1 **Title:** Milk of cow and goat, immunized by recombinant protein

2 vaccine ZF-UZ-VAC2001(Zifivax), contains neutralizing antibodies

3 against SARS-CoV-2 and remains active after standard milk

4 pasteurization.

5 **Authors: Victoria Garib[1,2], Stefani Katsamaki[3], Shahlo Turdikulova[3], Yuliya**

6 **Levitskaya[2,3], Nodira Zahidova[4], Galina Bus[4], Kristina Karamova[5], Manona**

7 **Rakhmedova[2], Nigora Magbulova[2], Alexander Bruhov[2], Firuz Y. Garib[5], Ibrokhim**

8 **Y. Abdurakhmonov[6*]**

9 **Affiliations:** [1]Division of Immunopathology, Department of Pathophysiology and Allergy

10 Research, Medical University of Vienna, Austria; [2]International Centre of Molecular

11 Allergology of the Ministry of Innovative Development of Republic of Uzbekistan;

12 [3]Centre of Advanced Technology, of the Ministry of Innovative Development of Republic

13 of Uzbekistan; [4]Scientific and Diagnostical Centre of Laboratory Technology "Defactum

14 Laboratories", Uzbekistan; [5]Russian Medical Academy of Continuous Professional

15 Education (RMAPE), Moscow, Russia; [6]Centre of Genomics and Bioinformatics,

16 Academy of Sciences of Uzbekistan.

17 **Corresponding author:** Correspondence to Ibrokhim Y. Abdurakhmonov, email:

18 i.y.abdurakhmonov@gmail.com. Centre of Genomics and bioinformatics, Academy of

19 Sciences of Uzbekistan, University street-2, Qibary region, Tashkent 111215, Republic

20 of Uzbekistan/

21

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Abstract

Here we report the first experimental validation of the possibility for obtaining immune milk with neutralizing antibodies against SARS-CoV-2 from vaccinated cow and goat using recombinant protein human vaccine, ZF-UZ-VAC2001. In the period of two weeks after first vaccination, we detected the neutralizing antibodies against coronavirus in the blood serum of vaccinated animals. The neutralizing activity, in its peak on the 21st days after receiving the third dose (77th day from first dose), was effective in Neutralization Test using a live SARS-CoV-2 in Vero E6 cells, even after 120-fold serum titration. Colostrum of the first day after 3rd dose vaccinated cow after calving had a greater activity to neutralize the SARS-CoV-2 compared to colostrum of subsequent three days (4.080 µg/ml vs 2.106, 1.960 and 1.126 µg/ml), goat milk (1,486 µg/ml), and cow milk (0.222 µg/ml) in MAGLUMI® SARS-CoV-2 neutralizing antibody competitive chemiluminescence immunoassay. We observed a positive correlation of receptor-binding domain (RBD)-specific IgG antibodies between the serum of actively immunized cow and milk-feeding calf during the entire course of vaccination (r = 0.95, p = 0.027). We showed an optimal regime for immune milk pasteurization at 62.5°C for 30 min, which retained specific neutralizing activity to SARS-CoV-2, potentially useful for passive immunization against coronavirus infection threats.

Key words: bovine immunoglobulins, SARS-CoV-2, immune milk, neutralizing antibody, passive immunization, vaccination, ZF-UZ-VAC2001.

Dear Editor,

In the global coronavirus disease pandemic, immunological studies proved that antibodies are the effective molecules for sanitizing the body from viruses. However, the formation of novel SARS-CoV-2 mutations is leading to a decreased effectiveness of vaccines. Moreover, the slow rate of massive vaccination process, due to poor public acceptance and/or insufficient vaccine supplies in some countries, are the one of the main factors for continuous reemergence of new virus variants of concern (VOC). The development and registration of new vaccines against constantly emerging mutations require additional time and funding. This underlies to explore new opportunities to establish a stable herd immunity, focusing on the development of highly effective neutralizing antibodies (nAbs) [1]. Apparently, nAbs against VOC can be quickly obtained by the vaccination of farm animals with the emergency use approved (EUA) human vaccines, covering the new mutations of importance. Here, we are to briefly communicate the first experimental validation report on obtaining immune milk with neutralizing antibodies (nAbs) against SARS-CoV-2 from cow and goat after vaccination with RBD-based recombinant protein subunit human vaccine, ZF-UZ-VAC2001 or (ZF2001 or Zifivax).

The idea, to study and validate if vaccinated farm animal milk contains nAbs or not, came from the evidence that antibodies to SARS-CoV-2 were found in the milk of women who have had COVID-19 or been vaccinated [2]. We also carefully read and acknowledge several publications on the possible benefit from the passive immunization using milk of vaccinated cow. Jawhara [3] first suggested that microfiltered raw immune milk or colostrum collected from SARS-CoV-2 vaccinated cows could provide short-term protection against SARS-CoV-2 infection in humans. Further, Arenas et al. [4] proposed to use of heterologous passive immunity using Bovine Coronavirus (BCoV) immune milk as an immunostimulant therapy to control SARS-CoV-2 infection because vaccination of

75 farm animals is a well-known and has been described in the literature to protect animals

76 from diseases, including BCoV [5]. Gallo et al. [6] reviewed the antiviral properties of

77 native and chemically modified whey proteins and their potential applications in human

78 health, focusing on their application in prevention and treatment of SARS-CoV-2

79 infection.

80 However, neither the detection of nAbs in the serum and milk of vaccinated household

81 farm animals using human vaccines approved for emergency use, nor the effect of

82 pasteurization of such immune milk on the SARS-CoV-2 virus-neutralizing activity, has

83 not yet been experimentally validated. To address these limitations, we first performed a

84 series of pilot experiments in 60 milk and serum samples of vaccinated household cows

85 and goat. In that, healthy pregnant as well as dairy cow with feeding calf were

86 vaccinated with the ZF-UZ-VAC2001 recombinant SARS-CoV-2 human vaccine,

87 containing a dimeric form of the receptor-binding domain (RBD) as the antigen [7].

88 We used this particular vaccine because it was successfully passed a 3rd phase clinical

89 trial in Uzbekistan and were approved for the massive vaccination of the population [8].

90 ZF-UZ-VAC2001 has shown its effectiveness against the diverged variants of the SARS

91 CoV-2 virus [8,9], namely Alfa (92.93%), Gamma (100%), Delta (77.47%), and Kappa

92 (90.0%). Although the neutralization activity of currently used human vaccines, has

93 shown a decreased effect against emerging new VOC B.1.1.529 (Omicron) variant [9],

94 the prolonged time between the second and third dose injections of ZF-UZ-VAC2001

95 (0,1, 2 vs 0, 1, 5 months) has revealed a better neutralization effect against Omicron

96 variant [10].

97 In our study, we performed vaccination of farm animals (cows and goat) with

98 revaccination carried after 28 and 56 days. Examination of the udder, palpation of the

99 lymph nodes, mucous membranes and measurement of rectal temperature as well as

100 the observation of the behavior of animals have not revealed any side effects during

101 daily monitoring of animals after vaccination.

102 Since the first dose vaccine injection period, we have regularly collected and evaluated

103 the virus neutralization activity of the immune milk and blood serum of the cow and

104 goat, measuring the inhibition level of viral RBD binding to the ACE2 receptor. For this

105 purpose, we used the MAGLUMI® SARS-CoV-2 Neutralizing Antibody competitive

106 chemiluminescence immunoassay (CLIA). Milk and blood serum from the non-

107 vaccinated cows were used as control samples. For blood serum samples, we

108 performed the direct neutralization test using a live SARS-CoV-2 proliferating in Vero E6

109 cells [S1] at the Medical University of Vienna, Austria. We also determined antigen-

110 specific antibody isotypes to the RBD of spike (S) and full-length S proteins in bovine

111 serum and milk samples using enzyme-linked immunosorbent assay (ELISA) analysis

112 (see Supplementary Materials and Methods).

113 We detected the neutralizing antibodies to the RBD domain and S-protein of SARS-

114 CoV-2 in the blood serum of a vaccinated animal as early as two weeks after the first

115 vaccination. Results showed that revaccination contributed to an increase in the effect

116 of inhibition. We noted maximum neutralization activity of blood serum (100%) and milk

117 (40%) on the 77th day from the date of the first vaccination. The correlation between

118 neutralization rate of cow sera and milk during the entire course of vaccination was

119 significant at $r = 0.96$, $p = 0.022$

120 Complete viral neutralization of the cytopathic effect on Vero E6 cells was detected

121 even with 120-fold serum titration. The blood serum and milk of the vaccinated cow

122 contained specific IgG to the RBD domain and S-protein of SARS-CoV-2.

123 We also found statistically significant correlation between the level of IgG specific to the

124 RBD domain and the neutralization rate of milk of vaccinated cows ($r = 1.0$, $p = 0.001$,

125 Supplementary Table 1).

126   When we vaccinated other group of "Simmental" cows during the dry period of the third

127   trimester of pregnancy, after calving we determined that that the colostrum of the first

128   day had a greater ability to neutralize the SARS-CoV-2 virus (4.080 µg/ml) compared to

129   colostrum of subsequent next days (2.106, 1.960 and 1.126 µg/ml) and milk [0.222

130   µg/ml that was within the range of 50% (or 0.30 µg/ml) neutralization activity].

131   In another pilot experiment in a domestic lactating goat (*Capra hircus*), it was sufficient

132   to use only 2 dose injection of the vaccine to achieve the effect of immunization and

133   obtain immune milk samples. The correlation between neutralization rate of goat sera

134   and goat milk in during the entire course of vaccination was high at r = 0.99, p = 0,003

135   (Supplementary Table 1). The maximum of virus-neutralizing activity of the immune

136   goat milk was 1.486 µg/ml on the 14 days of the third dose vaccine injection.

137   We further investigated the possibility of transferring of SARS-CoV-2 specific IgG

138   antibodies via milk from cow to its milking calf. Toward this goal, we performed the

139   analysis of sera from calf during active vaccination of cow. Results showed that there is

140   statistically significant correlation (r = 0.95, p = 0.027, Supplementary Table 1) between

141   the level of IgG specific to RBD of vaccinated cow and calf fed by milk of vaccinated

142   mother cow. This showed the possibility of passive immunization using milk of

143   vaccinated mother cows, containing specific IgG against SARS-CoV-2.

144   In perspectives, as highlighted above [2-6], there is a huge opportunity to use the

145   passive immunization property of immunized cow milk in humans. For this, there is a

146   need for determining the optimal pasteurization conditions of immune milk to keep its

147   biologically active neutralization against SARS-CoV-2. Toward this goal, we have

148   studied several pasteurization regimes of immunized milk while analyzing its

149   neutralization activity of SARS-CoV-2. We tested 20 milk samples obtained from

150   vaccinated cow and goat, pasteurizing them at different temperatures and regimes of

151   pasteurization according to State regulations (62.5-63°C for 30 min, 72°C for 5 min,

152   85°C for 5 sec).

153   As expected, temperature treatment for pasteurization of milk product negatively

154   correlated with neutralization activity (r =-1.0, p = 0.02). Compared to before-

155   pasteurization raw milk (0.5±0.2 μg/ml), pasteurization treatments at 72 ºC (for 5 min)

156   and 85 ºC (for 5 sec) temperatures showed sharp activity decreases (0.1±0.04 μg/ml

157   and 0.05±0.01 μg/ml respectively). However, the pasteurization at 62.5-63 ºC during 30

158   minutes retained sufficiently active neutralization property in nAbs chemiluminescence

159   immunoassay (0.3±0.1 μg/ml), small decrease observed between raw and pasteurized

160   immune milk at 62.5-63 ºC during 30 minutes was statistically non-significant (p = 0.24;

161   (Fig.1; Supplementary Table 2).

162   Thus, these first experimental validation results indicated that the ZF-UZ-VAC2001

163   human vaccine can be safely used for the vaccination of household farm animals to

164   obtain immuno-biologically active milk and serum, containing specific nAbs against

165   SARS-CoV-2. There is no need for the substantial corrections in dose calculations for

166   vaccination of the animals, which can be performed in accordance to the manufacturer's

167   instructions and protocols for human vaccination. Interestingly, we experimentally

168   observed that milk and serum of vaccinated cow not only have active neutralization

169   property against SARS-CoV-2, but also biologically active specific IgG antibodies to

170   RBD and S protein, transferred from mother cow milk to its milking calf, passively

171   forming offspring's immune protection.

172   We found that the most optimal condition for immunized milk pasteurization can be

173   achieved 62.5-63 ºC during 30 minutes, which retains efficient virus neutralizing

174   antibody activity against SARS-CoV, providing future opportunity for the passive

175   immunization of human by milk consumption. The limitation of this study is a small

176   number of farm animals included for vaccination experiments and future large-scale

177 experiments are required, which is in progress in our laboratories. However, early

178 results collectively showed that pasteurized immune milk obtained from vaccinated

179 household animals using human vaccine against SARS-CoV-2, administered herein,

180 potentially could be useful for passive immunization against coronavirus infection

181 threats. The use of immune colostrum, milk and dairy products with neutralizing

182 antibodies from vaccinated cows and goats seems to be a promising approach for the

183 preparation of safe and natural prophylactic agents against human and animal

184 infections.

185

Abbreviations

187 ACE2: angiotensin-converting enzyme 2; COVID-19: coronavirus disease; ELISA:

188 enzyme-linked immunosorbent assay; HRP: horseradish peroxidase; IgG, IgA, IgM:

189 immunoglobulin G, A, M; nAbs: neutralizing antibodies; NT: Neutralization test; OD:

190 optical density; RBD: receptor-binding domain; S: spike protein; S1: spike protein

191 receptor-binding subunit; SARS: severe acute respiratory syndrome; VOC: variants of

192 concern; CLIA: chemiluminescence immunoassay; EUA: emergency use approval; ZF-

193 UZ-VAC2001: Zhifei (ZF) – Uzbekistan (UZ) –Vaccine (VAC) project started in January

194 of 2020 (20/01).

Supplementary Information:

196 The online version contains supplementary material available at https://doi.org/xxx

197 **Supplementary Table 1.** Statistical analysis of correlations between parameters.

198 **Supplementary Table 2.** P-values of significant difference between nAbs levels of

199 immune milk at different pasteurization (n=20).

200 **Supplementary Materials and Methods.**

Acknowledgments

Authors' contributions

VG and **IYA**: project administration, conceptualization, methodology, investigation, data curation, and writing—original draft, review and editing; **FYG**: consultation, methodology, writing—review and editing; **YL** and **ST**: methodology, investigation, writing—review and editing; **SK GB, KK, MR, NM**, and **AB**: investigation, data collection and curation. All authors contributed to the article and approved the submitted version.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on a reasonable request.

Declarations:

Ethics approval and consent to participate

The studies reviewed and approved by the Ethics Committee Republic of Uzbekistan for SARS-CoV-2 research (authorization number 6/6 1449 from 13.10.2020)

Consent for publication

All authors consent to the publication of the manuscript in Nutrition Journal.

Competing interests

The authors declare that the study concept and results are filed for patenting at the Intellectual Property Agency under the Ministry of Justice of the Republic of Uzbekistan with pending applications № IAP 2021 0365 9 and IAP 2022 0054.

Additional Author Information

ORCID

Victoria Garib https://orcid.org/ 0000-0003-3855-217X

Shahlo Turdiqulova https://orcid.org/0000-0003-0764-2332

Firuz Garib https://orcid.org/0000-0003-3749-1950

Ibrokhim Abdurakhmonov https://orcid.org/0000-0001-9563-0686

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273   **Fig. 1 The box plots of SARS-CoV-2 Neutralizing Antibody levels (µg/mL).** Average

274   indices (y-axis) for before pasteurization (raw) and pasteurized immune milk samples at

275   the different regimes of pasteurization (x-axis) are shown. $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq$

276   0.01; ns: not statistically significant difference. Negative control represents milk sample

277   from non-vaccinated animal. The box plots were generated, using GraphPad Prism 8

278   (GraphPad Software Inc.).