

**PREFERENTIAL PHAGOCYTOSIS OF BACTERIA IN MIXED CULTURES BY BUFFALO (*BUBALUS BUBALIS*) NEUTROPHILS**INDIRA D. SILVA<sup>\*1</sup> and R.O. THATTIL<sup>2</sup><sup>1</sup> Department of Clinical Studies, Faculty of Veterinary Medicine & Animal Science, University of Peradeniya, Peradeniya.<sup>2</sup> Department of Crop Science, Faculty of Agriculture, University of Peradeniya, Peradeniya.

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**Abstract:** Comparative phagocytic activity of buffalo blood neutrophils incubated simultaneously with pure and mixed cultures of *Escherichia coli*, *Staphylococcus aureus*, and *Streptococcus agalactiae* was studied. Phagocytic activity for pure bacterial cultures of *Staph. aureus*, *Strep. agalactiae* and *E. coli* were 90.8%, 85.4% and 76.2%, respectively. The percent phagocytosis of neutrophils incubated with mixed bacterial cultures was similar to that with pure bacterial cultures. However, with mixed bacterial cultures, a majority of neutrophils showed preferential phagocytosis of one species of bacteria. Thus, neutrophils incubated with a mixed bacterial culture of *E. coli* and *Staph. aureus* showed a significantly higher phagocytosis of *Staph. aureus*, with a per cent phagocytosis of approximately 40%. Neutrophils incubated with a mixed bacterial culture of *E. coli* and *Strep. agalactiae* showed a significantly higher phagocytosis of *Strep. agalactiae*, with a percent phagocytosis of approximately 33%. These observations indicate that when buffalo neutrophils are exposed to a mixed bacterial culture the affinity for phagocytizing *E. coli* was much less in the presence of *Staph. aureus* than in the presence of *Strep. agalactiae*.

**Key words:** Buffalo, mixed bacterial cultures, neutrophil, phagocytosis.

**INTRODUCTION**

Mastitis is a major cause of economic loss in the dairy industry. Although the buffalo is becoming popular as a dairy animal in Asia, little information is available, in general, on mastitis in buffaloes. Since the buffalo udder is relatively pendulous with long teats, it is more liable to injury and infection than the udder of a cow.<sup>1</sup> Bacteria are the major cause of udder infections. Bacteriological studies on clinical mastitis in buffaloes have demonstrated that the most prevalent species isolated from mastitic quarters were staphylococci (*Staphylococcus aureus* & *Staphylococcus epidermidis*) and streptococci (*Streptococcus agalactiae* & *Streptococcus dysgalactiae*), while *E. coli* was the main gram-negative bacterium isolated.<sup>2-6</sup> Other than the defense mechanisms in the teat, phagocytic cells are the most important defense mechanism against ascending infections of the mammary gland.<sup>7</sup>

A simultaneous comparative evaluation of the phagocytic activity of buffalo blood neutrophils had revealed that the phagocytosis was highest for *Staph. aureus*, followed by similar phagocytic activity for *Strep. agalactiae* and *E. coli*.<sup>8</sup> Furthermore, neutrophils isolated from buffalo milk were as efficient as blood neutrophils in their phagocytic activity when allowed to interact with these three bacteria opsonized with serum.<sup>9</sup>

\* Corresponding author.

However, this property of the neutrophils was studied using the microorganisms in pure cultures. Common mammary pathogens namely, *Staph. aureus*, *Strep. agalactiae* and *E. coli* may cause mastitis in pure or mixed infections.<sup>2,10</sup> Previous workers have observed that 11% of the mastitic buffalo quarters tested had mixed infections.<sup>2</sup> In mastitis due to mixed infections, neutrophils may be required to phagocytose and kill more than one species of microorganisms. Previous studies have indicated that the phagocytic activity of cattle neutrophils for bacteria in mixed cultures was not identical to those in pure cultures.<sup>11</sup> Therefore, it is of clinical significance to compare and evaluate phagocytic activities of buffalo neutrophils for different micro-organisms in mixed cultures. We report here on a study of the phagocytic activity of buffalo blood neutrophils exposed to pure and mixed cultures of *E. coli*, *Staph. aureus* and *Strep. agalactiae*.

## METHODS AND MATERIALS

About 25 ml of blood was collected into EDTA vacutainers from each of 12 Lankan buffaloes (*Bubalus bubalis*) by jugular venepuncture according to the ethical guidelines. Blood was collected also into two 10 ml vacutainers without the anticoagulant to obtain fresh serum. The age of buffaloes used varied from 3 to 10y. Total and differential leukocyte counts were within the normal range for the buffalo.<sup>12</sup>

*Isolation of neutrophils from buffalo blood:* The neutrophils were isolated and the viability was tested as described earlier.<sup>13</sup> The viability of the isolated cell suspension varied from 94 to 100% and consisted of 80 to 90% neutrophils. A working suspension of  $5 \times 10^3$  viable neutrophils/ $\mu\text{l}$  in sterile 0.8% NaCl in 0.0132M phosphate buffer, pH 7.2 (PBS) with 2% bovine serum albumin was prepared and kept at 4°C until use.

*Preparation of bacterial cultures:* *E. coli* (ATCC 25922), *Staph. aureus* (ATCC 29213) and *Strep. agalactiae* were isolated from bovine mastitic milk. Separate suspensions of the three bacteria were prepared to obtain a concentration of  $1 \times 10^6$  organisms/ $\mu\text{l}$  in PBS<sup>13</sup> and the three bacteria were opsonized separately<sup>8</sup> using serum of the particular buffalo for each assay. A neutrophil : bacteria ratio of approximately 1:50 was used for optimum phagocytosis.

Four hundred  $\mu\text{l}$  of opsonized *E. coli*, *Staph. aureus* & *Strep. agalactiae* were aliquoted separately into three glass tubes. These three tubes constituted the pure bacterial cultures. Then 200 $\mu\text{l}$  each of opsonized *E. coli* & *Staph. aureus*, and 200 $\mu\text{l}$  each of opsonized *E. coli* & *Strep. agalactiae* were aliquoted separately into two glass tubes. These two tubes constituted the mixed bacterial cultures.

*Phagocytosis assay:* In a single experimental run, the five suspensions of microorganisms were incubated with 400 $\mu\text{l}$  of the neutrophil suspension prepared from one buffalo. In the negative control the bacterial aliquot was substituted with PBS. The phagocytosis assay was carried out as described earlier.<sup>8</sup>

**Staining and microscopic evaluation:** Smears stained with 2% methyl green and counter stained with Leishman stain<sup>8</sup> were examined microscopically under an oil immersion objective (x 1,250). The nuclei of leukocytes stained light green and the bacteria acquired different degrees of blue. The per cent phagocytosis (PP) or the percentage of phagocytically active neutrophils (cells with ingested bacteria) was determined by counting 500 neutrophils. Neutrophils incubated with mixed bacterial cultures were categorized into cells that had ingested only rods (*E. coli*), cells that had ingested only cocci (*Staph. aureus* or *Strep. agalactiae*), and cells that had ingested both rods and cocci (*E. coli* and *Staph. aureus* or *E. coli* and *Strep. agalactiae*). The neutrophils in the negative control tube (without bacteria) did not show ingested particles.

**Statistical analyses:** The means and standard errors (S.E.) of the means were calculated for the PP of neutrophils in pure cultures. To analyze the phagocytic activity in mixed cultures of *E. coli* + *Staph. aureus* and *E. coli* + *Strep. agalactiae*, the neutrophils were divided into three groups, namely, neutrophils which phagocytized *E. coli* only, neutrophils which phagocytized *Staph. aureus*/*Strep. agalactiae* only, and neutrophils which phagocytized both *E. coli* and *Staph. aureus* or *E. coli* and *Strep. agalactiae*. These three groups were subjected to the analysis of variance and the least significant difference was used in the comparison of means.

## RESULTS

Percentages of neutrophils showing phagocytosis or PP following incubation with either pure or mixed cultures of *E. coli* with *Staph. aureus* or *Strep. agalactiae* are shown in Tables 1 and 2. Neutrophils incubated with mixed bacterial cultures did not always phagocytize both species of bacteria. The tables give the means and the S.E. of the means of the PP in pure cultures. The mean PP of the three groups of neutrophils, namely, neutrophils which phagocytized *E. coli* only, neutrophils which phagocytized *Staph. aureus*/*Strep. agalactiae* only, and neutrophils which phagocytized both *E. coli* and *Staph. aureus* or *E. coli* and *Strep. agalactiae* and the least significant differences used to compare the means are also given in Tables 1 and 2.

### Phagocytosis of pure and mixed cultures of *E. coli* and *Staph. aureus*

The PP of *E. coli* in pure culture was 76.2%, and that of *Staph. aureus* in pure culture was 90.8%, and the total PP of *E. coli* and *Staph. aureus* in mixed culture was 87% (Table 1). In the mixed culture, the PP of *Staph. aureus* only (38.6%) was significantly higher ( $p < 0.01$ ) than that for *E. coli* only (8%) but was similar to the PP of both bacteria (40.5%).

**Table 1: Percent phagocytosis of buffalo neutrophils for *E. coli* and *Staph. aureus* in pure and mixed cultures.**

Organism	Percent phagocytosis	
	Mean	S.E. of mean
Pure cultures		
<i>E. coli</i>	76.2	1.9
<i>Staph. aureus</i>	90.8	0.9
Mixed culture		
<i>E. coli</i> only	8.0 <sup>b</sup>	
<i>Staph. aureus</i> only	38.6 <sup>a</sup>	
Both bacteria	40.5 <sup>a</sup>	
Total	87.0	

In mixed bacterial culture, the total represents combined percent phagocytosis for *E. coli* only, *Staph. aureus* only, and both bacteria. The least significant difference was 21.3. <sup>a,b</sup> Means with the same letters were not significantly different.

### Phagocytosis of pure and mixed cultures of *E. coli* and *Strep. agalactiae*

The PP of *E. coli* in pure culture was 76.2% and that of *Strep. agalactiae* in pure culture was 85.4%, and the total PP of *E. coli* and *Strep. agalactiae* in mixed culture was 82.4% (Table 2). In the mixed culture, the PP of *Strep. agalactiae* only (31.5%) was significantly higher ( $p < 0.01$ ) than that for *E. coli* only (10.8%) but was lower than the PP of both bacteria (40.1%). In the mixed cultures tested, the percentage of cells showing phagocytosis of both bacteria were similar, with a PP of 40.5% for *E. coli* + *Staph. aureus*, and 40.1% for *E. coli* + *Strep. agalactiae*.

**Table 2: Percent phagocytosis of buffalo neutrophils for *E. coli* and *Strep. agalactiae* in pure and mixed cultures.**

Organism	Percent phagocytosis	
	Mean	S.E.
Pure cultures		
<i>E. coli</i>	76.2	1.9
<i>Strep. agalactiae</i>	85.4	2.0
Mixed culture		
<i>E. coli</i> only	10.8 <sup>b</sup>	
<i>Strep. agalactiae</i> only	31.5 <sup>a</sup>	
Both bacteria	40.1 <sup>a</sup>	
Total	82.9	

In the mixed bacterial culture, the total represents combined percent phagocytosis for *E. coli* only, *Strep. agalactiae* only, and both bacteria. The least significant difference was 17.5. <sup>a,b</sup> Means with the same letters were not significantly different.

## DISCUSSION

Cell-mediated immunity (CMI), humoral immunity and non-specific resistance constitute the defense mechanisms of the mammary gland.<sup>14</sup> The role of the CMI in the pathogenesis of mastitis is not well understood. However, lymphokines secreted by the primed lymphocytes located in the mucous membrane of the gland enhance the attraction of leukocytes resulting in mobilization of phagocytic cells from the blood vessels in the region, thus providing resistance against most mammary pathogens.<sup>14-16</sup> The well known California mastitis test (CMT) indirectly measures the concentration of these cells in mastitic milk. The specific activity of phagocytes and their bactericidal action can be enhanced by the presence of opsonins and cytophilic antibody which comprise the humoral immunity.<sup>17</sup> Other than the defense mechanisms in the teat, phagocytic cells are the most important defense mechanism against ascending infections of the mammary gland.<sup>7</sup> However, the teat sphincter in the buffalo contains more smooth muscle fibres and blood vessels, and might therefore function as a better barrier against infection than the cow udder.<sup>18</sup>

A simultaneous comparative evaluation of the phagocytic activity of buffalo neutrophils revealed that the phagocytic activity was highest for *Staph. aureus*, followed by similar phagocytic activity for *Strep. agalactiae* and *E. coli*.<sup>8</sup> When compared with cattle neutrophils, the percent phagocytosis of buffalo neutrophils was 22.5% higher for *Staph. aureus*, 8% lower for *E. coli*, and similar to that of cattle neutrophils for *Strep. agalactiae*.<sup>8</sup> This may explain the finding that buffaloes are more resistant to mastitis caused by *Staph. aureus* than cattle.<sup>19</sup>

Similar studies on cattle neutrophils and mixed bacterial cultures, demonstrated a low preference for phagocytizing *E. coli* in the presence of *Strep. agalactiae*, but not in the presence of *Staph. aureus*.<sup>11</sup> The present study demonstrates that the buffalo neutrophils also show a poor preference for phagocytizing *E. coli*, but the affinity of buffalo neutrophils for internalizing *E. coli* was much less in the presence of *Staph. aureus* than in the presence of *Strep. agalactiae*. Thus, it was interesting to note that the buffalo neutrophils preferentially phagocytosed *Staph. aureus*, whereas, the cattle neutrophils preferentially phagocytosed *Strep. agalactiae* over *E. coli*.

Several studies have reported on the inhibition of phagocytic activity of phagocytes by micro-organisms or their metabolic products.<sup>20-23</sup> Factors concerned with preferential phagocytosis of an organism in a mixed culture need elucidation. Preferential phagocytosis may be partly due to a stimulation or inhibition imposed by one particular species of organism against the other, possibly through induction of changes in appropriate membrane receptors. This influence on phagocytic activity did not seem to affect the total neutrophil population, since a proportion of neutrophils phagocytized both species of bacteria. It is possible that the age of the neutrophils may also play a role in the potential of their phagocytic activity. The number of bacteria available for phagocytosis was not a factor because the neutrophil:bacteria ratio was 1:50 in all experiments achieving optimum phagocytosis.

The findings of the present study warrant studies on the resistance of buffaloes against clinical mastitis due to a mixed infection including *Staph. aureus* and the appropriate treatment regime for mastitis due to mixed bacterial infections.

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