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HEMAGGLUTINATION AND HEMOLYSIS BY *ESCHERICHIA COLI* ISOLATED FROM CASES OF MASTITIS

By

M. EL-SAGHEER AHMED

National Research Center, Dokki, Cairo, Egypt.

SUMMARY

A total of 64 isolates of *Escherichia coli* secured from cases of intramammary infections were tested for their ability to hemagglutinate bovine erythrocytes; of these 37 (58%) were hemagglutination positive. Only 2 of 12 fecal *Escherichia coli* isolates (17%) obtained from healthy controls were hemagglutination positive. This significant association of hemagglutinating *Escherichia coli* and intramammary infections indicates the likelihood that hemagglutination is a marker of virulence. Only 16% (3 of 19) of *Proteus* species and 13% (1 of 8) of *Klebsiella pneumoniae* mastitis isolates were hemagglutination positive. There was a significant correlation ($P < 0.025$) between hemolysin production and hemagglutination; 67% (16 of 24) of the isolates that produced hemolysin also hemagglutinated bovine erythrocytes. There was no significant correlation between hemagglutination and motility, as there was a trend for flagellated organisms to be non hemagglutinators.

INTRODUCTION

Escherichia coli belonging to the normal flora of the animal intestine are generally considered to be non-pathogenic; but in fact are opportunistic pathogens; the initiation of extraintestinal infection depends upon both predisposing host factors and possession of one or more virulence factors by individual strains of *Escherichia coli* composing the contaminating fecal inoculum.

Hemagglutination and hemolysis of erythrocytes are considered virulence factors of *Escherichia coli* strains causing extraintestinal infections in humans. Hemagglutination was associated with the binding capacity of bacterial cells to epithelial cell surface (Green and Thomas, 1981). Hemolysin production was related to the release of iron into the bacterial environment and cytotoxic effect on neutrophils (Cooke and Ewins, 1975 and Cavalieri and Snyder, 1982). In addition, hemolysin production and mannose-resistant hemagglutination (MRHA) were genetically linked in human isolates (Low, et al., 1984).

Escherichia coli that produces these virulence factors may also have greater pathogenicity toward bovine mammary glands infections, because establishment and severity of *Escherichia coli* infections were related to speed of response of neutrophils into the gland (Hill, 1981) and availability of iron in mammary secretion (Todhunter, et al., 1990).

This study, furtherly tests the hypothesis that hemagglutination and/or hemolysis of bovine erythrocytes are markers of virulence. *Escherichia coli; Klebsiella pneumoniae* and *Proteus* species,

mastitis pathogens and fecal *Escherichia coli* isolated from normal cows and buffaloes were compared in their hemagglutinating ability.

The frequency of hemagglutination by *Escherichia coli* isolated from cases of clinical and subclinical mastitis were compared to determine their significance in mammary invasion. In addition a comparison was made of the hemagglutinating ability, hemolysin production and motility of *Escherichia coli*, also the sensitivity of hemagglutination reaction to blocking by D-mannose was tested.

MATERIAL AND METHODS

Bacterial isolates:

The bacteria concerned in this study were isolated from cases of clinical and subclinical mastitis of dairy cows and buffaloes, quarter milk samples collection and microbiological procedures were performed according to El-Sagheer et al. (1992) Normal-flora *Escherichia coli* strains were isolated from stool of healthy female cows and buffaloes. All bacteria were subcultured in Todd-Hewitt broth and stored at -70°C.

Hemagglutination test:-

Blood was collected from healthy cow into tubes containing 1 ml of 3.8% citric acid in distilled water per 9 ml blood and diluted 1:1 with phosphate-buffered saline (PBS) to test for hemagglutination and 1:1 with PBS containing 1% D-mannose for mannose resistant hemagglutination (MRHA). Blood was freshly collected periodically from the same animal throughout the trial and tested the day of collection.

Bacterial isolated were subcultured on colonization factor antigen agar for 14 hours at 37°C (Evans et al., 1977). An isolated colony was mixed into 20µL of diluted blood on a glass microscopic slide using a sterile wooden applicator stick. Negative controls were blood mixed with a sterile wooden applicator stick. The slide was gently rotated for 2 minutes and macroscopic clumping of erythrocytes recorded. All isolates were tested for hemagglutination and mannose resistant hemagglutination.

Detection of hemolysin production:-

Escherichia coli isolates were tested for hemolysin production on 5% bovine blood agar. Isolates were inoculated into a chemically defined medium described by Synder and Koch (1966), and incubated for 14 hours at 37°C. Bacteria from the broth cultures were streaked for isolated colonies on trypticase Soy agar containing 1.5 g/L of Ca Cl and 5% washed bovine erythrocytes. Erythrocytes were washed three times on PBS. Blood agar plates were incubated at 37°C for 24 hours and observed for hemolysin production as indicated by a clear zone surrounding the growth.

Motility:-

Escherichia coli strains were tested for motility by stabbing the isolates into motility test medium (BBL) containing 0.05 gm of triphenyltetrazolium chloride per liter, which allowed better visualization of the spread. After overnight incubation at 37°C, organisms were reported as motile when their growth spread out the stab, non motile strains and weakly motile strains were retested.

Statistical analysis:

Significance levels were determined with the Chi-square test, using the Yates continuity correction.

RESULTS

Hemagglutination by *Escherichia coli* intramammary infection and normal fecal *Escherichia coli* isolates:-

Results showed that *Escherichia coli* isolated from mastitic cases hemagglutinated bovine erythrocytes significantly more often ($P < 0.001$) than did normal fecal *Escherichia coli* isolated from healthy cows and buffaloes. Of the *Escherichia coli* isolated from mastitic cases, 58% (37 of 64) hemagglutinated bovine erythrocytes. Versus to 17% (2 of 12) of the normal fecal *Escherichia coli* isolates.

Studies have shown that hemagglutination of erythrocytes by *Escherichia coli* may be inhibited by D-mannose, indicating the possible role role of this sugar as receptors on the bacteria or erythrocyte surface. All the hemagglutinating *Escherichia coli* strains were tested to determine whether hemagglutination was blocked by D-mannose or not. It is shown that hemagglutination by these strains of *Escherichia coli* was not inhibited with 1% D-mannose treatment.

Hemagglutination of bovine erythrocytes by other members of family *Enterobacteriaceae:*

The question was posed as to whether hemagglutination is characteristic of *Enterobacteriaceae* mastitis pathogens other thean *Escherichia coli.* Results showed that *Escherichia coli* mastitogenic isolates hemagglutinated bovine erythrocytes much more frequently than did *Proteus* species, and *Kelbsiella pneumoniae,* (Table I). Only 4% of the *Proteus* species and 1% of the *Kelbsiella pneumoniae* mastitogenic isolates hemagglutinated bovine erythrocytes.

Table (1) Hemagglutinaton of bovine erythrocytes by *Enterobacteriaceae* mammary pathogens.

Tab. 1,2

Organism [a]		No.of isolates showing bacterial hemagglutination of bovine erythrocytes (%)					
		Positive			Nagative		
		clinical mastitis	Subclinical mastitis	Total	clinical mastitis	Subclinical mastitis	Total
Escherichia coli	(64)	12	25	37(58)[b]	4	23	27 (42)
Proteus species	(19)	1	2	3 (16)	6	10	16 (84)
Kelbsiella pneumoniae	(8)	-	1	1 (13)	2	5	7 (87)
Total	(91)	13	28	41(45)	12	38	50 (55)

[a] Number of isolates is given within parentheses.

[b] The significance of the difference between hemagglutination by *Escherichia coli* compared with other *Enterobacteriaceae* is expressed as a P value for chi-square analysis ($P< 0.001$).

Table (2) Hemagglutination and hemolysis of bovine erythrocytes by *Escherichia coli* mammary pathogens.

Hemolysin production	No. of isolates (%) showing hemagglutination		Total No(.%)
	Positive	Negative	
Positive	16(67)[a]	8 (33)	24(38)
Negative	21 (52)	19(48)	40(62)

[a] Chi-square value; $P<0.025$

Table (3) Hemagglutination of bovine erythrocytes by *Escherichia coli* mammary pathogens compared with motility

Motility	No. of isolates (%) showing hemagglutination		Total No(.%)
	Positive	Negative	
Positive	23(55)[a]	19(45)	42(66)
Negative	14(64)	8(36)	22(34)

[a] Chi-square value; P>0.05

Hemolysin production and hemagglutination:-

Escherichia coli strains isolated from mastitic cases were more frequently hemolytic (p <0.001) for bovine erythrocytes than were normal fecal *Escherichia coli* i.e. 39% (25 of 64) compared with 8% (1 of 12). Table (2) shows a correlation between hemolysis production and hemagglutination, 67% of the mammary gland pathogens that were hemolytic also hemagglutinated bovine erythrocytes.

Hemagglutination and motility:-

There was no relationship between pathogenicity and motility (p > 0.2), both mammary pathogens and normal fecal *Escherichia coli* isolates were frequently motile, i.e, 67% (42 of 64) compared with 75% (9 of 12). Whether the presence of flagella inhibited or enhanced hemagglutination was determined Table (3). Results showed a tendency for flagella to be associated with organisms that were hemagglutination negative, although this association was not statistically significant.

DISCUSSION

This study questions the relationship between hemagglutination and virulence of *Escherich coli*. Previous studies by Hogan et al. (1990) ha shown that the ability of *Escherichia coli* hemagglutinate was characteristic of strains th cause disease rather than normal-flora strains th are unassociated with disease. In this study 58 of the *Escherichia coli* isolated fro intramammary infection hemagglutinated bovin erythrocytes, whereas only 17% of normal fec *Escherichia coli* isolated from healthy animal hemagglutinated bovine erythrocytes. Thi marked association of hemagglutinatin *Echerichia coli* with infection indicates th likelihood that hemagglutination of bovine erythrocytes is a marker of microbial virulence Hemagglutination is an indicator of the bindin capacity of bacterial cells for membrane surfaces and may be related to the ability of strains to attach to epithelial cell surfaces enhancing colonization. However, the actual relationship of the capacity of an organism to hemagglutinate bovine erythrocytes and to attach to mammary epithelial cells is not known.

D-mannose was used as inhibitor of the bacterium-erythrocyte interaction because studies have shown that pilus-mediated hemagglutination is mannose sensitive (Ottow, 1975). In this study D-mannose did not inhibit hemagglutination.

Thus hemagglutination of bovine erythrocytes by *Escherichia coli* isolated from bovine mastitis is generally mannose resistant and may be unrelated to pili and these results indicated that the simple sugar tested is not part of the bacterium or erythrocyte receptor site, it is possible that a more complex form of the sugar is a part of the receptor site which can not be blocked by the simple sugar. The use of blood types other than bovine erythrocytes may give different results. Hogan, et al. (1990) used bovine, sheep and guinea pig erythrocytes, and found that most mammary glands isolates demonstrate mannose sensitive hemagglutination.

the presence of hemagglutinin may convey invasive characteristics to *Escherichia coli* strains. There was an increased frequency of hemagglutination positive *Escherichia coli* isolates from clinical mastitis 75% (12 out of 16) compared with hemagglutination positive isolates from subclinical cases 52% (25 out of 48). These results are not surprising when the fact that most mammary glands infections occur by the ascending route is considered. bacteria probably first colonize the teat canal before the mammary tissue. The hemagglutinin may aid the colonization of the teat canal and cistern by the organism and may further give the organism a selective advantage in ascending to the all mammary tissues.

Escherichia coli is the most common, but not the only Gram-negative bacterium that causes mastitis. Therefore it was of interest to determine the capacity of other Gram-negative mammary pathogens to hemagglutinate. Results showed that *Escherichia coli* isolates hemagglutinated bovine erythrocytes much more frequently (58%) than did other mastitis pathogens tested including *Proteus* species (16%) and *Kelbsiella pneumoniae* (13%). Thus the fact that *Escherichia coli* is, by far, the most common Gram-negative cause of mastitis and is also the most efficient hemagglutinator may be related to the greater ability of the organism to attach to membrane surfaces as compared with other *Enterobacteriaceae.*

Hemagglutination is one of several potential virulence factors described for *Escherichia coli.* Until now, no one has defined the relationship of hemagglutination to these other factors, including hemolysin production and motility. Several studies have suggested that hemolysin production by *Escherichia coli* might be an important virulence factor for strains that cause mastitis (Cooke and Ewins, 1975 and Hill, 1981). The finding of a significant association between hemolysin production and hemagglutination further substantiates the role of these factors in the virulence of *Escherichia coli* mammary pathogens. Although hemagglutination and hemolysin production appear to be related phenomena, each factor can occur independently. The relationship of these two characteristics could be explained if they were controlled by the same plasmid or lysogenic bacteriophage. Studies have shown that plasmids may control the ability of an organism to hemagglutinate or hemolyze erythrocytes. Low et al. (1984) found that, hemolysin production and mannose-resistant hemagglutination were genetically linked in human isolates.

Motility has been suggested as a possible virulence factor in mammary gland infections. However, there was no association between motility and virulence, since most of *Escherichia coli* mastitis isolates and normal fecal isolates were motile. There was a tendency for flagella to be associated with organisms that were hemagglutination negative. Therefore, flagella may interfere with hemagglutination, possible by sterically hindering the bacterium-erythrocyte interaction or blocking the hemagglutinin present on the bacterial cell surface.

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