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Comparative Antibacterial Effects of the Whole and Diluted Human Breast Milk on *Some* PCR Detected Diarrheagenic *Escherichia coli* (DEC)

Anthony A. Adegoke

Department of Biochemistry and Microbiology, University of Fort Hare, Alice 5700, South Africa.

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This study was conducted to assess the comparative perspective effects of whole and diluted human breast milk in the face of other changing medical verdicts. The comparative antimicrobial effects of whole and diluted (50%) concentrations of human breast milk were assessed using conventional methods in Microbiology and polymerase chain reaction (PCR). Ten human breast milk samples were collected and tested against 4 isolates of diarrheagenic *Escherichia coli* (DEC) from stool samples of diarrheal patients. Activity indices of the breast milk with respect to ofloxacin as standard drug were determined. None of the sterility plates for the breast milk samples showed any growth of potential pathogen, but very scanty colonies of Lactic Acid Bacteria (LAB). Whole breast milk showed activity indices that ranged from 0.61 to 1.28 while diluted (50%) showed lower activity indices that ranged from 0 to 0.63. The test isolates, DEC exhibited resistance (percentage resistance) to ciprofloxacin (25%), gentamycin (50%), augmentin (50%), penicillin (75%), cotrimoxazole (100%) and chloramphenicol (100%). Multidrug resistance was also observed among all the test isolates. The whole (exclusive) concentration of breast milk showed much higher antimicrobial effects on the DEC than the diluted ($p < 0.01$). Due to the high activity in whole breast milk as observed in this study, the need for exclusive breast feeding for safe motherhood, to reduce infantile diarrhoea is once again re-established.

Keywords: Human breast milk, antimicrobial and diarrheagenic *Escherichia coli*.

Breast feeding has overbearing advantages over the infant-formula because of the presence of its rich constituents which guarantee early nourishment and protection against childhood killer diseases[1]. The human milk serves as the source of immunoglobulin and other protective factors to ward off foreign infective cells[2]. So, a child is supplied with an array of bioactive factors from milk which includes hormones, growth factors and colony stimulation factors, thus increasing the possibilities for survival and reduction of infantile mortality[1].

Breastmilk affords a child the availability of factors necessary in prompt maturation of gastrointestinal mucosa and reduction of infection[3], and other factors that interfere with disease pathogenesis. The protective effects of breastfeeding transcend the lactation period, because it serves as the baseline protection while the immune system begins to learn or develop following subsequent exposure[2]. So, it is generally believed that the breastfed infants are usually better protected than those fed with infant formula. Also, advocacy has been intensified globally that

* To whom all correspondence should be addressed.
E-mail: aadegoke@ufh.ac.za

protective breastfeeding is guaranteed when it is exclusive: without water supplement as it has sufficient water a child needs. The protective factor provided include specific antibodies to pathogens, inhibiting gut colonization by gut-philia[4]. Such antibodies involve involve B-lymphocytes, plasma cells, immunoglobulous and antibodies[5].

One of the gut-philic organisms and a threat of gastroenteritis in children and adult is *Escherichia coli*[6,7]. The *E. coli* strains classified as Diarrheagenic *Escherichia coli* (DEC) are known for their roles in diarrhoea among [6-8]. Infant mortality due to diarrhoea accounts for 0.3 of 3.6 million in Africa[9]. Enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), and enterohemorrhagic *E. coli* (EHEC) are known threat in infantile diarrhoea with high mortality[9,10].

This study becomes very important because of the emergence of total antibiotic resistant member of Enterobacteriaceae, in which the bacteria was resistant to the existing 26 antibiotics in the United States[11], leading to the death of the patient. The World Health Organization, WHO[12] placed some Enterobacteiaceae in the critical priority of those requiring new R and D drugs. The neonates, infants and children who are infected by these types of *E. coli* may have low survival probability, because the administration of some antibiotics in the last line of defence may be unsafe for them due to toxicity[13-15].

While breast feeding has long been known for effective way to check infantile diarrhoea, the practice is on the decline due to misconceptions and lack of recent reference materials on the antibacterial importance of breastfeeding[16]. This informed the research objectives to evaluate the perspective microbiological quality of the human breast milk.

MATERIALS AND METHODS

Identification/typing of the test isolates

The DEC was earlier identified, typed and reported in our previous reports[17] using the primer in table 1 below, as the same isolates have been used for that other research.

Antibiotic susceptibility testing (AST) of DEC

The standard disc diffusion method following the scheme of Clinical and Laboratory Standard Institute, CLSI[18] was employed for AST of the test isolates, DEC using conventional antibiotics. The antibiotics used include those inhibiting the synthesis of the cell wall (penicillin, cotrimoxazole, oxacillin, ceftazime, augmentin), protein (chloramphenicol, gentamycin) and nucleic acid (ofloxacin, ciprofloxacin). Young cultures at log phase were carefully transferred into peptone water which was adjusted to 0.5 McFarland standards (1.5×10^8 CFU/100 mL). The bacterial suspension was carefully spread on solidified Mueller-Hinton (MH) agar (Oxoid, UK) in Petri plate with the aid of sterile cotton-tipped applicator (swab sticks). The resulting culture was place in incubator for 10-15 minutes to ensure the bacterial suspension dries up into the agar. Antibiotic disc were carefully placed on the agar surface at equidistant from each other, using sterile forceps. This was place in the chiller at 4 ºC for 10-15 min to ensure proper diffusion of the disc. The resulting cultures were then removed and were incubated at 35 ºC ± 2 ºC overnight, after which the zones of inhibition were measured. The zones were interpreted using the interpretive manuals of the British Society for Antibiotic Chemotherapy, BSAC[19] and minimal inhibitory concentration (MIC) breakpoints for Enterobacteriacea[18].

Antimicrobial susceptibility testing of DEC to human breast milk

The agar well diffusion method of Perez et al.,[20] and BSAC[19] were used with modifications. The test isolates, DEC containing 2 strains ETEC, EIEC and EHEC were used. Mueller-Hinton agar (Oxoid, UK) and a turbidity of the test isolates equivalent to 0.5 McFarland standards (1.5×10^8 CFU/100 mL) were prepared. The bacterial suspension was carefully spread on solidified MH and wells bored using 6 mm cork borer. An exact measure of 0.2 ml of whole (100 %) and diluted (50 %) of the breast milk was introduced in separate wells on the inoculated agar. The resulting cultures were incubated for 35 ºC ± 2 ºC overnight. After incubation, the zones of inhibition were measured and the activity index with respect to positive control (standard antibiotic) determined as the ratio of zone of inhibition by breast milk zone of inhibition by antibiotics / zone of inhibition by standard antibiotics.

Statistical Analysis

One way anova (SPSS, Version 17) was used to determine the significance ($p < 0.01$) of the

difference in the antibacterial activity of the whole and diluted concentration of human breast milk.

RESULTS

The molecular identification whose primers are listed in table 1 shows 2 to be ETEC and the other 2 to be EIEC and EHEC. Human breast milk exhibited high antibacterial activity. Whole breast milk showed activity indices that ranged from 0.61 to 1.28 while diluted (50%) showed lower activity indices that ranged from 0 to 0.63. The whole (exclusive) concentration of breast milk showed much higher antimicrobial effects on the DEC than the diluted (Tables 2 and 3).

None of the sterility plates for the breast milk samples showed any growth of potential pathogen, but very scanty colonies of Lactic Acid Bacteria (LAB). The test isolates, *E. coli* exhibited resistance (percentage resistance) to ciprofloxacin

Table 1. Primers for species-specific identification of *Escherichia coli* and typing Diarrheagenic *Escherichia coli* (DEC)[17]

DEC	Genes	Primer	Amplicon size (bp)
E. coli	*Alr* gene	CTGGAAGAGGCTAGCCTGGACGAG AAAATCGGCACCGGTGGAGCGATC	366
ETEC	*sta*	ATTTTTCTTTCTGTATTGTCTT CACCCGGTACAAGCAGGATT	180
EIEC	*ial*	CTGGTAGGTATGGTGAGG CCAGGCCAACAATTATTTCC	320
EHEC	*hlyA*	GCATCATCAAGCGTACGTTCC AATGAGCCAAGCTGGTTAAGCT	534

Table 2. Zone of Inhibition of Breast Milk against Test Isolates 2 ETECs (1 and 2)

Undiluted Human Breast (Whole)			50 % Diluted Breast Milk			Diameter of Standard Antibiotics (mm)
Sample codes	Zone of Inhibition (mm)	Activity Index	Sample codes	Zone of Inhibition (mm)	Activity Index	
UBM 1-01	12	0.67	DBM 1-01	10	0.56	
UBM 1-02	18	1.00	DBM 1-02	10	0.56	
UBM 1-03	14	0.78	DBM 1-03	6	0.33	
UBM 1-04	16	0.89	DBM 1-04	7	0.38	
UBM 1-05	14	0.78	DBM 1-05	6	0.33	18
UBM 1-06	18	1.00	DBM 1-06	8	0.44	
UBM 1-07	11	0.61	DBM 1-07	9	0.50	
UBM 1-08	16	0.89	DBM 1-08	8	0.44	
UBM 1-09	15	0.82	DBM 1-09	10	0.56	
UBM 1-10	17	0.94	DBM 1-10	9	0.50	
UBM 2-01	18	1.13	DBM 1-01	0	—	
UBM 2-02	17	1.06	DMB 2-02	0	—	
UBM 2-03	15	0.93	DMB 2-03	0	—	
UBM 2-04	18	1.13	DMB 2-04	0	—	
UBM 2-05	15	0.93	DMB 2-05	0	—	16
UBM 2-06	16	1.00	DMB 2-06	9	0.56	
UBM 2-07	11	0.68	DMB 2-07	10	0.63	
UBM 2-08	12	0.75	DMB 2-08	7	0.44	
UBM 2-09	18	1.13	DMB 2-09	6	0.37	
UBM 2-10	12	0.75	DMB 2-10	10	0.63	

(25%), gentamycin (50%), augmentin (50%), penicillin (75%), cotrimoxazole (100%) and chloramphenicol (100%) (Table 4). Multidrug resistance was also observed among all the test isolates (Table 5). Two strains of ETEC showed different profile to conventional antibiotics and human breast milk. EC – 01 (ETEC) showed resistance to five antibiotics including penicillin, ofloxacin, augmentin, chloramphenicol, oxacillin, and cotrimoxazole in that order, while EC – 02 (ETEC) showed resistance to only four: ofloxacin, chloramphenicol, oxacillin and cotrimoxazole. EIEC and EHEC showed resistance to seven and eight antibiotics respectively.

DISCUSSION

The observed antibacterial activities of human breast milk on DEC from diarrheal patients explains the reason why it is still the preferred prophylaxis for infantile diarrhoea (Lorget *et al*., 2002). It is also in line with the reports of Berkhout *et al*. (2003) that the human milk contain antibacterial effect but the quality depends on the

Table 3. Zone of Inhibition of Breast Milk against EIEC (3) and EHEC (4)

Undiluted Human Breast (Whole)			50 % Diluted Breast Milk			Diameter of
Sample codes	Zone of Inhibition (mm)	Activity Index	Sample codes	Zone of Inhibition (mm)	Activity Index	Standard Antibiotics (mm)
UBM 3-01	11	0.73	DBM 3-01	8	0.53	
UBM 3-02	12	0.80	DBM 3-02	0	--	
UBM 3-03	16	1.06	DBM 3-03	0	--	
UBM 3-04	14	0.93	DBM 3-04	8	0.53	
UBM 3-05	13	0.86	DBM 3-05	9	0.60	15
UBM 3-06	14	0.93	DBM 3-06	8	0.53	
UBM 3-07	17	1.13	DBM 3-07	0	--	
UBM 3-08	18	1.20	DBM 3-08	9	0.60	
UBM 3-09	16	1.06	DBM 3-09	7	0.46	
UBM 3-10	13	0.86	DBM 3-10	8	0.53	
UBM 4-01	18	1.13	DBM 3-01	8	0.44	
UBM 4-02	12	0.75	DMB 4-02	10	0.56	
UBM 4-03	16	1.00	DMB 4-03	0	--	
UBM 4-04	17	1.06	DMB 4-04	0	--	
UBM 4-05	15	0.93	DMB 4-05	6	0.33	
UBM 4-06	15	0.93	DMB 4-06	6	0.33	16
UBM 4-07	17	1.06	DMB 4-07	0	--	
UBM 4-08	14	0.87	DMB 4-08	0	--	
UBM 4-09	13	0.81	DMB 4-09	7	0.44	
UBM 4-10	18	1.13	DMB 4-10	0	--	

Table 4. Antibiotic Profile of the four DEC

Isolates' Code	Antibiotics' Profile								
	PN	CEP	CN	OFL	AU	CHL	CPX	OX	SXT
EC – 01 (ETEC)	R	S	S	R	R	R	S	R	R
EC – 02 (ETEC)	S	I	S	R	S	R	S	R	R
EC – 03 (EIEC)	R	R	I	R	S	R	R	R	R
EC – 04 (EHEC)	R	R	R	R	R	R	S	R	R

KEY: Penicillin (PC), Ceftazidime (CEP), Gentamycin (CN), Ofloxacin (OFL), Augmentin (AU), Chloramphenicol (CHL), Ciprofloxacin (CPX), Oxacillin (OX), Cotrimoxazole (SXT)

Table 5. Multidrug Resistance Pattern

Isolate's code	Multidrug Resistance Pattern
EC – 01 (ETEC)	PN-OFL-AU-CHL-0X-SXT
EC – 02 (ETEC)	OFL-CHL-OX-SXT
EC – 03 (EIEC)	PN-CEP-OFL-CHL-CPX-OX-SXT
EC – 04 (EHEC)	PN-CEP-CN-OFL-AU-CHL -OX-SXT

human health. This probably explains the reason why few breast milk samples here showed no activity.

Higher antibacterial activity than ofloxacin on some multidrug resistant test (DEC) isolates gives further credence to the place of breast feeding in appropriate motherhood, irrespective of civilization. The higher antibacterial effects in whole breast milk than 50 % concentration is in agreement with retrospective observation of Berkhout *et al*.[21]. This is evident in the activity indices that ranged from 0.61 to 1.28 to whole concentration compared to 0 to 0.63 for 50 % concentration. This difference was statistically significant ($p < 0.01$) and it gives a perspective voice in support of advocacy for exclusive breast feeding[22, 23].

While human breast milk is expected as the ideal food for the new born during the first six months of life, it effect will be pronounced if it is exclusively without adding water. This is because of complete nutrition, early protection against illness, safe and healthy food are guaranteed in it [24]. Igumbor et al.[25] observed that freshly expressed human milk may increase the microbial flora of the neonate and these non-pathogenic microbial flora compete favourably with pathogenic diarrheagenic *E. coli*. This might be the reason for the presence of very scanty colonies of Lactic Acid Bacteria (LAB) in the human breast milk when it was tested for sterility.

Earlier studies from milk establishes two main classical mechanisms for immunological protection in form of IgA, IgG, IgM and other antimicrobial ligands[26]. High natural adaptiveness to the intestinal mucosal sites where they act and to the sharp changes in pH around the gut (due to ptyalin in saliva, acid in stomach, pancreatic juice /bile salt that sharply changes acidity to alkalinity in duodenum etc), unlike some conventional antibiotics whose metabolisms are partly affected by these biochemical dynamics[27].

CONCLUSION

Whole (exclusive) concentration of breast milk showed higher antimicrobial effects on the multiple antibiotic resistant Diarrheagenic *Escherichia coli* than the 50 % diluted concentration. The effect compares more favourably than few conventional antibiotics. This reiterates the need for exclusive breast feeding for safe motherhood, to prevent infantile diarrhea.

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