpression of proteins as an adaptive mechanism or release of OMP in the extracellular medium due to a weakening of membrane structure. Since the MKC ECP was not concentrated in this study, an important consideration in future research should be concentration of the MKC ECP and an evaluation of the antigenic properties of the ECP from MKC. The MKC vaccine may be an alternative to FKC in mass vaccination strategies. Although microwave killing of the *S. agalactiae* cells did not generally improve vaccine efficacy in 5 g Nile tilapia and did not improve *S. agalactiae* BI vaccination under the conditions tested in this study, given the practical, time and energy savings and less labor intensive use of microwave irradiation as compared to formalin fixation in the preparation of killed vaccines, further research is needed to assess the antigenic components and efficacy of a MKC vaccine using a mass immunization technique, bath immersion, in older immune-competent fish.

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