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Role of water chemistry and stabilizers on the Vero-cells-based infectivity of Newcastle disease virus live vaccine

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Primary Audience: Researchers, Flock Supervisors, Poultry Farm Managers, Poultry Veterinarians.

SUMMARY

Newcastle disease virus (NDV) live vaccines are supplied in lyophilized form and usually administered through conventional routes (drinking water, spray, or eye drop) following reconstitution in a diluent. Virus inactivation due to physico-chemical properties of the diluent at the time of administration may lead to vaccine failure. The present study aimed to evaluate the survival of NDV live vaccine strain immersed in 5 pH-amended water samples (pH 5.00, pH 6.00, pH 7.00, pH 8.00, and pH 9.00) by sequential determination of virus infectivity on Vero cells for 3 hours. Minimum reduction in virus infectivity was recorded in the water with neutral or slightly alkaline pH, while the virus was relatively less stable at extreme pH conditions. Maximum reduction of infectivity was observed in the water with pH 9.00 in which the virus was completely inactivated within 3 hours. Addition of stabilizers (Cevamune® or skimmed milk) slightly altered the pH and total dissolved solids (TDS) values of the virus-charged water samples. In the stabilizer-added water samples, minimum reduction in infectivity was observed in the water with neutral pH, followed by the ones with a pH of 8.00, 6.00, 5.00, and 9.00. In all types of water samples, T-90 values (time required for 90% reduction in virus infectivity) were highest (485 minutes) at neutral pH (pH 7.00) and lowest (102 to 134 min) at an extreme alkaline condition (pH 9.00). Results of the present study indicate that water with a pH range of 7.00 to 8.00 is suitable for administration of NDV live vaccines. However, the addition of Cevamune® or skimmed milk may have beneficial effects on preserving the infectivity of the virus, even at extreme pH conditions.

Key words: infectivity, NDV, pH, stabilizers, water

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DESCRIPTION OF PROBLEM

Newcastle disease (**ND**) is an important viral disease of poultry worldwide and causes

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enormous economic losses to the poultry industry. ND is generally endemic in Pakistan, but most severe outbreaks with heavy mortality (90 to 100%) in broilers and broiler breeders have occurred in recent years [1]. ND virus (**NDV**) is an enveloped virus containing a single-stranded genomic RNA of negative polarity and is supposed to be highly sensitive to physical (extreme pH, temperature, and radiations) and chemical (disinfectants) insults [2–4]. Lentogenic NDV strains usually cause subclinical infection with mild respiratory or enteric symptoms, which are common reactions to live virus vaccines [5]. In ND endemic regions, vaccination strategy involves the use of 2 or 3 doses of NDV live vaccines through conventional route. During severe ND outbreaks, killed vaccines are commonly used in Pakistan as an emergency vaccination against field virus challenges [1].

Preservation of virus infectivity in live virus vaccine is extremely important for such vaccines to be effective. In addition to several other factors, efficacy of live virus vaccines can be negatively affected through improper handling [6]. Live virus vaccines are usually supplied in freeze-dried form and need to be reconstituted in water or other appropriate diluent before administration. Chemical properties of the water such as pH, hardness, and salinity may have a significant impact on virus infectivity. Previous studies have demonstrated that extreme pH, elevated temperature, and high salinity of the water negatively affect virus infectivity [7]. Drinking water quality at poultry farms is usually improved by using water sanitizers such as chlorine, detergents, and organic acids. These agents, if not properly neutralized, may negatively affect the infectivity of the virus and lead to vaccine failure.

It has been observed that despite routine vaccination at farms, severe ND outbreaks still occur in Pakistan. Mutation in the genetic makeup of the virus has been suggested as a reason for such vaccine outbreaks [8], but virus inactivation in NDV live vaccines due to water chemistry also may contribute to such conditions. The present study has therefore been designed to evaluate the effect of water pH on the infectivity of NDV vaccine strain “LaSota” and the role of a commercially available stabilizer and skimmed milk in preserving the infectivity of the virus.

MATERIALS AND METHODS

Virus and Fertile Chicken Eggs

NDV LaSota strain [9] was inoculated into 9-day-old embryonated chicken eggs [10] via the allantoic cavity for virus propagation [11]. The inoculated eggs were incubated at 37 °C, and allantoic fluid (**AF**) containing the virus was harvested at 72 h post inoculation. Virus propagation in each egg was confirmed by the spot hemagglutination (**HA**) test of the harvested AF. All of the AF showing positive results were pooled and stored at -80 °C.

Vero Cells and Virus Titrations

Vero cells [12] were propagated using growth medium: Dulbecco modified eagle medium plus F-12 (**DMEM+F-12**) with L-glutamine [13] supplemented with 5% fetal bovine serum (**FBS**) [14] as a confluent monolayer in T-75 cell culture flasks by incubating at 37 °C and 5% CO_2 cell culture incubator. Virus infectivity as tissue culture infective dose 50% (**TCID50**) was measured as described earlier [15]. Briefly, Vero cells were sub-cultured to a final concentration of $10^{6.00/}$mL and dispensed in a volume of 100 µl per well of the 96 well cell culture plate. The plates were incubated at 37 °C and 5% CO_2 in a cell culture incubator for 24 h to attain 90% confluency. The virus samples were diluted 10-fold in the maintenance medium: DMEM+F-12 supplemented with 1% FBS containing penicillin (100 IU/mL), streptomycin (100 µ g/mL), gentamicin (50 µ g/mL), and amphotericin B (2.5 µ g/mL) all from the same manufacturer [13]. Growth medium was discarded from the plates, and 100 µl of the maintenance medium containing the appropriate virus dilution were dispensed in the respective wells. A total of 4 wells was inoculated with each dilution of the virus sample. The cell control wells were fed with 100 µl of the maintenance medium. The plates were incubated at 37 °C and 5% CO_2 in a cell culture incubator for 96 hours. The results were recorded by checking cytopathic effects under a light microscope. A complete destruction of the cell monolayer was regarded as positive for virus growth. Infectivity data thus obtained were recorded and

processed to calculate TCID50/mL by using the Reed and Muench formula [15].

Preparation of Water–virus Mixture in 5 pH Values

Water used in the present experiment was purchased from a local supplier [16] and contained a TDS value of 244 parts per million (**PPM**). The water was transferred to a glass bottle and autoclaved for use in this study. In each experiment, the freshly propagated NDV in AF suspension was mixed with water at a ratio of 1:10 (v/v) in a 100 mL glass bottle. Five bottles of such NDV and water mixture were prepared, and their pH values were adjusted to 5.00, 6.00, 7.00, 8.00, and 9.00, respectively, by using sterile 1 M NaOH and 1 M HCl. Virus infectivity was measured immediately, and samples were placed at room temperature (25 °C ± 2). Residual virus infectivity was measured afterwards at 30, 60, 120, and 180 min post storage. The entire experiment was repeated 3 times. Each of the samples was filtered through a 0.20 µm pore size syringe filter [17] before processing for virus infectivity.

Commercial Stabilizer and Skimmed Milk in Water–virus Mixtures

The role of a commercial stabilizer and skimmed milk to preserve virus infectivity also was evaluated. Cevamune® [18] commercial stabilizer was used at a concentration of one tablet per 100 liters of water. Each Cevamune® tablet was dissolved in sterile PBS to make a 10% stock solution that was then mixed with the pH-amended water samples (5.00, 6.00, 7.00, 8.00, and 9.00) to have a final concentration of 0.1 mL/10 mL of water. The Olpers light [19] was used at a concentration of 2% in the water, and 0.20 mL of the skimmed milk was mixed with 9.8 mL of the pH-amended water samples. The virus suspension was added to the water samples containing the Cevamune® and skimmed milk and placed at room temperature (25 °C ± 2). Virus infectivity was measured immediately after mixing and subsequently at 30, 60, 120, and 180 min of storage. Each of the samples was filtered through 0.20 µ m pore size syringe filter [17] before processing for measuring virus infectivity. In order to test the possible effect of Cevamune® or skimmed milk on the shift of pH and TDS values of the virus-charged water samples, the pH and TDS values also were tested after 3 h of incubation. The entire experiment was repeated 3 times independently.

Statistical Analysis

Sequential data as a measure of virus infectivity were statistically analyzed with the linear regression model to calculate T-90 values (time required for 90% reduction in the virus infectivity).

RESULTS AND DISCUSSION

Mean infectivity titers of NDV reconstituted in the water samples adjusted to various pH conditions (5.00, 6.00, 7.00, 8.00, and 9.00) without any stabilizer, as well as following the addition of Cevamune® and skimmed milk, are presented in Table 1 (experimental data of triplicate samples are provided as supplementary material). In water samples without any stabilizers, minimum reduction in virus infectivity was observed at pH 7.00 (from a titer of 6.75 ± 0.00 to 6.33 ± 0.14), while maximum reduction was observed at pH 9.00 (from a titer of 2.58 ± 0.12 to an undetectable level) after 3 h of incubation at room temperature. A similar trend was observed in the Cevamune®-added water samples in which minimum reduction of virus infectivity was observed at pH 7.00 (from a titer of 6.75 ± 0.00 to 6.25 ± 0.14), while maximum reduction was observed at pH 9.00 (from a titer of 3.75 ± 0.00 to 0.58 ± 1.01). Virus infectivity was predominantly preserved in the water samples following addition of skimmed milk. Virus infectivity at pH 5.00 with a starting virus titer of 4.58 (±0.14), at pH 6.00 with a starting virus titer of 4.58 (±0.14), at pH 7.00 with a starting virus titer of 6.58 (±0.14), at pH 8.00 with a starting virus titer of 5.25 (±0.25), and at pH 9.00 with a starting virus titer of 4.25 (±0.00) was reduced to 2.75 (±0.66), 3.33 (±.38), 6.17 (±0.14), 4.33 (±0.29), and 1.75 (±0.31), respectively, after 3 h of incubation at room temperature.

Linear regression models showing a decline in the infectivity of NDV in the pH-amended water samples with and without the addition

Table 1. Infectivity titers of NDV suspended in pH-amended water samples mixed with Cevamune® and skimmed milk at various time intervals after storage at room temperature.

	Virus infectivity as $\log_{10}$ $TCID_{50}$/mL in the pH-amended water samples														
	Simple water					Cevamune®-containing water					Skimmed-milk-containing water				
Time (Min)	5:00	6:00	7:00	8:00	9:00	5:00	6:00	7:00	8:00	9:00	5:00	6:00	7:00	8:00	9:00
0	4.58 (±0.29)	6.42 (±0.29)	6.75 (±0.00)	6.58 (±0.14)	2.58 (±0.14)	5.75 (±0.00)	5.58 (±0.14)	6.75 (±0.00)	6.75 (±0.25)	3.75 (±0.00)	4.58 (±0.14)	4.58 (±0.14)	6.58 (±0.14)	5.25 (±0.25)	4.25 (±0.00)
30	3.83 (±0.14)	6.50 (±0.00)	6.67 (±0.14)	6.33 (±0.14)	2.33 (±0.14)	5.50 (±0.25)	5.50 (±0.25)	6.42 (±0.29)	6.25 (±0.50)	3.42 (±0.14)	4.67 (±0.14)	4.5 (±0.25)	6.33 (±0.38)	4.58 (±0.14)	3.67 (±0.20)
60	3.33 (±0.38)	6.75 (±0.00)	6.58 (±0.14)	5.25 (±0.25)	2.5 (±0.25)	3.67 (±0.14)	5.50 (±0.25)	6.42 (±0.38)	6.67 (±0.14)	3.08 (±0.38)	4.50 (±0.25)	3.67 (±0.76)	6.50 (±0.25)	4.42 (±0.38)	3.33 (±0.35)
120	2.17 (±0.14)	5.17 (±0.14)	6.25 (±0.25)	5.17 (±0.14)	2.00 (±0.00)	3.58 (±0.14)	5.25 (±0.25)	6.58 (±0.14)	6.5 (±0.00)	2.08 (±0.38)	3.67 (±0.76)	3.33 (±0.29)	6.17 (±0.14)	4.33 (±0.29)	2.50 (±0.25)
180	2.00 (±0.00)	5.00 (±0.00)	6.33 (±0.14)	5.08 (±0.29)	0.00 (±0.00)	3.75 (±0.00)	3.75 (±0.00)	6.25 (±0.00)	5.75 (±0.00)	0.58 (±1.01)	2.75 (±0.66)	3.33 (±0.38)	6.17 (±0.14)	4.33 (±0.29)	1.75 (±0.31)

Note: Values in parenthesis are standard deviations.

$TCID_{50}$/mL: Tissue culture infective $dose_{50}$/mL.

of stabilizers are presented in Figures 1 to 3. Trend lines show a minimum decline at pH 7.00, while an abrupt decline was observed at extreme acidic (pH 5.00) or alkaline (pH 9.00) pH conditions. For a better comparison of virus inactivation rates for all treatments, T-90 values were calculated (Table 4). In all types of water samples, T-90 values at pH 7.00 are significantly higher than all of the other pH conditions. The Cevamune®-added, virus-charged water sample adjusted to pH 7.00 displayed a maximum T-90 value (485 ± 64 min). The addition of Cevamune® or skimmed milk in water at pH 8.00 resulted in significantly higher T-90 values than the water without stabilizers at pH 8.00 or the water with or without stabilizers at pH 5.00, 6.00, and 9.00 (Table 3). The Nestle bottled water has a pH of 7.37 and a TDS value of 244 PPM. A negligible change was observed in the pH and TDS values of the virus-charged water samples following the addition of Cevamune® or skimmed milk (Tables 2 and 3) except at acidic conditions in which the addition of skimmed milk slightly raised the pH values of pH 5.00 and 6.00 adjusted water samples to 5.69 ± 0.04 and 6.49 ± 0.03, respectively.

Lentogenic strains of NDV are universally used as live virus vaccines in domestic and commercial poultry. These vaccines are administered through conventional routes that include aerosol (spraying), drinking water, and eye drops. However, due to cost and convenience, administration of NDV vaccines is mostly performed through the drinking water route. The efficacy of NDV live vaccines is highly dependent upon the infectivity of the vaccine virus, which can be lost by improper cold-chain management and inadequate storage conditions [20]. The presence of a virus-inactivating substance in the water also may lead to vaccine failures [21]. A model virus system was developed in the present study to evaluate the effect of water pH on the survival of the LaSota strain of NDV. However, due to identical virus morphology, the experimental findings might be applicable to other virus strains as well. It was observed that extreme pH conditions (5.00 and 9.00) negatively affect virus survival rates. At a neutral pH (7.00), a negligible decrease in the virus titer was recorded, while this effect was more pronounced at pH 6.00 and 8.00 (Table 1, Figure 1). Previous studies on the

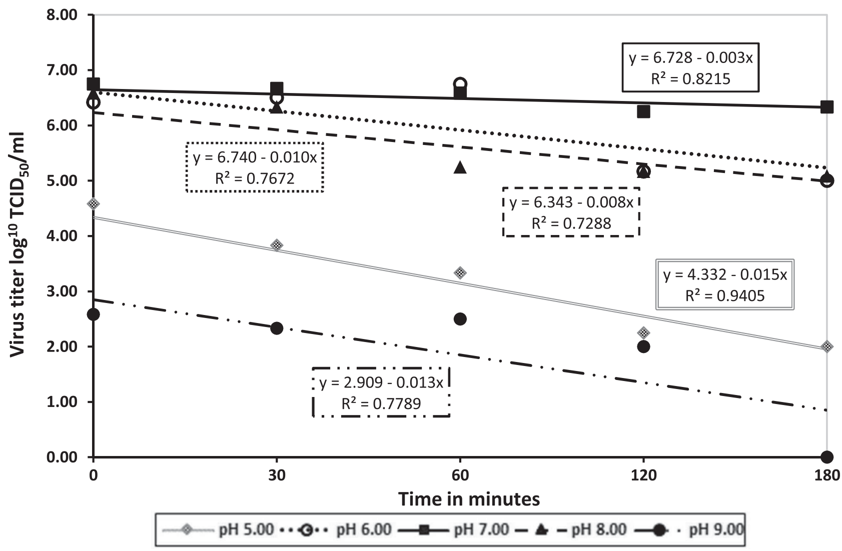

Figure 1. Linear regression model showing reduction in the infectivity of NDV suspended in pH-amended water samples.

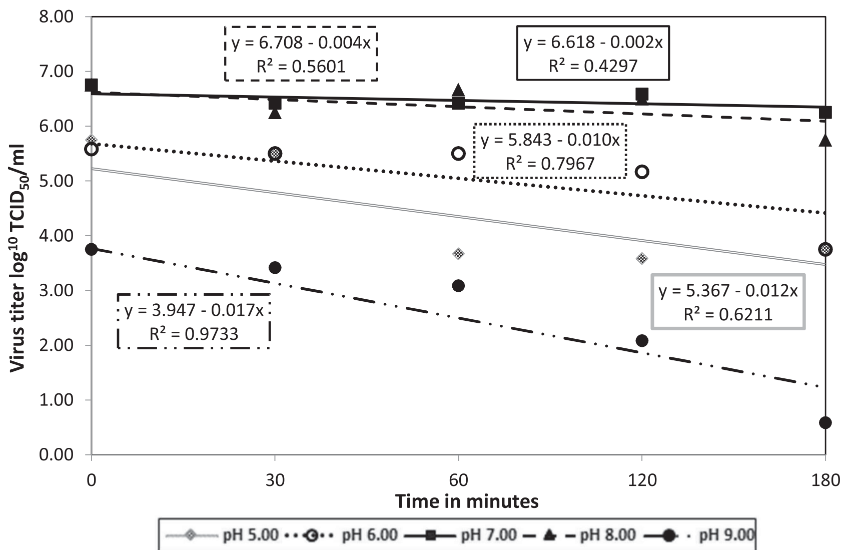

Figure 2. Linear regression model showing reduction in the infectivity of NDV suspended in pH-amended water samples containing Cevamune®.

environmental inactivation of viruses show that the pH of the medium primarily affects virus infectivity. It was observed that at low pH, enveloped viruses display conformational changes in their spike glycoproteins, which distort their ability to bind the cell surface receptors [22]. An *Orthomyxovirus* was completely inactivated by exposure to pH 4.00 for 30 min, while infectivity was reduced by more than 90% at pH 11 immediately after exposure [23]. The present study also demonstrates that extreme acidic (pH 5.00) or alkaline (pH 9.00) conditions were detrimental to virus infectivity (Table 1). Similarly, while testing various natural water sources as a diluent of live NDV vaccines, the ones with acidic pH induced minimum antibody titers in the vaccinated birds [24], indicating a negative effect on the efficacy of that vaccine. In all of the experiments,

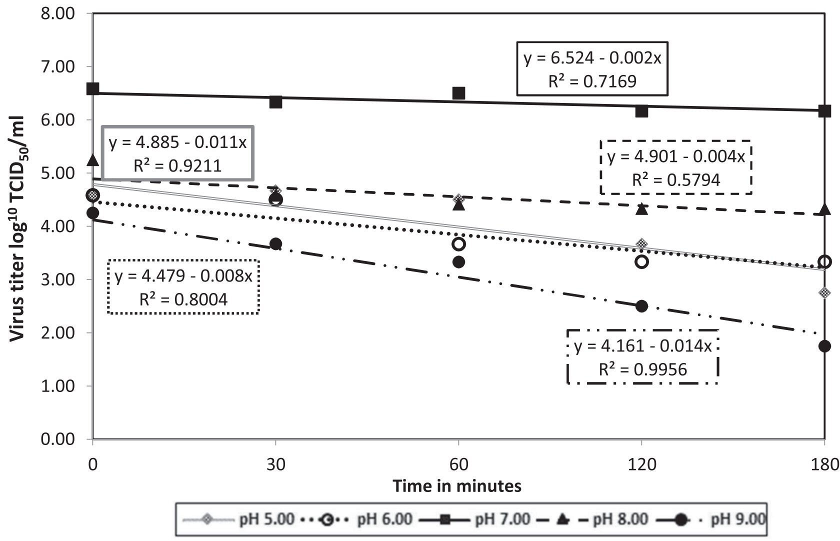

Figure 3. Linear regression model showing reduction in the infectivity of NDV suspended in pH-amended water samples containing skimmed milk.

Table 2. Shift in pH of the virus-charged water samples after addition of Cevamune® and skimmed milk.

pH of the water without stabilizers	pH of water after addition of Cevamune®	pH of water after addition of Skimmed milk
5.00	4.81 (±0.07)	5.69 (±0.04)
6.00	5.95 (±0.03)	6.49 (±0.03)
7.00	6.81 (±0.04)	7.08 (±0.02)
8.00	7.99 (±0.01)	8.04 (±0.01)
9.00	9.10 (±0.05)	8.88 (±0.03)

Table 3. Alteration in the total dissolved solvent values of the virus-charged water samples after addition of Cevamune® and skimmed milk.

	Total dissolved solids (TDS) vales in parts per million (ppm)		
pH of the water sample	Without stabilizer	Cevamune®	Skimmed milk
5.00	245 (±5)	247 (±3)	259 (±4)
6.00	244 (±1)	248 (±7)	261 (±5)
7.00	248 (±10)	251 (±11)	263 (±3)
8.00	246 (±5)	261 (±12)	278 (±18)
9.00	244 (±4)	248 (±9)	259 (±3)

the same virus stock with equivalent biological activity was used, but virus-charged water samples adjusted to extreme pH conditions displayed low infectivity titers in comparison to the ones with neutral pH values at the beginning of the experiment. This shows that extreme pH conditions resulted in drastic virus inactivation (Table 1). In the case of Cevamune®- and skimmed-milk-added samples, this trend was relatively less. Despite the fact that the addition of commercial stabilizer or skimmed milk did not have any effect on water pH, the high protein content of skimmed milk may have protected the virus from the damaging effects of extreme pH conditions [25]. Skimmed milk proved to contain better stabilizing properties for administration of live bacterial and viral vaccines [26], especially for the drinking water vaccine regimen

Table 4. Comparison of T-90 values of the NDV in the water containing various stabilizers adjusted to different pH conditions.

pH	T-90 values		
	Without stabilizers	Addition of Cevamune®	Addition of skimmed milk
5.00	69 (±3)^{A}	85 (±6)^{A}	95 (±19)^{A}
6.00	102(±10)^{A}	106 (±10)^{A}	134 (±25)^{A}
7.00	381 (±64)^{B}	485 (±64)^{C}	455 (±69)^{C}
8.00	119 (±17)^{A}	282 (±84)^{B}	292 (±133)^{B}
9.00	68 (±16)^{A}	65 (±22)^{A}	84 (±7)^{A}

Notes: T-90 values: Time in min required for one log reduction in the virus infectivity.
Values in parenthesis indicate mean standard deviation of T-90 values.
$^{A–C}$Mean values having similar superscripts are not significantly different ($P < 0.05$).

of infectious bronchitis and NDV vaccines of poultry [2,27]. Likewise, Cevamune® increases virus survival in water, as it neutralizes chlorine and other water sanitizers as well as binds heavy metals that may be present in the water [28].

In a biological system, it was not possible to maintain the initial virus concentration at the same level, as several other factors such as pH may have resulted in an abrupt change in virus infectivity. This is why, for better comparison of the virus survival rates, a measure of 90% reduction in virus infectivity (T-90 value) was used. At a neutral pH (7.00), the highest T-90 values (around 8 h) were recorded. All of the experiments were conducted at ambient temperature (25 °C ± 02), and the decrease in virus infectivity even at pH 7.00 may be attributed to the effect of temperature, as virus infectivity can be measured in h at 20 °C [29]. Temperature-based inactivation of a *Metapneumovirus* (*Paramyxoviridae*) in 24 h duration was reported to be one and 2 log10 reduction at 21 and 37 °C, respectively [30]. In a study by Nazir et al. [7], it took 8 d for one log10 reduction in the infectivity of NDV at 20 °C, which is quite higher than the time calculated in the present study. In the study by Nazir et al., avian influenza virus was adsorbed onto germ carriers. It is known that adsorbed virus particles have a higher survival rate than the suspended virus particles in the same medium [7,31]. Statistical analysis shows that the addition of Cevamune® or skimmed milk did not have any significant effect on the survival of the virus in the water adjusted to 5.00, 6.00, or 9.00 pH, while in the water with pH 7.00 and 8.00, virus survival was significantly increased after adding either Cevamune® or skimmed milk (Table 4). This is in line with the findings of a previous study [25] in which dried skimmed milk helped to stabilize the NDV vaccine virus in containers and water pipes. In the present study, the role of only 2 water-quality parameters, i.e., pH and salinity, was tested, but under field-like conditions, several other factors, such as heavy metals, chlorine, or disinfectants in the water, organic matter, and microbial biofilms in storage tanks or water distribution systems, also may affect virus survival rates. Therefore, the role of all these factors needs to be evaluated before drawing a final conclusion about the role of water quality in the effective delivery of the live NDV vaccines through drinking.

CONCLUSIONS AND APPLICATIONS

1. Infectivity of NDV live vaccine can be effectively preserved for up to 3 h following reconstitution in the water adjusted to pH 7.00, even without the addition of any stabilizer. Hence, regular water with pH 7.00 can be used as an optimum medium for effective delivery of the vaccine.
2. The addition of Cevamune® to the ND virus water mixture enhances the virus survival rate, while the addition of skimmed milk preserves virus infectivity to a maximum level and protects the virus from the damaging effects of extreme acidic and alkaline pH conditions.

SUPPLEMENTARY DATA

Table S-1. Infectivity titers of NDV suspended in pH-amended water at various time intervals after storage at room temperature.

Table S-2. Infectivity titers of NDV suspended in pH-amended water samples containing Cevamune® at various time intervals after storage at room temperature.

Table S-3. Infectivity titers of NDV suspended in pH-amended water samples mixed with skimmed milk at various time intervals after storage at room temperature.

Supplementary data are available at *The Journal of Applied Poultry Research* online.

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