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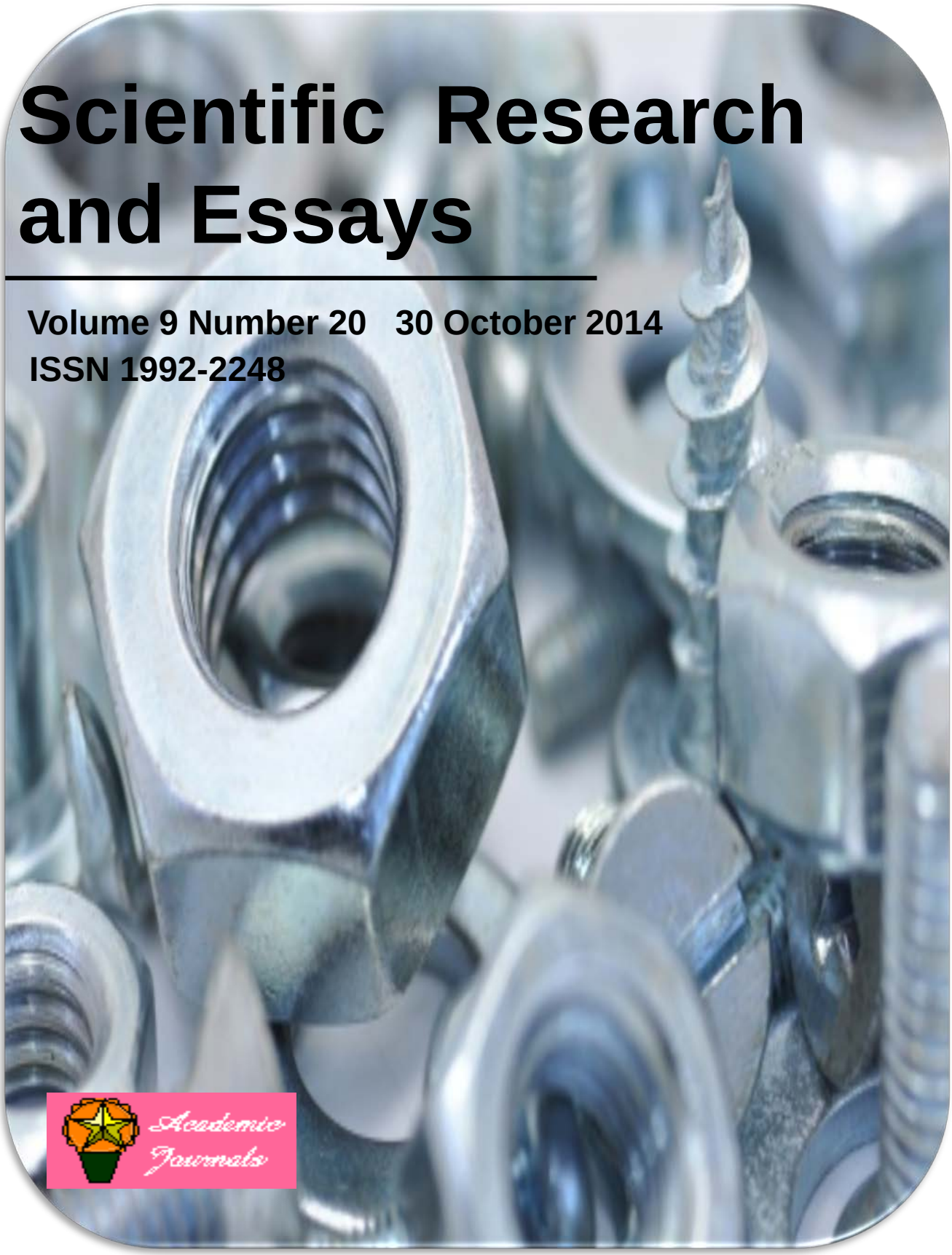

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Scientific Research and Essays

Full Length Research Paper

Isolation and identification of *Staphylococcus aureus* from bovine and the detection of its coagulase gene (*coa*) using polymerase chain reaction (PCR)

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Two hundred and fifty different samples were collected from bovine and examined for the presence of staphylococcal bacteria. 189 isolates were able to grow on the mannitol salt agar (MSA), known as staphylococci. Coagulase test revealed that 165 isolates were able to produce this enzyme; 138 of these isolates were *Staphylococcus aureus which* appeared in 55.2% of the isolates. Deoxyribonuclase (DNAase), urase and beta haemolysis activities of the isolates were also investigated and it showed 90.69, 86.23, and 87.86% of the isolates respectively. An enzymatic examination of the isolates was combined in numerous tests like catalase test, coagulase test, non- producing oxidase, sugar fermentation, oxidative and fermentation test, liquefaction of gelatin and MR-VP test. The polymerase chain reaction (PCR) amplification of *coa* gene products of *S. aureus* showed the following: gene product of 500 bp (22.5%); 650 bp (15%); 800 and 850 bp (25% for each); and 600 bp (12.5%).

Key words: *Staphylococcus aureus*, bovine, coagulase, coa gene, polymerase chain reaction (PCR).

INTRODUCTION

Staphylococcus aureus can infect any part of the body; it causes some diseases in humans and animals, ranging from skin infection, food poisoning, brain abscesses and outbreak in post operative wound infection (Kenneth, 2008). In cows, it causes some important diseases; for example, mastitis (clinical and sub clinical) and respiratory tract infection, skin sepsis, tick pyemia in lamb and contagious skin necrosis (Kinight, 1999). *S. aureus* is one of the major causes of serious infections, passively colonizing human skin and nasal passages of healthy individuals; although this opportunistic pathogen colonizes without causing diseases (Kloos et al., 1992). One of the specific features of *S. aureus* is its ability to acquire resistance to antibiotics (Ohta et al., 2004). The coagulase protein is an important phenotypic determinant and is accepted as a major virulence factor of *S. aureus*. In coagulase-negative staphylococci (CoNS) originating from bovine mastitis, methicillin resistance is more common (Gindonis et al., 2013). The analysis of coagulase encoding *S. aureus* DNA *coa* gene has demonstrated variable sequences in the 3´ end coding region (Goh et al., 1992). This region contains a polymorphism repeat region that can be used to differentiate *S. aureus* isolates. This characteristic has

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Table 1. Numbers and percentages of bovine's samples that gave growth on MSA and positive coagulase result *(Staphylococcus)*.

Sample /No	Growth on MSA		Coagulae negative	Coagulase positive		Other coagulase +ve Staphylococcus		Suspected *S .aureus*	
	No.	%	No.	No.	%	No.	%	No.	%
Milk/70	44	62.9	4	40	90.1	6	8.6	34	48.6
Nasal Swabs/70	55	78.6	10	45	81.8	8	11.4	37	52.9
Vaginal Swabs/70	50	71.1	3	47	94	6	8.6	41	58.6
Urine/40	40	100	7	33	82.5	7	17.5	26	65
Total/250	189	75.6	24	165	87.3	6.75 ±0.957		34.5 ± 6.351	

X^2=20.06, P < 0.01.

been used to type *S. aureus* isolates of human and bovine origin (Guler et al., 2005). The aim of this study is to investigate the presence of virulence (coa) gene in *S. aureus* that is isolated from bovine.

MATERIALS AND METHODS

Samples collection

Two hundred and fifty samples were obtained from pathogenic and non pathogenic cases of bovine (70 milk sample from clinical and subclinical mastitis, 70 vaginal swabs, 70 nasal swab, and 40 urine sample) from AL- Tathamine Bovine Station in the Waste Province. Each sample was collected from different animals. The specimens were transported to the laboratory directly and inoculated onto plates of mannitol salt agar (MSA); they were incubated at 37°C for 24 h. All colonies from primary cultures were purified by subculturing onto MSA medium and incubating at 37°C for 24 to 48 h (Talan et al., 1989).

Biochemical testes

Different tests were performed for bacterial identification of *S. aureus*. The tests were catalase test; oxidase test; coagulase; clumping factor test; free coagulase test; Vogas- Proskauer test; ONPG ; Latex agglutination (MASTSTAPH); heamolysin production; NAase production test; urease test; O\F test; gelatin test; methyl red test and sugar fermentation test. The tests were done using the methods of Treagan and Pulliam (1982), Finegold and Baron (1986), Baron et al. *(*1994) and Macfaddin, (2000).

API Staph test was done for the conformity of the identification of isolates. Homogenous bacterial suspension was prepared with a turbidity equivalent to 0.5 McFarland stander.

Molecular study using polymerase chain reaction (PCR) technique

DNA kit was used for isolating DNA from bacterial cells based on the method of Sambrook et al. (1989) (Promega USA). Forty isolates from bovine (20 from milk, 10 from vaginal samples, 10 from nasal samples) were subjected to molecular study by using PCR technique. DNA from the 40 *S. aureus* isolates was extracted by purification using Promega kit to detect the presence of *coa* gene. The successful binding of gene appeared as clear band under U. V. light. The *coa* gene was studied according to the protocol of Hookey et al. (1998). Genomic DNA was amplified by using the primers given below:

5´-ATA GAG ATG CTG GTA CAG G-3´.
5´-GCT TCC GAT TGT TCG ATG C-3´.

PCR products were detected by electrophoresis on the agarose gel at 1%. 10 µl from PCR product was inoculated in each well from agarose gel. DNA ladder marker was used to measure the amplification molecular weight from PCR product compared to DNA marker. After 30 min examination was done under UV light (Sambrook et al., 1989).

RESULTS

The results showed that 189 out of 250 bovine samples (75.6%) gave positive result for *Staphylococcus* (able to grow on mannitol salt agar) and 165 isolates out of 189 (87.3%) were coagulase positive *Staphylococcus*. The urine samples revealed high percentage of isolation on MSA (100%), while nasal samples gave the lower percentage (62.9%). Coagulase results show that the high percentages of positive test were from vaginal isolates 47/50 (94%), while 45/55(81.8%) of nasal swabs isolates were coagulase positive. There were significant differences (P < 0.01) between numbers of isolation in different samples (Table 1).

Bacterial identification

Biochemical tests were used to confirm the identity of *S. aureus* isolates. *S. aureus* isolates were similar in some biochemical tests like catalase, oxidase, coagulase, O/F, ONPG, VP, MR, sugar fermentation, gelatin liquefaction, latex agglutination and API Staph. All tests were positive at 100%. On the other hand, *S. aureus* isolates differ in other tests like DNAase, urease and haemolysis, with average of 90.8, 87.23 and 87.86%, respectively. Most coagulase positive *S. aureus* isolates gave DNAase positive results. On the other hand, *S. aureus* isolates of bovine origin produced beta- haemolysis (Table 2).

Molecular genetics study results (PCR on *coa* gene)

The results of DNA amplification of *coa* gene in bovine

Table 2. Percentage of DNAase, Urease, Haemolysin reactions in the bovine *S. aureus* isolates.

Test	Milk	Nasal swabs	Vaginal swabs	Urine	Average	
DNAase	97.05% 33/34	91.89% 34/37	85.36% 35/41	88.46% 23/26	90.80%	X2= 3.19, P =0.363
Urease	91.17% 31/34	83.78% 31/37	85.36% 35/41	84.61% 22/26	87.23%	X2= 0.91, P = 0.808
Haemolysis	94.11% 32/34 X2=1. 063, P = 0.587	81.08% 30/37 X2=1.899, P = 0.386	87.80% 36/41 X2= 137, P =0.933	88.46% 23/26 X2=0.299, P =0.891	87.86%	X2= 2.81, P = 0.421

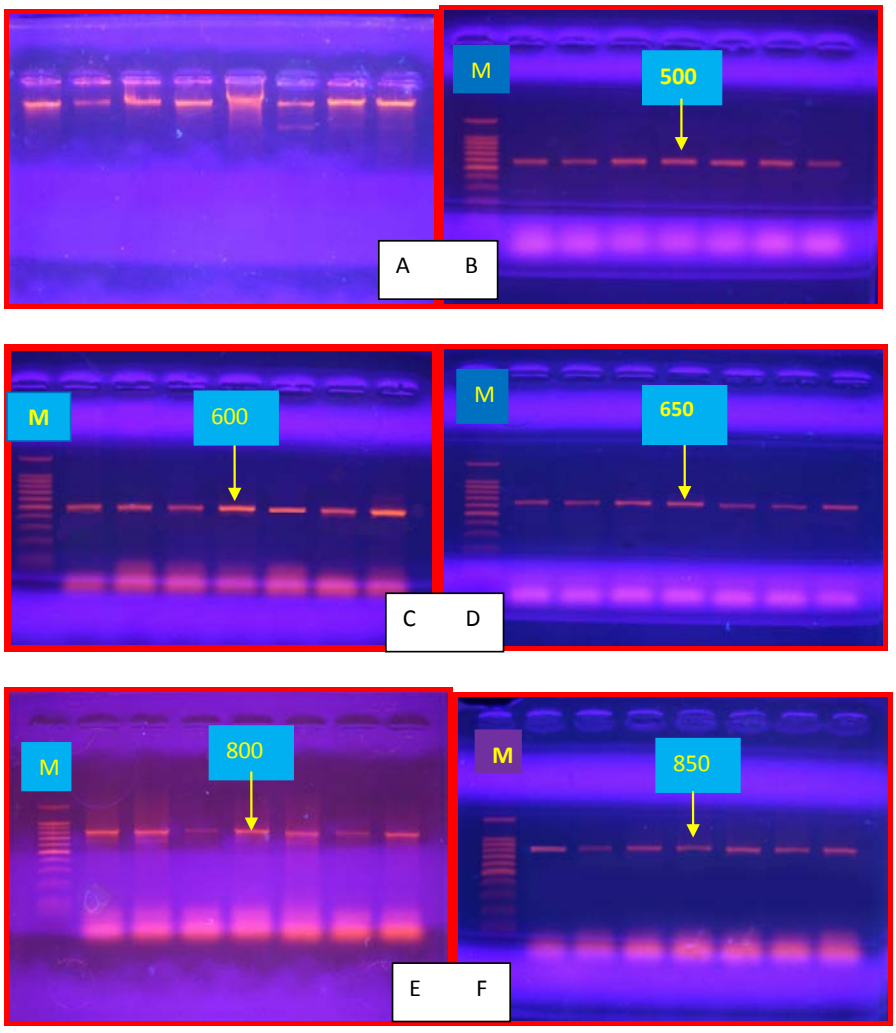

Figure 1. Electrophoresis in 1% agarose for *S. aureus* whole DNA (A) and *coa* gene showing different size, (B) 500bp; (C) 600 bp; (D) 650 bp; (E) 800 bp; (F) 850 bp; M= DNA ladder.

isolates revealed that PCR products were 500, 600, 650, 800, and 850 bp with 22.5, 12.5, 15, 25, and 25% respectively as a single band in 38/40 (95%) of isolates (Figure 1). From a total of forty studied isolates, there were significant differences (P< 0.01) between *coa* gene products in all the isolates (Table 3).

Table 3. Number and size product of *coa* gene in the bovine *S. aureus* isolates.

Sample	No. of isolates	Size of product bp					Total
		500	600	650	800	850	
Milk	20	9	0	1	10	0	20
Vaginal swabs	10	0	5	5	0	0	8
Nasal swabs	10	0	0	0	0	10	10
Total	40	9	5	6	10	10	38
Percentage		22.5%	12.5%	15%	25%	25%	95%

P > 0.05.

DISCUSSION

In this present study, different samples were collected from bovine (healthy and infected) for the isolation of *S. aureus.* This was done to study the properties of one of the most important pathogens in different samples of this microorganism responsible for economic loss and public health problems. This microorganism was isolated in 52.2% (milk, 48.57%; nasal swab, 52.85%; vaginal swab, 58.75% and urine, 52%). Kav et al. (2011) investigated the presence of *S. aureus* and staphylococcal enterotoxin (SE) genes in cheese samples. From a total of 127 cheese samples, 53 isolates (41.7% of the samples) were identified.

S. aureus isolates from bovine were similar in some biochemical tests results at 100%, and differ in other tests like, DNAase, Urease and heamolysis at 90.80, 87.23 and 87.86%, respectively. This result showed that *S. aureus* isolates from bovine are able to produce beta-haemolysin. Most of *S. aureus* isolates were able to produce urease enzyme at different percentages. Other study indicated that *S. aureus* strains were isolated in 481 (39%) samples. Of the 481 isolates of *S. aureus* tested, 255 (53%) were positive for one or more SE genes, and thirty-five different enterotoxin gene profiles were distinguished among the isolates; thus suggesting that the pathogenic potential of *S. aureus* may be of greater importance than previously thought (Bianchi et al., 2014).

Associations between bacterial genotype and outcome of bovine clinical *S. aureus* mastitis were investigated (Lundberg et al., 2014). The results of the presence of *coa* gene revealed that polymorphism phenomena of this gene resulted in different molecular weights of 500, 600, 650, 800 and 850 bp. The PCR test was used to detect the gene coded for producing coagulase enzyme that accounts for virulence of bacteria. *S. aureus* is able to produce different molecular weights found in the polymorphism phenomena and this enzyme is used to classify isolates depending on the different molecular weights in outbreak of epidemiological studies (Da Silva and Da Silva, 2005). Based on the detection of the most prevalent clones in a herd or region, appropriate antibiotic therapy and specific immunization can be used for the treatment and control of staphylococcal mastitis (Silveira et al., 2014). The prevalence of coagulase-positive staphylococci (CPS) was studied among 390 samples of ewe milk. Fifty-seven (14.85%) samples of tank milk and all samples (6) of silo milk gave a positive result. The detection of the coa gene from milk samples could help to assess the microbiological safety of raw milk intended for direct use in the dairy industry (Linage et al., 2012).

Conflict of Interests

The authors have not declared any conflict of interests.

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Scientific Research and Essays

Full Length Research Paper

Charaterization of galactose-specific lectin from the skin mucus of African catfish *Clarias gariepinus* [a]Burchell, 1822

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A galactose-specific lectin was purified from the skin mucus of African catfish (*Clarias gariepinus*) and the physicochemical properties determined. Phosphate buffered saline extract of the skin mucus of African catfish specifically agglutinated erythrocytes of rabbit and human blood group B, but did not agglutinate bat, rat, hen and human blood A and O erythrocytes. The haemagglutinating activity of the lectin was completely inhibited by lactose while galactose and melibiose inhibited the activity to some extent and was calcium-independent. The purified lectin has a native and subunit molecular weight of 63, 000 and 20, 000 Daltons, respectively suggesting a homotrimeric structure for the protein. The protein contained 126 amino acid residues per subunit. This was characterized by large amount of polar amino acids that constituted about 60% of the total amino acids. The lectin showed maximum activity over the pH range 6 – 9 and was heat stable up to 50°C. Ethylenediaminetetraacetic acid (EDTA) had no inhibitory effect on its haemagglutinating activity. Periodic acid–Schiff (PAS) staining showed that the lectin was not a glycoprotein. Chemical modifications of serine and arginine residues of the protein did not affect its haemagglutination activity while modifications of cysteine, tryptophan and histidine residues led to total loss of its activity. The study concluded that African catfish skin mucus lectin exhibited similar physicochemical properties with lectins from other fish skin mucus.

Key words: African catfish, Lectin, haemagglutination, skin mucus, galactose-specific, *Clarias gariepinus*

INTRODUCTION

Lectins are heterogeneous class of proteins that bind specifically and reversibly to carbohydrates (Lis and Sharon, 1998). They are widely distributed in nature and can be found in almost all living organisms including bacteria, fungi, plants, invertebrates and vertebrates and may be either soluble or membrane-bound (Lis and Sharon, 1986; Drickamer, 1995; Khan and Khan, 2011). However, majority of the studies on lectin have been carried out on plant lectins, particularly on the seeds of legume species (Sharon and Lis, 1990; Chrispeels and Raikhel, 1991). They have attracted immense interest because of the various biological activities such as cell agglutination, antiviral, antitumor, antiproliferative, antifungal and immunomodulatory (Wang et al., 1996;

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Fang et al., 2010; Melo et al., 2011; Souza et al., 2011). They have become important class of proteins with a wide variety of biochemical uses which include their role in bioseparation and reversible immobilization.

Since the discovery of animal lectins, most animal lectins studied occur inside the body like in the plasma, cell cytoplasm or at the cell surface (Tasumi et al., 2004). However, some lectins outside the body have also been studied especially in fish. A large numbers of researchers have intensively purified lectins from skin mucus of various fish species (Alexander and Ingram, 1992; Suzuki et al., 2003; Tsutsui et al., 2007). Skin mucus lectins of several species of fish have been shown to bind microorganisms (Tasumi et al., 2002; 2004: Tsutsui et al., 2006, 2007). These findings suggest that lectins contribute to the self-defense mechanism against these microorganisms and also support the report that fish skin secretions contain immunoglogulins as well as various innate-defense factors such as complements, C-reactive protein, lysozyme, hemolysin, anti-microbial peptides and lectins (Suzuki et al., 2003; Argayosa et al., 2011; Benhamed et al., 2014).

African catfish (*Clarias gariepinus*) is essentially an omnivorous bottom feeder; however they are known to be very tolerant of extreme environmental conditions. To fight off pathogenic microorganisms, the epidermis and its secretion, the mucus acts as a barrier between the fish and the environment (Benhamed et al., 2014). The composition and rate of mucus secretion has been observed to change in response to microbial exposure or to environmental perturbations such as hyperosmolarity and acidity (Ellis, 2001; Subramanian et al., 2008). Consequently, study of the physical and biological properties of the lectin in the skin mucus of African catfish (*C. gariepinus)* would give insight to the roles of this molecule in host defense and in the known adaptability of the catfishes to diverse environmental conditions. Hence, this study reports the purification of a galactose-specific lectin from the skin mucus of the African catfish *C. gariepinus* and physicochemical properties of this lectin.

MATERIALS AND METHODS

African catfish (*C. gariepinus* Burchell, 1822) was obtained from the Fishery Unit, Osin Farm Ltd, Yakoyo, via Ile Ife, Osun State, Nigeria. Fresh human blood was obtained from healthy donors and animal blood from rabbits, hen and Wistar albino rats supplied by the Animal Science, Department of Obafemi Awolowo University, Ile-Ife. Red blood cells were obtained from the blood samples and fixed with glutaraldehyde according to the method of Kuku and Eretan (2004). Sepharose 4B, divinyl sulphone, sugars and molecular weight standards were from Sigma Chemical Company, St. Louiz, MO, USA. All other reagents used were of analytical grades.

Extraction of crude lectin from fish skin mucus

Four African catfishes (each between 300 to 400 g body weights) were used per run of the purification procedure. The skin mucus was collected from the ventral part of the fish by gentle scraping of the skin using a soft rubber spatula with enough care not to damage the skin to avoid contamination with blood or secretion of epithelial cells/peripheral circulation and homogenized in five volumes of 10 mM phosphate buffer, pH 7.2 containing 50 mM NaCl. The homogenate was centrifuged for 30 min at 10,000 rpm using refrigerated centrifuge. The supernatant obtained constituted the skin mucus crude extract.

Protein concentration determination

Protein concentration of the crude extract and other fraction were determined by method of Lowry et al. (1951) using Bovine Serum Albumin (BSA) as standard.

Purification of Lectin

The crude extract of the skin mucus of the African catfish was applied on to a Sephadex G-150 column (2.5 x 40 cm) previously equilibrated with 10 mM phosphate buffer, pH 7.2 containing 50 mM NaCl. The protein was eluted with the same buffer. Fractions of 4 ml were collected, elution was monitored at 280 nm and the fractions were assayed for hemagglutinating activity. The peak with lectin activity was pooled, dialysed and chromatographed on a column (0.5 x 20 cm) of lactose-sepharose 4B equilibrated with 10 mM phosphate buffer containing 50 mM NaCl (pH 7.0). The unbound proteins were washed off from the column with 100 ml of equilibrating buffer. The adsorbed protein was eluted with 0.2 M lactose in 10 mM phosphate buffer after which it was exhaustively dialyzed to remove the bound sugar.

Haemagglutinating activity

Agglutination of the red blood cells by the crude extract and the various fractions that were obtained during purification was estimated as described by Pattanapanyasat et al. (2010). A serial two-fold dilution of the lectin solution in U-shaped microtitre plates (100 µl) was mixed with 50 µl of a 2% suspension of human and various animals erythrocytes in phosphate buffered saline, pH 7.2 at room temperature (All erythrocytes were fixed with 1% glutaraldehyde). The plate was left undisturbed for 1 h for agglutination to take place. The haemagglutination titre of the lectin expressed as the reciprocal of the highest dilution exhibiting visible agglutination of erythrocytes was reckoned as one haemagglutinating unit. Specific activity was the number of haemagglutination units per mg protein.

Blood group specificity

The blood group specificity of the crude extract was established using erythrocytes from different blood groups of the ABO system and other animals.

Sugar inhibition test

The hemagglutination inhibition tests to investigate inhibition of lectin-induced hemagglutinations by various carbohydrates were performed in a manner analogous to the hemagglutination test (Tsivileva et al., 2001). A serial dilution of the sample was made until the end-point causing haemagglutination was obtained. 50 µl of the 0.2 M sugar solution was added to each well and allowed to stand for 30 min at room temperature and then mixed with 50 µl of

2% rabbit erythrocyte suspension. The haemagglutination titres obtained were compared with a non-sugar containing blank. 0.2 M of each sugar in PBS was prepared. The sugars tested include: D(+)-glucose, D(+)-mannose, D(+)-arabinose, D(+)-glucosamine hydrochloride, D(-)-sorbose, sorbitol, mannitol, maltose, sucrose, fructose, lactose, 1-O-methyl-α-glucopyranoside, rhamnose, raffinose, galactose, dulcitol, cellobiose, 2-deoxy-α-D-glucose, melibiose, L-fucose, melezitose and N-acetylglucosamine.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) was performed on 10% gels in Tris-glycine buffer, pH 8.9. The proteins were stained with Coomassie Brilliant Blue R, while the presence of covalently bound sugar in the lectin was detected by staining the gels with periodic acid Schiff reagent (PAS staining) (Packer et al., 2002).

Molecular weight determination

The purified lectin was subjected to Sodium Dodecyl Sulfate – Polyacrylamide gel electrophoresis (SDS–PAGE) for subunit molecular weight determination in accordance with the procedure of Weber and Osborn (1975) using the following protein markers: Bovine serum albumin (Mr 66,000), Ovalbumin (Mr 45, 000), Pepsin (Mr 38,000), Trypsinogen (Mr 24, 000), β-Lactoglobulin (Mr 14, 000). Gel filtration on a Sephadex G-100 column (2.5 × 100 cm), which had been calibrated with molecular weight markers was carried out to determine the native molecular weight of the lectin. 5 ml of each standard protein was applied to the column and ran separately using a 10 mM phosphate buffer, pH 7.2 as eluants at a flow rate of 10 ml/h. Fractions of 5 ml were collected and the elution of the standard proteins was monitored at 280 nm. The void volume (V_o) of the column was determined using Blue dextran (elution monitored at 620 nm). The molecular weight markers used were: Creatine phosphokinase (Mr 81,000; 5 mg/ml), bovine serum albumin (Mr 66,000; 5 mg/ml), Ovalbumin (Mr 45,000; 5 mg/ml), Chymotrysinogen (Mr 25,000; 5 mg/ml), and Lysozyme (Mr 14,000; 5 mg/ml).

Effect of temperature on haemagglutinating activity

The effect of temperature on the haemagglutinating activity was determined as described by Sampaio et al. (1998). Aliquots of lectin were incubated at different temperatures (30 to 90°C) for 30 min then rapidly cooled in ice and assayed for agglutinating activity. Agglutinating activity of the lectin sample kept at 20°C for 30 min was used as control.

Effect of pH on haemagglutinating activity

The effect of pH on the haemagglutinating activity was measured by incubating the samples in the following buffers at different pH values; 0.2 M citrate buffer, pH 3 to 6; 0.2 M Tris-HCl buffer, pH 7 and 8; and 0.2 M glycine-NaOH buffer, pH 9 to 11. After 1 h, the haemagglutination activity of the lectin was determined. The control values were the agglutination titre of the lectin in PBS, pH 7.2.

Effect of EDTA and divalent cations

The effect of ethylenediaminetetraacetic acid (EDTA) and divalent cations on the lectin activity was carried out as described by Wang et al. (1996). The purified lectin sample was dialysed against 10 mM EDTA for 24 h and the hemagglutination activity of the demetallized lectin was determined. The treated lectin was then incubated with 50 µl each of the following cations: 10 mM $MgSO_4$, $BaCl_2$, $MnCl_2$, $FeCl_3$, $CaCl_2$ and $SnCl_2$ for 2 h in order to evaluate their capacity to restore haemagglutination.

The Ouchterlony double diffusion experiment

1.5% (w/v) agar solution in PBS containing 0.01% (w/v) sodium azide was prepared. The solution was slowly heated until the agar had completely dissolved and poured into clean Petri dishes. A well was made at the centre of each Petri dish and four other wells equidistant from the centre were made around it. 50 µl of the lectin sample was placed in the centre well, and 50 µl of the polysaccharide (250, 100, 50 and 10 mg/ml of each polysaccharide) was placed in the surrounding wells. The polysaccharides tested were, inulin, dextrin, glycogen, starch and the polysaccharide (galactomannan) from Afzelia africana seeds.

Amino acid analysis

The purified lectin was subjected to analysis of amino acid content. The amino acid composition of the lectin sample was determined using methods described by Spackman et al. (1958). The sample was hydrolyzed, evaporated in a rotary evaporator and loaded into the Technicon sequential Multi-sample Amino Acid Analyzer (TSM). The nitrogen content of the sample was determined by Kjeldhal method.

Effect of chemical modification of amino acid residues on hemagglutinating activity

Modification of tryptophan residues with N-bromosuccinimide (NBS) was carried out according to the method of Spande and Witkop (1967). 10 µl aliquot of 10 mM NBS (in water) was added to 100 µllectin sample (1 mg/ml in 50 mM sodium acetate buffer, pH 6.0) with rapid mixing, 10 µl of the reagent was then added to the lectin sample every 10 min at 20°C for 1 h. Excess reagent (NBS) was removed by dialyzing the solution against distilled water after which 100 µl aliquot was removed from the dialyzed solution and assayed for residual hemagglutinating activity. Lectin incubated with PBS in the absence of NBS served as control.

Reduction of the thiol groups was carried out by incubating 100 µl of the lectin (1 mg/ml) in 50 mM phosphate buffer (pH 8.0) with 10 µl of 0.1 mM 5, 5'- dithiobis-(2-nitrobenzoic acid) (DTNB) at 27°C (10 µl of the reagent was added at 15 min intervals for 1 h). Excess reagent was removed followed by determination of residual hemagglutinating activity. Lectin incubated in the absence of DTNB served as control.

For serine modification, the lectin (100 µg) in 100 µl of 50 mM Tris-HCl buffer (pH 7.4) was incubated with10 µl of 5 mM phenyl methyl sulfonyl fluoride (PMSF) at 27°C (10 µl of the reagent was added at 15 min intervals for 1 h). Excess reagent was removed followed by determination of residual hemagglutinating activity. Lectin incubated in the absence of PMSF served as control (Habeeb, 1972).

Phenylglyoxal was used for modification of arginine residues (Riordan, 1979). 100 µl of 1 mg/ml lectin sample in PBS, pH 7.5 was incubated with 10 µl of 10 mM phenyglyoxal (in 0.1 M sodium carbonate, pH 8.0) at room temperature for 1 h (with addition of 10 µl of reagent every 15 min). The modified lectin sample was dialyzed exhaustively to remove excess reagent and assayed for hemagglutinating activity as described above.

Histidine residues were modified with diethyl pyrocarbonate according to the method of Ovaidi et al. (1967). The lectin (100 µl, 1

Table 1. Blood group specificity of phosphate buffered saline extract of African catfish skin mucus.

Erythrocyte	Blood group	Haemagglutination titre
Human		
	A	2^0
	B	2^2
	O	2^0
Bat		2^0
Rabbit		2^6
Rat		2^0
Hen		2^0

Table 2. Inhibition of haemagglutinating activity of African catfish skin mucus lectin by different sugars.

Sugar	Minimal inhibitory concentration (mM)
Sorbose	-
Galactose	6.25
Glucose	-
Mannose	-
Fructose	-
Lactose	0.5
Maltose	-
Sucrose	-
Dulcitol	-
Sorbitol	-
Mannitol	-
Melibiose	6.25
Cellobiose	-
L-Rhamnose	-
L-Fucose	-
Raffinose	-
Melezitose	-
Arabinose	-
N-Acetylglucosamine	-
Glucosamine-HCl	-
Methyl-α-O-glucopyranoside	-
2-deoxy-glucose	-
Dashes indicate no inhibition at a concentration of 200 mM for sugar	

mg/ml) in 0.1 M phosphate buffer (pH 7.2) was mixed with 20 mM diethyl pyrocarbonate freshly prepared in absolute ethanol for 1 h. Excess reagent was removed from the solution by dialysis followed by determination of residual haemagglutinating activity. Lectin sample in the absence of diethyl pyrocarbonate served as control.

RESULTS

It was observed that African catfish skin mucus lectin agglutinated only erythrocytes from human blood Group B, but exhibited no haemagglutinating activity when it was checked against human A and O blood cells. The haemagglutinating activity was more pronounced with rabbit blood erythrocytes but showed no specificity for rat, bat and hen erythrocytes (Table 1).

The hapten inhibition studies to define the sugar specificities of the crude extract showed that lactose completely inhibited the haemagglutinating activity with minimum inhibitory concentration of 0.5 mM (Table 2). Other galactose–containing sugar like melibiose and

Table 3. Summary of purification procedures.

Fraction	Total protein (mg)	Total activity (HU)	Specific activity (HU/mg)	Percentage yield	Purification fold
Crude extract	128.9	64	0.50	100	1
Gel filtration of crude extract	21.3	16	0.75	16.5	1.50
Affinity chromatography of active gel filtration peak	3.8	4	1.10	2.95	2.20

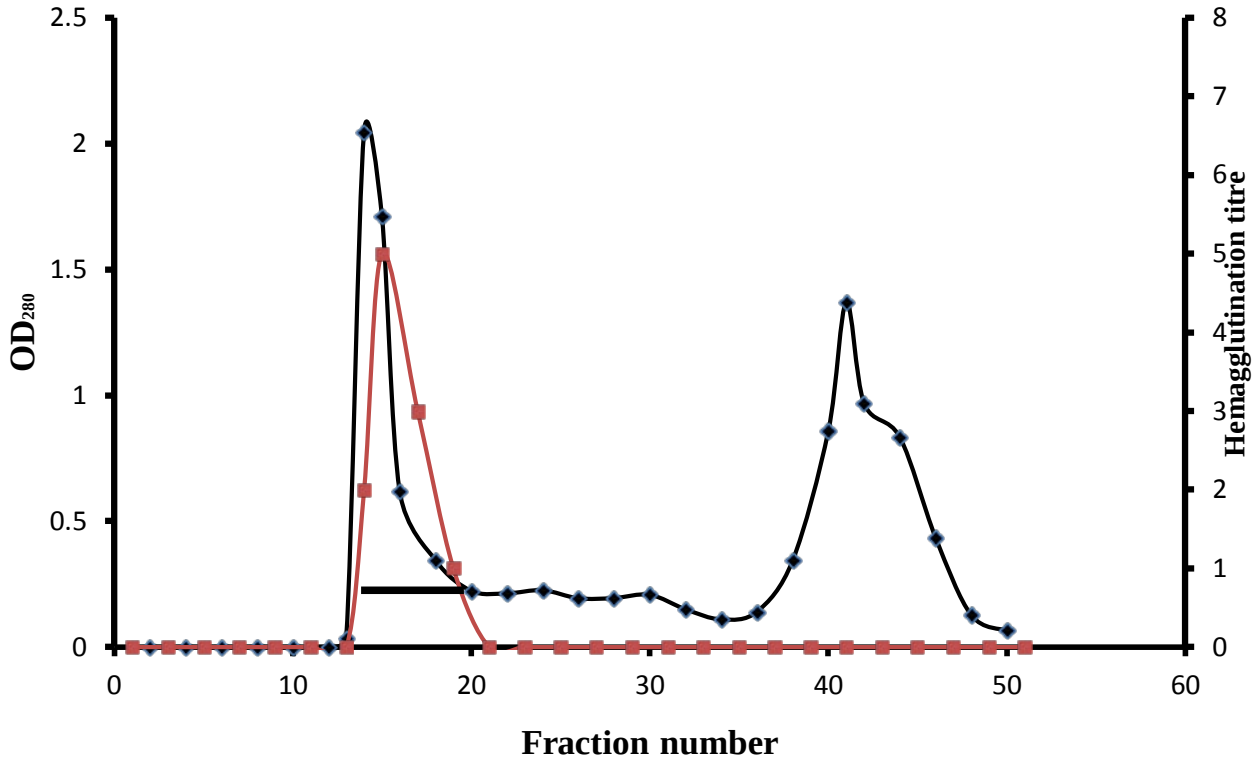

Figure 1. Gel filtration of crude extract of African catfish skin mucus on sephadex G-150 column. The column (2.5 x 40 cm) packed with Sephadex G-150 was equilibrated with 10 mM Phosphate buffer pH 7.0 containing 50 mM NaCl. 6 ml of crude extract (55.7 mg) was layered on the column and lectin was eluted with the same buffer at a flow rate of 18 ml/hr and fractions of 4 ml were collected. Legend: Absorbance at 280 nm —◆—, Haemagglutinating Activity —■—. The bar indicates the fractions pooled.

galactose also inhibited the lectin activity significantly. Dulcitol, mannitol, cellobiose, L-fucose, raffinose, melezitose, arabinose and methyl-α-O-glucopyranoside slightly inhibited the lectin activity. However, sucrose, glucose, maltose, cellibiose, glucosamine hydrochloride, 2-deoxy-glucose, sorbose, mannose, fructose, sorbitol, L-rhamnose and N-acetylglucosamine showed no inhibitory activity against African catfish skin mucus lectin.

The lectin was isolated and purified to homogeneity from the skin mucus by a two step purification procedure comprising of gel filtration on Sephadex G-100 and affinity chromatography on Lactose-Sepharose 4B and the bioactivity of the lectin was measured by haemagglutination during each purification step. The level of purification was monitored by increase in its specific activity (Table 3). A typical elution profile on gel filtration is as shown in Figure 1. Two protein peaks were obtained, only the first peak exhibited haemagglutinating activity. The lectin active peak was further purified by affinity chromatography on Lactose-Sepharose 4B column (Figure 2). The lectin activity resided in the fraction adsorbed in the immobilized sugar, which was eluted with 0.2 M lactose in 10 mM phosphate buffer pH 7.2. The final preparation gave a distinct single protein band in SDS-PAGE.

The molecular weight of the native protein was estimated to be 63,000 Daltons by gel filtration while the subunit molecular weight of 20,000 Daltons was obtained by SDS-PAGE suggesting a trimeric structure for the lectin. The minimum chemical molecular weight estimated

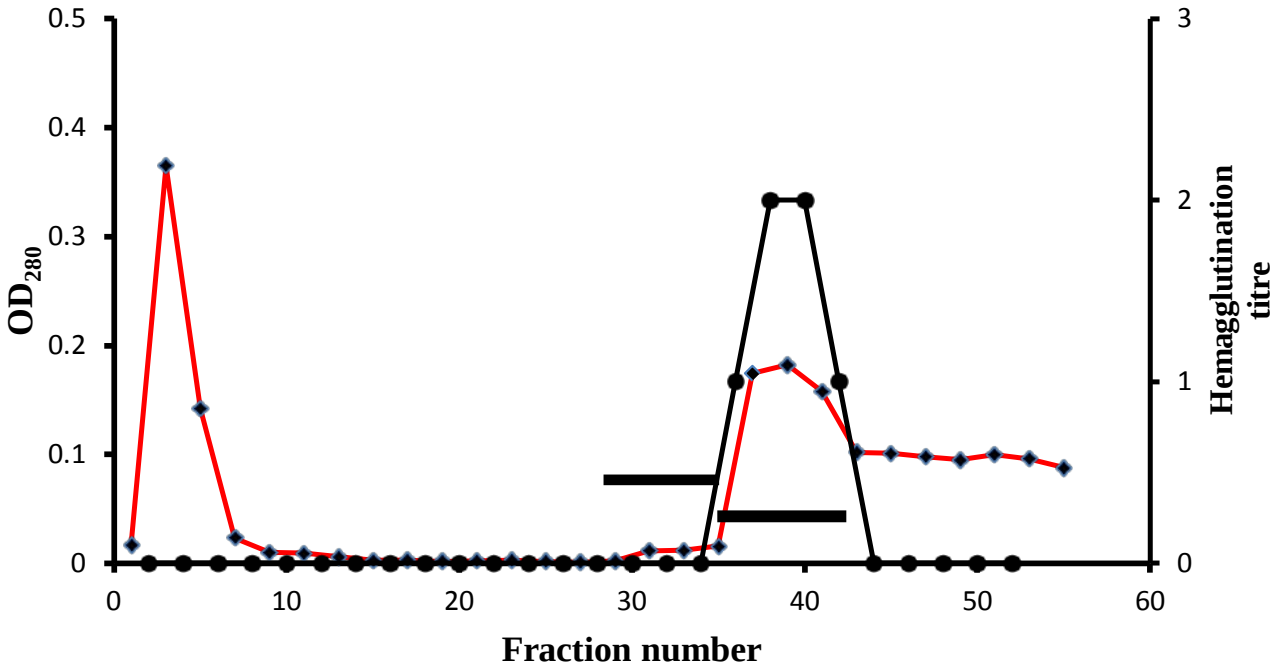

Figure 2. Affinity chromatography of active peak from gel filtration on lactose-sepharose 4B column. The column (0.5 x 20 cm) packed with Lactose-Sepharose 4B was equilibrated with 10 mM Phosphate buffer pH 7.0 containing 50 mM NaCl. 1 ml sample (8.7 mg) was layered on the column and first washed with 100 ml of the same buffer to remove the unadsorbed proteins then the adsorbed lectin was eluted with 0.2 M lactose in the same buffer. The flow rate was 12 ml/h and 1 ml fractions were collected. All operations were carried out at temperatures between 0-4°C in the cold box. Legend: Absorbance at 280 nm —◆— , Haemagglutinating activity —●—. The bar indicates the fractions pooled.

Table 4. Amino acid composition of African catfish skin mucus lectin.

Amino acid	Calculated residues to the nearest integer	Residue weight
Lysine	8	1168
Histidine	9	1395
Arginine	3	522
Aspartic acid	4	532
Threonine	4	476
Serine	4	420
Glutamic acid	11	1617
Proline	14	1610
Glycine	4	300
Alanine	5	445
Cysteine	9	1089
Valine	4	468
Methionine	5	745
Isoleucine	4	524
Leucine	4	524
Tyrosine	22	3982
Phenylalanine	12	1980
Total	126	≈18,000

from the amino acid composition was approximately 18,000 Daltons. SDS-PAGE revealed a homogenous single band of protein stain. The amino acid composition analysis revealed that the purified lectin is made up of 126 amino acids residues (Table 4).

The haemagglutinating activity of the African catfish skin

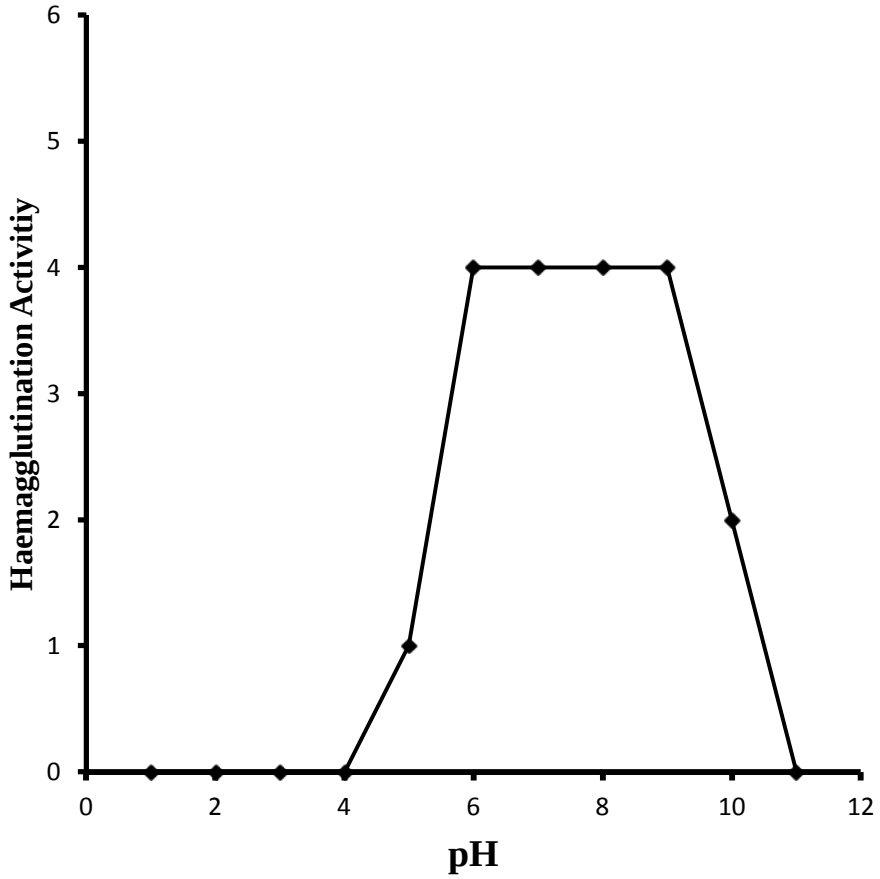

Figure 3. Effect of pH on the haemagglutinating activity of African catfish skin mucus lectin. Effect of pH on the haemagglutinating activity of the lectin was determined by incubating the lectin sample at different pH. The following buffers were used: 0.2 M Citrate buffer (pH 3 to 6), 0.2 M Tris-HCl buffer (pH 7 to 8), 0.2 M Glycine-NaOH buffer (pH 9 to 11).

mucus lectin was stable between pH 5 and 10. The highest haemagglutinating activity was obtained at pH 6-9. Below pH 5, 25% of the activity was retained while above pH 10, the activity was reduced by 50% (Figure 3). The effect of temperature on the haemagglutinating activity of African catfish skin mucus lectin is shown in Figure 4. The activity of the lectin was stable when heated up to 40°C for 30 min but activity reduced to 75% when heated at 50°C for 30 min. The activity was completely lost at 60°C.

The haemagglutinating activity of African catfish skin mucus lectin was not affected by EDTA even at high concentration. Also, addition of divalent metal ions showed no effect on the activity of the lectin either before or after dialysis against EDTA.

African catfish skin mucus lectin was negative with Periodic acid-Schiff's reagent staining, suggesting that it is not glycosylated. The lectin when examined for possible interaction with some polysaccharides by Ouchterlony double diffusion experiment did not form any precipitin line with any of the polysaccharides/glycoprotein used.

The involvement of some amino acids residues in the haemagglutinating activity of African catfish skin mucus lectin was investigated using specific modifying reagents. Phenylglycoxal and Phenylmethane sulphonyl fluoride (PMSF) did not produce any significant alteration in the haemagglutinating activity of the mucus lectin. However, total loss of haemagglutinating activity was observed when African catfish skin mucus lectin was treated with 5, 5'–dithiobis-(2-nitro benzoic acid) (DTNB), N-bromosuccinimide (NBS) and Diethyl pyrocarbonate (DEPC) (Table 5).

DISCUSSION

Recently, a number of lectins have been purified from the skin mucus of several species of fish including Japanese eel (*Anguilla japonica*) (Tasumi et al., 2002), Ponyfish (*Leiognathus* nuchalis) (Okamoto et al., 2005), Torafugu (*Takifugu rubripes*) (Tsutsui et al., 2006) and Conger eel

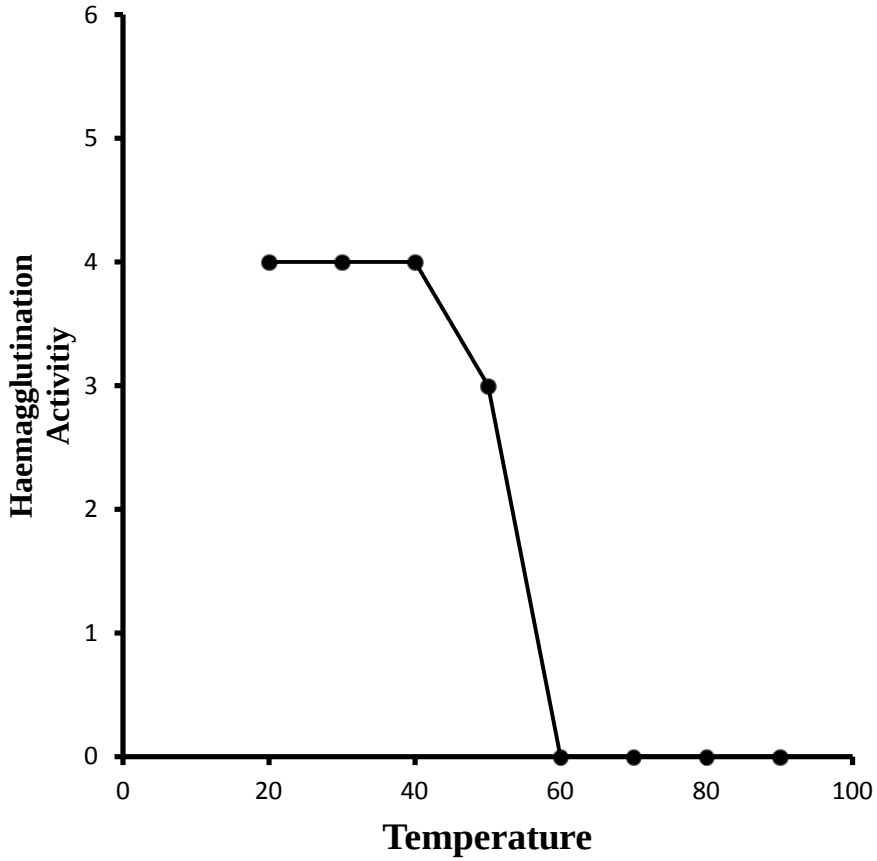

Figure 4. Effect of temperature on the haemagglutinating activity of African catfish skin mucus lectin. Aliquots of lectin were incubated at different temperatures (20 to 90°C) for 30 min then rapidly cooled in ice and assayed for agglutinating activity. The control was agglutinating activity of lectin sample kept at 20°C for 30 min.

Table 5. Effect of chemical modification on haemagglutinating activity of African catfish skin mucus lectin.

Treatment	Modified group/amino acid	% Haemagglutinating activity remaining
Phenylmethylsulfonyl fluorde (PMSF)	Serine	100
5, 5' – Dithiobis-(2-nitrobenzoic acid) (DTNB)	Cysteine	0
N- Bromosuccinimide (NBS)	Tryptophan	0
Phenylglyoxal	Arginine	100
Diethyl-pyrocarbonate (DEPC)	Histidine	0

(*Conger myrister*) (Tsutsui et al., 2007). In addition, studies have also shown that skin mucus contains a number of novel lectins (Suzuki et al., 2003; Tasumi et al., 2004; Tsutsui et al., 2005).

We successfully purified and characterized a lectin from the skin mucus of African catfish, an important commercial tropical freshwater fish, by a combination of gel filtration on Sephadex G-150 and affinity chromatography on Lactose-Sepharose 4B gels. It was noted that the skin mucus lectin exhibited blood group specificity similar to that of lectin found in Pufferfish and Japanesse eel (Tsutsui et al., 2003; Tasumi et al., 2004). African catfish skin mucus lectin could not agglutinate erythrocytes from all blood samples tested. However, it showed strong specificity towards blood Group B erythrocyte but could not agglutinate other human erythrocytes. The lectin could not agglutinate erythrocytes from hen, rat and bat but showed high preference for the rabbit erythrocytes.

The inhibitory analysis showed that glucose and its derivatives had no inhibitory effect on the skin mucus lectin activity. Among the carbohydrates tested, galactose, lactose and melibiose strongly inhibited the haemagglutinating activity. This inhibitory effect on lectin

activity gives support to the classification of this lectin as a member of galactose-specific lectins. Similarly, lectins from Conger eel and Japanesse eel have also been classified as galactose-specific (Tasumi et al., 2004; Muramoto et al., 1999). The strong inhibition by lactose and melibiose over galactose indicated that catfish skin mucus lectin may possess an extended sugar-binding site. The result also suggested that this lectin does not differentiate between α and β-galactoside since lactose and melibiose inhibited the lectin activity to a similar extent. The importance of C-4 hydroxyl group orientation is indicated by the failure of mannose or glucose to act as inhibitor.

The activity of the skin mucus lectin does not require Ca^{2+} or any other divalent cation for its haemagglutinating activity. It is therefore calcium-independent lectin. Tsutsui et al. (2003) reported that the haemagglutinating activity of pufflectin-s (skin mucus lectin of Pufferfish, *Takafugu rubripe*) was not altered by the addition of either EDTA or $CaCl_2$, indicating that pufflectin-induced agglutination does not require calcium. The skin mucus lectins of conger eel and Japanese eel have also been reported to be calcium-independent (Tasumi et al., 2002; Suzuki et al., 2003; Tsutsui et al., 2007).

Based on the SDS-PAGE and gel filtration results we conclude that African catfish skin mucus lectin is a homomeric protein with three identical subunits. The molecular weight calculated from the deduced amino acid composition was 18,000 Daltons. This calculated value is in good agreement with that measured by SDS-PAGE. This is in conformity with what was obtained for starfish lectin (Kakiuchi et al., 2002) and salmon serum lectin (Ewart et al., 1999). Though, many of the skin mucus lectins isolated so far have been shown to be homodimer (Tasumi et al., 2004), our result indicated that skin mucus lectin from African catfish is a unique one with three subunits of identical size. The amino acid composition analysis revealed that the purified lectin is made up of 126 amino acids residues, which is very close to what was obtained for skin mucus lectins from pufferfish and conger eel, (Tsutsui et al., 2003, 2007). The lectin from African catfish skin mucus was characterized by low content of arginine, aspartic acid, serine, glycine, valine, isoleucine and leucine. The number of half-cystine is considerably high compared to other sulphur containing amino acid (methionine) suggesting the presence of disulphide bridges within the native structure of the African catfish skin mucus. There is also abundance of charged and uncharged polar amino acids like tyrosine, glutamic acid, lysine, histidine and cysteine, which together represents about 60% of the total amino acids of the lectin. In general, the hydrophobic amino acid content of this lectin is very high and is more than half of the total amino acids suggesting that the protein may be more of a globular protein than a fibrous.

The thermostability of various lectins appears to differ widely. Some are relatively stable while others are much less so. The lectin isolated was thermally stable over a wide range of temperature between 25 and 50°C; however after 50°C, it started losing activity very rapidly. At 60°C, the lectin completely lost its activity. Also, the lectin was found stable between pH 5.0 and 10.0 but maximally active between pH 6.0 to 9.0. Some of the fish skin mucus lectins that have been isolated were heat and pH stable over the same range of pH and temperature obtained for African catfish skin mucus lectin (Tateno et al., 1998; Tasumi et al., 2004; Dutta et al., 2005).

The carbohydrate content varies from lectin to lectin and in some cases, it could be as high as 30%, however in some cases it is totally absent. The lectin was not glycosylated according to the Periodic acid-Schiff's staining which is comparable to the result obtained by Tsutsui et al. (2003) for Pufflectin-s. The agreement of calculated and measured molecular weights also indicates that this lectin lacks attached carbohydrate. The lectin did not show any precipitin line with any of the polysaccharides/glycoprotein used. This could however be due to many factors such as the concentration of the protein as well as the molecular size of the polysaccharides. This could also be explained based on the theory that a lectin may either fail to precipitate a polysaccharide or form precipitin bands in agar gel because the lectin may not be specific for that polysaccharide.

Identification of specific amino acids involved in the biological activity of proteins provides information about the relationship between its structure and the role played by amino acid side chains in its activity. A common strategy for identifying the amino acids is to treat the protein with specific affinity modifying reagents. This investigation provides clues about the amino acids involved in the biological activity. The outcome of this investigation indicated that arginine and serine are not playing any important role in the activity of the lectin. The results also strongly suggest that cysteine, tryptophan and histidine were either located at the sugar-binding site or were involved in the maintenance of the lectin active conformation. Previous studies have shown that tryptophan is indispensable for the haemagglutinating activity of some lectins especially galectins. Tryptophan residue was implicated in sugar binding activity of both Congerin I and II because its modification by 2-nitrophenyl sulfenyl chloride (NPS-CL) led to about 87% loss of the haemagglutinating activity of the congerins (Muramoto et al., 1999). The loss of activity may also be attributed to deprotonation of some reactive groups at the active site like indole group of tryptophan at low pH, consequently leading to the disruption of binding forces and the loss of activity. Also, congerins haemagglutinating activity decreased significantly when histidine residue was modified with DEPC (Muramoto et al., 1999). The cysteine residue seems to play a structural role rather than sugar binding considering the homotrimeric structure of the lectin.

Conclusions

The lectin from skin mucus of African catfish showed similar biological and molecular properties with the skin mucus lectins from several species of fish. However, some minor differences still exist which give each lectin unique characteristic. Further line of research should involved deducing the structure of this lectin for proper classification and also investigation of the physiological function of this lectin to clarify its involvement in the mucosal defense mechanism of African catfish.

Conflict of Interests

The authors have not declared any conflict of interests.

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Scientific Research and Essays

Full Length Research Paper

Semi-analytic solutions to Riemann problem for one-dimensional gas dynamics

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This work deals with the implementation of reduced differential transform method (RDTM) for solving the Riemann problem for gas dynamics in one dimension. The RDTM is an analytical method that can be applied to many linear and nonlinear partial differential equations and is capable of reducing the size of computations. Using this method, the solution is calculated in the form of convergent power series with easily computable components. The definition and basic properties of RDTM are investigated. Some new generalized formulas of reduced differential transforms are derived. The Riemann problem that describes the isentropic flow of an inviscid gas is considered to demonstrate the effectiveness and promising of the proposed algorithm.

Key words: Reduced differential transform method, gas dynamics, isentropic flow of an inviscid gas equations, conservation law.

INTRODUCTION

For one dimensional flows, the state U of a perfect gas is specified by two dependent variables, the velocity $u = u(x,t)$ and the density $v = v(x,t)$ of gas, and two convenient constants which are the ratio of specific heats γ and gas constant a. Hence the state U is completely defined by u, v, γ and a.

In this paper, the recently analytic technique, namely the reduced differential transform method (RDTM) (Keskin and Oturanc, 2009, 2010), is presented for approximating the solutions to one-dimensional gas dynamics equations

$$\begin{aligned} u_t + uu_x + c^2 v^{-1} v_x &= 0, \\ v_t + uv_x + vu_x &= 0, \end{aligned} \tag{1}$$

That describes the isentropic flow of an inviscid gas (IFIG) (Gottlieb and Groth, 1988). Where c is the local speed. Given $c^2 = c^2(v) = a\gamma v^{\gamma-1}$ for perfect gas and the ratio of specific heats $\gamma = 1.4$ for air, we have:

$$c^2 = 1.4av^{0.4} \tag{2}$$

The system in Equation (1) is an example of a system of hyperbolic conservation laws for $a > 0$ and elliptic for $a < 0$, but never to be of mixed type.

The initial value or Riemann problem will be expressed in the form of two discrete initial states as:

$$u(x,t_0) = \begin{cases} u_- \\ u_+ \end{cases}, \; v(x,t_0) = \begin{cases} v_- & , x < t_0 \\ v_+ & , x \geq t_0 \end{cases}. \tag{3}$$

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In the recent years, many authors have devoted their attention to study solutions of nonlinear partial differential equations and systems using various methods. Among these attempts are the Adomian decomposition method (ADM) (Jebari et al., 2013; Dhaigude and Birajdar, 2014), the homotopy analysis method (HAM) (Gupta and Gupta, 2012; Zubair et al., 2012; Abdul Wahab et al., 2014). the homotopy perturbation method (HPM) (Al-Sharifi and Mohammed, 2014; Abdul Wahab et al., 2014; Manaa et al., 2014), the variational iteration method (VIM) (Borhanifar and Sadri, 2014; Hilal and Elzaki, 2014), the differential transform method (DTM) (Ndlovu and Moitsheki, 2013; Jafariet al., 2012) and the RDTM (Az-Zo'bi, 2013; Vineet et al., 2014; Rawashdeh and Obeidat, 2014; Al-Amr, 2014).

THE REDUCED DIFFERENTIAL TRANSFORM METHOD

To overcome the demerit of complex calculations of DTM, the RDTM was presented. This method was used by many mathematicians and engineers to solve various partial differential equations. The RDTM presents an efficient improvement in solving nonlinear partial differential equations since the amount of computations required is much less than that in other existing techniques. Its rapid convergence, gives exact solution with small number of iterations.

Consider the analytic and continuously differentiated function of two variables $u(x,t)$ and suppose that it can be represented as a product of two single-variable functions, that is, $u(x,t) = f(x)g(t)$. Based on the properties of differential transform, the function $u(x,t)$ can be represented as

$$u(x,t) = \sum_{i=0}^{\infty} F(i)\, x^i \sum_{j=0}^{\infty} G(j)\, t^j = \sum_{k=0}^{\infty} U_k(x)\, t^k, \qquad (4)$$

Where $U_k(x)$ is the transformed function, called t-dimensional spectrum function of $u(x,t)$, and defined by:

$$U_k(x) = \frac{1}{k!}\left[\frac{\partial^k}{\partial t^k} u(x,t)\right]_{t=0} \qquad (5)$$

Combining Equations (4) and (5) implies the differential inverse transform:

$$u(x,t) = \sum_{k=0}^{\infty} \frac{1}{k!}\left[\frac{\partial^k}{\partial t^k} u(x,t)\right]_{t=0} t^k. \qquad (6)$$

One can easily obtained that this transform is derived from the power series expansion. Next, some basic theorems and generalized formulas of reduced differential transform are listed.

Theorem 1: The reduced differential transform is linear.

Theorem 2: If $u(x,t) = x^m t^n$ then:

$$U_k(x) = x^m \delta(k-n),\ \delta(k) = \begin{cases} 1 & ,k=0 \\ 0 & ,k \neq 0 \end{cases}. \qquad (7)$$

Theorem 3: If $u(x,t) = x^m t^n\, v(x,t)$ then,

$$U_k(x) = x^m\, V_{k-n}(x). \qquad (8)$$

Theorem 4: If $u(x,t) = \frac{\partial^r}{\partial t^r} v(x,t)$ then:

$$U_k(x) = (k+1)\ldots(k+r)V_{k+r}(x). \qquad (9)$$

Theorem 5: If $u(x,t) = \frac{\partial^r}{\partial x^r} v(x,t)$ then,

$$U_k(x) = \frac{\partial^r}{\partial x^r} V_k(x). \qquad (10)$$

Next theorems are new generalized reduced differential transforms.

Theorem 6: If $u(x,t) = v^n(x,t)$, $n \in N$, then,

$$U_k(x) = \sum_{r_1=0}^{k} \sum_{r_2=0}^{k-r_1} \cdots \sum_{r_{n-1}=0}^{k-\sum_{t=1}^{n-2} r_t} V_{r_1}(x) V_{r_2}(x) \cdots V_{k-\sum_{t=1}^{n-1} r_t}(x). \qquad (11)$$

Corollary 7: If $u(x,t) = v(x,t)\, w(x,t)$ then,

$$U_k(x) = \sum_{r=0}^{k} W_r(x) V_{k-r}(x) = \sum_{r=0}^{k} V_r(x) W_{k-r}(x). \qquad (12)$$

Corollary 8: If $u(x,t) = v(x,t)\, w(x,t)\, \omega(x,t)$ then:

$$U_k(x) = \sum_{r=0}^{k} \sum_{t=0}^{k-r} V_r(x) W_t(x) \Omega_{k-r-t}(x). \qquad (13)$$

Theorem 9: If $u(x,t) = v^{n-m}(x,t)\left(\frac{\partial}{\partial x} v(x,t)\right)^m$, $n,m \in N$, then:

$$U_k(x) = \sum_{r_1=0}^{k} \sum_{r_2=0}^{k-r_1} \cdots \sum_{r_{n-1}=0}^{k-\sum_{t=1}^{n-2} r_t} V_{r_1}(x) V_{r_2}(x) \cdots V_{r_{n-m}}(x) \frac{\partial}{\partial x} V_{r_{n-m+1}} \cdots (x) V_{k-\sum_{t=1}^{n-1} r_t}(x) \qquad (14)$$

Corollary 1: If $u(x,t) = v(x,t) \frac{\partial}{\partial x} v(x,t)$ then:

$$U_k(x)=\sum_{r=0}^{k}V_r(x)\frac{\partial}{\partial x}V_{k-r}(x). \quad (15)$$

Theorem 10: If $u(x,t)=\frac{\partial^{n+m}}{\partial x^n\partial t^m}v(x,t)$ then:

$$U_k(x)=(k+1)\ldots(k+m)\frac{\partial^n}{\partial x^n}V_{k+m}(x). \quad (16)$$

ANALYTIC SOLUTIONS OF ONE DIMENSIONAL PERFECT GAS EQUATIONS

The RDTM is designed to deal with continuous initial data. So, we will use the following transforms for our Riemann problem:

$$u(x,t_0)=\frac{u_-e^{-\alpha x}+u_+e^{\alpha x}}{e^{-\alpha x}+e^{\alpha x}},$$
$$v(x,t_0)=\frac{v_-e^{-\alpha x}+v_+e^{\alpha x}}{e^{-\alpha x}+e^{\alpha x}}. \quad (17)$$

In this part, the reduced differential transform technique is applied to solve IFIG model. Taking into account the local speed of gas flow in Equations (3) and (1) can be written in more compact form as:

$$u_t+\left(0.5u^2+3.5av^{0.4}\right)_x=0,$$
$$v_t+(uv)_x=0. \quad (18)$$

Operating the reduced differential transform for system in Equation (18), and using related facts in the previous section gives

$$(k+1)U_{k+1}(x)=-P(V_k(x))-\sum_{r=0}^{k}U_r(x)\frac{\partial}{\partial x}U_{k-r}(x)$$
$$(k+1)V_{k+1}(x)=-\sum_{r=0}^{k}U_r(x)\frac{\partial}{\partial x}V_{k-r}(x)-\sum_{r=0}^{k}V_r(x)\frac{\partial}{\partial x}U_{k-r}(x) \quad (19)$$

, $k\geq 0$

With starting transformed initial data $U_0(x)$ and $V_0(x)$. $P(V_k(x))$ is the transformed formula of the nonlinear function $\partial_x v^{0.4}(x)$. For the easy to flow of reader, the first few terms of the sequence $P(V_k(x))$, are:

$$P(V_0(x))=\frac{2\partial_x V_0}{5V_0^{0.6}},$$

$$P(V_1(x))=\frac{2\partial_x V_1}{5V_0^{0.6}}-\frac{6V_1\partial_x V_0}{25V_0^{1.6}},$$

$$P(V_2(x))=\frac{2\partial_x V_2}{5V_0^{0.6}}-\frac{6(V_2\partial_x V_0+V_1\partial_x V_1)}{25V_0^{1.6}}+\frac{24V_1^2\partial_x V_0}{125V_0^{2.6}},$$

$$P(V_3(x))=\frac{2\partial_x V_3}{5V_0^{0.6}}-\frac{6(V_3\partial_x V_0+V_2\partial_x V_1+V_1\partial_x V_2)}{25V_0^{1.6}}+\frac{24(2V_1V_2\partial_x V_0+V_1^2\partial_x V_1)}{125V_0^{2.6}}-\frac{104V_1^3\partial_x V_0}{625V_0^{3.6}}$$

Other transforms of $P(V_k(x))$ can be generated in the same way. Substituting into Equation (19) and using straight forward recursive calculations, we get the following $U_k(x)$ and $V_k(x)$ values. The n^{th} order approximate solutions are:

$$u_n(x,t)=\sum_{k=0}^{n}U_k(x)t^k,$$
$$v_n(x,t)=\sum_{k=0}^{n}V_k(x)t^k. \quad (20)$$

And the exact solutions are given by:

$$u(x,t)=\lim_{n\to\infty}u_n(x,t),$$
$$v(x,t)=\lim_{n\to\infty}v_n(x,t), \quad (21)$$

Provided the series converge and have closed forms.

NUMERICAL EXPERIMENT

In order to assess the advantages and accuracy of RDTM for solving IFIG equations, Equation (18). Let $a=\frac{2}{7}$, and the converted initial data

$$u(x,0)=\tanh x,$$
$$v(x,0)=1. \quad (22)$$

Following recurrence relations in Equations (19), and up to $n=5$ in Equation (20), approximate solutions of order five are found with help of *Mathematica*. The obtained soliton solutions of velocity and density of gas are represented in Figures 1 and 2 respectively.

In view, the methodology appears to be very promising for solving this system, it is convergent and stable. In this example, we cannot determine the errors in comparative to the exact solutions since we do not know these solutions. However, many terms can be calculated in order to achieve a high level of accuracy of the RDTM.

Conclusions

Many non-linear partial differential equations and systems that arising in various physical, chemical and

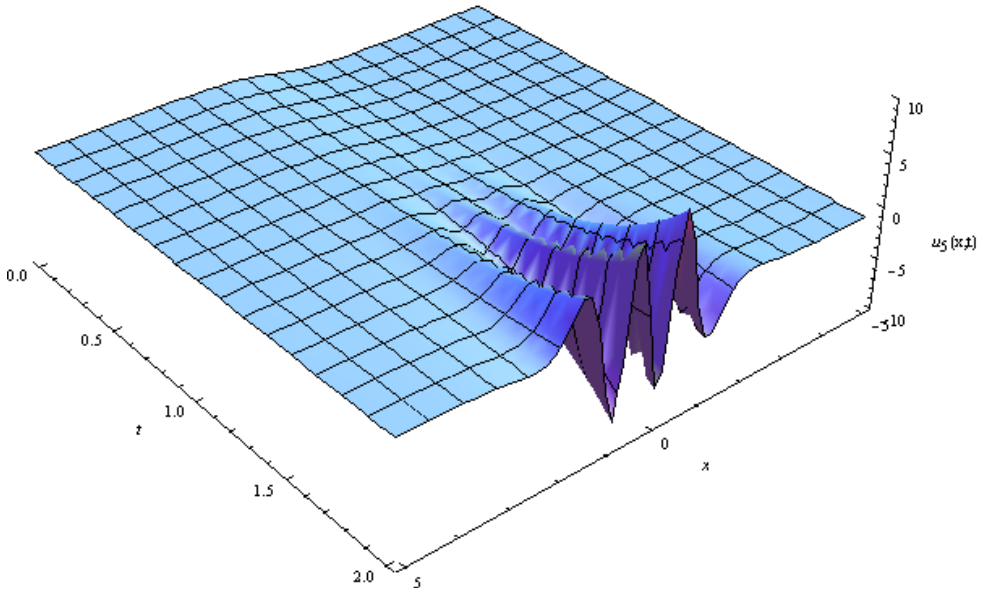

Figure 1. The fifth order approximate velocity of the gas for $0 \le t \le 2$, $|x| \le 5$

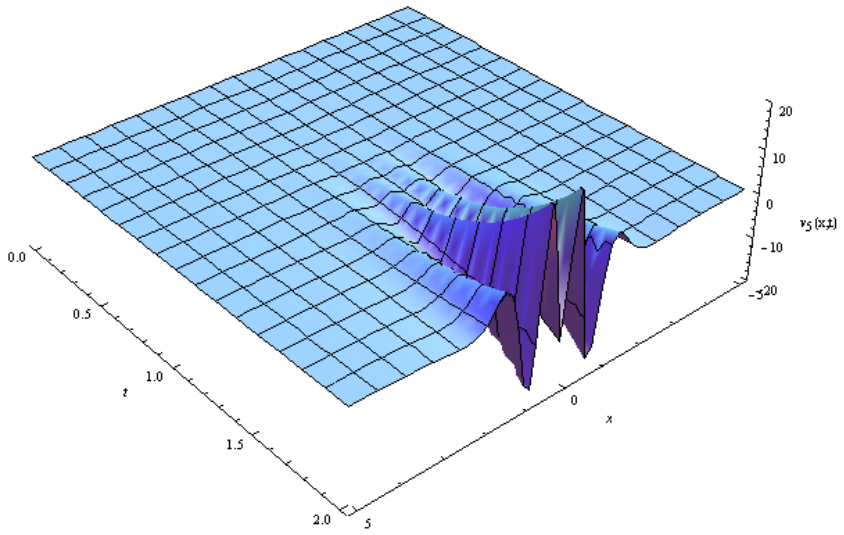

Figure 2. The fifth order approximate density of the gas for $0 \le t \le 2$, $|x| \le 5$

engineering applications have no exact solution. So, semi-analytic solution for these equations is very important. In this paper, we found successfully approximate semi-analytic solutions for nonlinear one dimensional gas dynamics equations, namely the isentropic flow by an inviscid gas equations. The example presented demonstrates the fast convergence of the method. Another benefit of the reduced differential transform methodology is that it does not need any discretization to get numerical solutions. It introduces simple and straight forward calculations over other existing method. The numerical results obtained in this study show that the reduced differential transform method is powerful tool for solving linear and nonlinear partial differential equations and systems.

Conflict of Interest

The authors have not declared any conflict of interest.

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Scientific Research and Essays

Full Length Research Paper

Evaluation of risk factors for surfactant re-dosing in neonates with respiratory distress syndrome

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Respiratory distress syndrome (RDS) is a common cause of mortality and complications in the preterm neonates. Specific treatment of this disease is endotracheal surfactant administration. Some neonates need more than one dose of drug (re-dosing) so that in addition to the cost, avoidace of treatment complications and, if necessary, timely prescribtion are of high importance. In this study, the effects of the contributing factors, including prescription or non-prescription of corticosteroid to the mother, the gestational age, fetal gender, birth weight, the first and fifth minute APGAR score, respiratory distress score, time of first dose of surfactant administration, type of delivery, type of surfactant, in the case of re-dosing of surfactant in neonates with RDS, and in NICU of Motahari Maternity-Children Hospital, Urmia, Iran were compared and the positive and negative effects of each of the above-mentioned factors were examined. Few studies have been conducted on the effect of the administration of the second dose of surfactant and its subsequent doses compared with the used doses. All hospitalized neonates, who were diagnosed with RDS after birth during 2011 to 2013, were included in the study. Data was collected from a designed form and analyzed by SPSS version 22. They included 213 (60.9%) male and 137 (39.1%) females with a mean weight of 1782.03 ± 387.04 g. 66 males (31%) and 32 females (23.4%) received more than one dose of surfactant. 227 neonates (64.9%) were discharged with a recovery and 123 neonates (35.1%) died. Findings of our study showed that neonates with the low birth weight have received more than one dose of surfactant. Moreover, neonates born between 32 to 36 weeks of gestation needed re-dosing of more surfactant. Neonates with first dose of surfactant for 6 to 24 h after birth need significantly more re-dosing of surfactant. There was a significant difference between the re-dosing of surfactant and type of delivery. In addition, re-dosing of more surfactant was needed with the increasing numbers of neonate per delivery. In contrast; the need to re-dosing of surfactant in the neoantes whose mothers had received one dose of prenatal corticosteroid was greater than the neoantes whose mothers had received two doses of prenatal corticosteroid. Moreover, the type of the surfactant products (curosurf and Survanta) had no effect on the re-dosing of doses of surfactant in the studied infants. The percentage of the use of more than one dose of surfactant was higher in male neonates than the female ones. Inaddition, the first and fifth minute APGAR scores were significantly lower in re-dosing group. In this study, the need for re-dosing of surfactant was significantly greater in the neonates with the higher respiratory distress score (>8) than the neonates with the mild to moderate respiratory distress scores (≤8).

Key words: Respiratory distress syndrome, surfactant, re-dosing.

INTRODUCTION

Respiratory distress syndrome (RDS), which formerly was known as Hyaline membrane disease (HMD), is a common cause of mortality and complications in preterm neonates. RDS is a defect in lung maturation that is usually seen in the preterm births and is caused due to surfactant deficiency. Preterm birth is defined as the birth before the end of week 37 from the first day of the last menstrual period, which occurred in 5 to 15% pregnancies (Asnafei et al., 2004; Mahoney and Jain, 2013; Colin et al., 2010; Gortner and Tutdibi, 2011). Percentage of preterm births in the United States has been reported to be 11% whereas it includes 5 to 7% in European countries (Robert, 2002). This rate was reported to be 28% in a study conducted in Iran (Moravedji et al., 2005). A major cause of premature neonate birth is the premature rupture of the membrane. Premature birth has increased in the past twenty years due to the increased special attention to the obstetrical care, the improved status of evaluation and research in preterm birth, the increasing use of ultrasound to estimate the gestational age and pregnancies resulting from the infertility treatment (Roberts and Dalzell, 2006). Premature birth causes a higher incidence of neonatal disease, of which HMD or RDS and intraventricular hemorrhages (IVH) are the most important ones (Committee on Obstetric Practice, 2002; Colin et al., 2010; Gortner and Tutdibi, 2011). Pulmonary surfactant deficiency leads to massive atelectasis, loss of pulmonary residual capacity, and collision of the ventilation to perfusion ratio (Jobe, 1993; Gortner and Tutdibi, 2011). Consequences of this condition are decrease in lung compliance, decreased oxygenation with cyanosis, respiratory and metabolic acidosis that results in severe hypoxemia with the increased pulmonary vascular resistance and right-to-left shunt through the ductus arteriosus (Rodriguez et al., 2006). Neonates who are born preterm suffer from RDS due to the lack of enough surfactant concentration in the alveoli. These neonates have lungs with low compliance that need to try and spend a lot of energy for their distension in every breath and their alveoli always tend to collapse. If treatment does not start immediately, about 50% of them die. RDS is a common and deadly disease, which is inversely related to the gestational age (Shahfarhat et al., 2006; Engle, 2008; Mahoney and Jain, 2013; Colin et al., 2010; Gortner and Tutdibi, 2011) so that it occurs in 60 to 80% of neonates less than 28 weeks, 15 to 30% of neonates of 32 to 36 weeks, and 5% of neonates of more than 37 weeks gestational ages (Halliday, 2005). The discovery of the key role of surfactant in the pathophysiology of RDS by the researchers in 1959 led them to think about the prescription of surfactant aerosol for premature neonates with RDS, theory that has been introduced again recently (Avery and Mead, 1959; Pohlmann et al., 2013). Surfactant decreases the surface tension of alveolar lining liquid layer in the lung and prevents the smaller alveolar collapses. Human surfactant is a mixture of lipoproteins, such as palmytioyl phosphatidyl choline. Surfactant is synthesized by alveolar type 2 cells and secreted into the alveoli (Schurch et al., 1992). Normally, surfactant synthesis begins around week 25 of the gestation under the influence of several hormones. Surfactant production usually reaches a sufficient level about 32 to 34 weeks of pregnancy (Roberts and Dalzell, 2006; Colin et al., 2010). With the introduction of exogenous surfactant in recent decades for the treatment of RDS in patients under artificial ventilation, an obvious improvement in the mortality and air leak syndrome was observed due to the decreased need to oxygen therapy and ventilator pressures (Fanaroff and Martin, 2006; Ramanathan, 2008; Polglase et al., 2009; Fujiwara and Maeta, 1980; Robertson and Halliday, 2009). In general, surfactant reduces mortality due to the RDS if, particularly, it is accompanied by the administration of corticosteroids to the mother before birth (Behrman, 2004; Eriksson et al., 2012). The most important role of surfactant in the prevention and treatment of RDS is to reduce the surface tension in the alveoli (Dolfin et al., 1994; Iarukuva et al., 1999). The use of surfactants is increasing in other respiratory disorders, such as meconium aspiration syndrome (Fanaroff, 2001). There are two types of surfactants to treat; exogenous surfactant derived from animal resources or natural surfactant and synthetic surfactants. Natural surfactant contains palmytioylphosphatidyl choline with surfactant protein-B, sp-C, and without sp-A, sp-D. Synthetic surfactant is a mixture of surface active phospholipids and the releasing agents (Goldsmith, 2003; Verhagen et al., 2001; Rangasamy, 2009). In the early 80s, Fujiwara and Maeta. prescribed a mixture of natural and synthetic surfactant for the preterm neonates with RDS. They found a high decrease in oxygen consumption and the ventilator pressure (Stevens et al., 2007). At present, surfactant is given to the preterm neonates with a dose of 100 or 200 mg per kg of body weight and may need to prescribe other doses. Response to surfactant therapy depends on several factors, including quality of the produced surfactants (Liechty et al., 1991), the time and manner of administration (Dijk et al., 2012), the recommended dose (Cogo et al., 2009), and finally the condition of the resuscitation of neonate in the delivery room (Bjorklund et al., 1997; Chong-Woo and Won-Ho, 2009; Colin et al., 2010). Results of the conducted clinical trials in this area

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Table 1. APGAR scoring for the neonates at birth.

Sign	0	1	2
Heart rate	No	Less than 100	More than 100
Respiratory effort	No	Slow and irregular	Good-crying
Muscular tone	flappy	Brief flexion of limps	Active movement
Response to the stimulations	No	Face grimase	Coughing or sneezing
The color of the neonate's body	Blue-pale	Pink body- blue face	Completely pink

Table 2. Downes scoring system.

Score	0	1	2
Percentage of inspiratory oxygen (FiO_2) required to maintain PO_2>50 mm/Hg	Room air (21%)	< 40%	> 40%
Intercostal retraction	No	Mild to moderate	Severe
Expiratory sighs (Grunting)	No	Is heard with stethoscope	Is heard without stethoscope
Auscultation of lung sounds	Heard well	Decreased	Hardly heard
Number of breath per minute	< 60	60-80	> 80

indicated that the use of different doses of surfactant significantly decreased the rate of mortality compared with single dose or placebo (Hoekstra et al., 1991; Corbet et al., 1995; Kotecha and Kotecha, 2012). Most studies conducted in this area investigated and compared the effect of synthetic and animal surfactants based on the recommended doses in the related protocols (Rangasamy et al., 2009). Few studies have been done on the effect of the administration of the second dose of surfactant and the subsequent doses compared to the used doses (Chong-Woo and Won-Ho, 2009; Dunn et al., 2008; Speer et al., 1992; Soll and Ozak, 2009). Some meta-analysis studies have been performed on the relationship between two surfactants. The results showed that the greater improvement in the oxygenation status, the need for ventilators, the reduced risk of pneumothorax, and higher survival rate existed in the neonates with the respiratory distress group without pneumonia who received some more doses of surfactant than the group with a single dose of surfactant (Chong-Woo and Won-Ho, 2009; Soll and Ozak, 2009; Kattwinkel et al., 2008). According the great costs of re-dosing of surfactant, its possible complications, the complications of delayed surfactant therapy, and a lack of studies conducted on the risk factors for re-dosing of surfactant doses, this study was conducted to examine the risk factors in order to gain a timely recognition of risk factors, prevention and early intervention so that the the complications of delayed treatment could be prevented. This may have a significant effect in the duration of the neonate's hospitalization, decrease in the costs and the complications that result from hospitalization, decrease in complications of late prescription of surfactant doses and decrease in use of more aggressive methods. Knowledge of these risk factors could be a clinical guide to be used in NICU.

MATERIALS AND METHODS

In this applied-analytic study, which was approved by the research committee of the University, all hospitalized neonates diagnosed with RDS after birth in NICU of Motahari maternity-children Hospital, Urmia, Iran during 2011 to 2013 were included in the study. Time of injection of the first dose, re-dosing, the number and frequency of prescription, the type of surfactant received for each child, birth weight, mode of delivery (normal vaginal delivery or cesarean), age of pregnancy, gender, prescription of the pre-natal steroid to mothers (two intramuscular doses of 12 mg, betamethasone with an 24 h interval), the score of RDS (based on the number of breath per minute – the intensity of intercostal retraction - quality of breath sounds on auscultation – expiratory sighs or grunting - the percentage of the required oxygen), the first and fifth minute APGAR scores (based on the heart rate - respiratory condition – muscular tone - response to stimulation – the color of body) were recorded for each neonate (Table 1). Two types of surfactant (Curosurf- with a pocrine origion that was made by Chiesi Fctory in Italia and survanta- with a bovine origin that was by Abbot Factory in America) were used by endotracheal administration (100 mg/kg dose). Some patients needed to prescribe the second and third dose in 6 to 12 h after the first dose. Breathing of patients after surfactant administration was conducted by mechanical ventilation or Nasal-CPAP. The results of the treatment were recorded as recovery, death, or complications in neonates. Finally, the impact of the intended risk factors on re-dosing of surfactant dose was measured. The following criteria were used to diagnose RDS: tachypnea (respiratory rate above 60/min), expiratory grunting and intercostal retraction, cyanosis in room air, a view of reticulonodular and bronchogram in Chest X-Ray, hypoxia and hypercapnia in ABG. All patients were examined in terms of sepsis and monitored continuously including; cardio-respiratory, body temperature, arterial oxygen saturation and therapeutic efforts were conducted for hypoxia, acidosis, and hypothermia. Indications for the intubation and mechanical ventilation included persistent apnea, arterial pH less than 7.20, arterial Pco_2 greater than 60 mm Hg, arterial PO_2 less than 50 mm Hg in 70 to 100% inspirated oxygen concentration (Table 2).

Neonates with fatal congenital anomalies, congenital heart defects, those patients, in whom the tracheal intubation was not possible for reasons such as abnormalities of the airways and

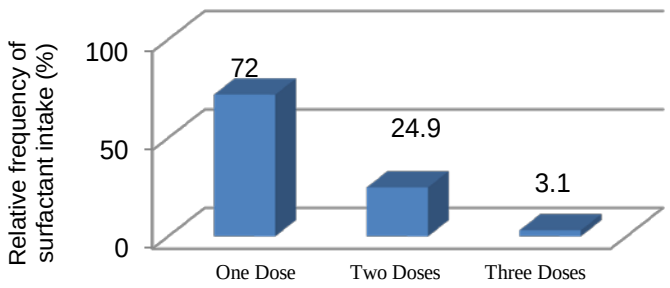

Figure 1. Distribution of relative frequency of surfactant intake in hospitalized neonates with RDS.

Table 3. Distribution of absolute and relative frequency of complications after treatment.

Type of complication	Percentage	Cases (n)
Pneumothorax	26	33
IVH	23.6	30
Co-multiple complications	17.3	22
DIC	12.6	16
Pulmonary hemorrhage	10.2	13
Sepsis	8.7	11
Pneumonia	1.6	2
Total	100	127

patients with the lung diseases other than RDS, were excluded from the study. According to the preliminary studies (pilot study), it was found that approximately P=0.36% (referral rates) had re-dosing surfactants, at 95% level of confidence and error d= 0.05. 350 patients, who had received at least one dose of surfactant, were studied. Non-probability sampling was used. Therefore, all neonates with RDS admitted to our hospital were selected for the purposes of the study according the inclusion and exclusion criteria from the beginning of the study. SPSS v 21 was employed to analyze and process the data obtained from the patients through statistical indices of descriptive analysis and chi-square test. The P-value less than 0.05 were statistically considered significant. Neonates with indication of surfactant therapy were not excluded from the study. Thus, there is no control group in this study. All information of the patients would be confidential with the researcher and the researcher would be faithful to the Helsinki Convention.

RESULTS

In this study, 350 neonates with the diagnosed RDS were hospitalized and treated by surfactant. Of them, 213 neonates were male (60.9%) and 137 were female (39.1%). The average weight of the neonates was 1782.03 ± 783.04 gr (the minimum weight was 500 gr and the maximum weight was 4250 gr). Of 350 neonates hospitalized, 239 (68.3%) were singletone, 79 (22.6%) twin, 24 (6.9%) triplet, and 8 (2.3%) quadruplet. The average gestational age of neonates with the RDS was 31.44 ± 3.74 weeks (the minimum 22 weeks and the maximum 40 weeks). Of 350 studied neonates, 241 cases (68.9%) were cesarean section (C/S) and 109 cases (31.1%) were normal vaginal delivery (NVD). 203 mothers (58%) received prenatal corticosteroids and 147 mothers (42%) did not receive corticosteroids. The number of mothers receiving one dose of corticosteroids before delivery was 144 cases (70.9%) and 59 mothers (29.1%) of them received two doses. Of 350 neonates hospitalized, the reported RDS Score was mild (<5) in one case (0.3%), moderate (5 to 8) in 228 cases (65.13%), and severe (>8) in 121 cases (34.57%). The degree of the first minute APGAR was high (7 to 10) in 133 neonates (38%), moderate (4 to 6) in 174 neonates (49.7%), and low (0 to 3) in 43 neonates (12.3%). The degree of the fifth minute APGAR was high in 238 neonates (68%), moderate in 94 neonates (26.9%), and low in 18 neonates (5.1%). Time of receiving the first dose of surfactant was less than 2 h after birth in 97 cases (27.7%) less than, 2 to 6 h after birth in 149 neonates (42.6%), 6 to 24 h after birth in 76 cases (21.7%), and more than 24 h after birth in 28 cases (8%). Of 350 studied neonates, 252 cases (72%) received one dose of surfactant and 98 cases (28%) received more than one dose of surfactant. Of 98 neonates that received more than one dose, 87 neonates (24.9%) received two doses, and 11 neonates (3.1%) received three doses (Figure 1).

Assisted ventilation after receiving surfactant was Oxy-Hood in 3 (0.3%) of 35 neonates, N-CPAP in 249 neonates (71.1%), and different types of ventilation with endotracheal intubation in 98 neonates (28%). Side effects of surfactant was observed in 127 (36.3%) of 350 hospitalized neonates with RDS. There was no complication of surfactant in 223 neoantes (63.7%). Of 127 neonates that suffered from the side effects of surfactant, pneumothorax was reported in 33 cases (26%), IVH in 30 cases (23.6%), more than one complication in 22 cases (17.3%), DIC in 16 cases (12.6%), pulmonary hemorrhage in 13 cases (10.2%), sepsis in 11 cases (8.7%), and pneumonia in 2 cases (1.6%) (Table 3).

Of 22 neonates who suffered from co-multiple complications after treatment, IVH+ DIC was reported in 10 neonates (45.47%), pulmonary hemorrhage + IVH in 5 cases (22.74%), pneumothorax + IVH in 1 case (4.54%), IVH + DIC + pulmonary hemorrhage in 1 case (4.54%), pneumothorax + sepsis in 1 case (4.54%), pneumothorax + pulmonary hemorrhage in 1 case (4.54%), and pulmonary hemorrhage + DIC in 1 case (4.54%) (Table 4).

Of 350 neonates with RDS who were hospitalized, 227 (64.9%) were discharged in a recovery status and 123 cases (35.1%) died. Of 213 male neonates, 138 cases (64.8%) were discharged with the recovery status and 75 cases (35.2%) died. Of 137 female neonates, 89 cases (65%) were discharged in a recovery status and 48 cases

Table 4. Distribution of absolute and relative frequency of co-multiple complications after treatment.

Co-multiple complications	Percentage	Cases (n)
IVH and DIC	45.47	10
Pulmonary hemorrhage + IVH	22.74	5
Pneumothorax+ DIC	4.54	1
Pneumothorax+ IVH	4.54	1
IVH+ DIC + pulmonary hemorrhage	4.54	1
Pneumothorax+ IVH + Pneumonia	4.54	1
Pneumothorax+sepsis	4.54	1
Pneumothorax+ Pulmonary hemorrhage	4.54	1
Pulmonary hemorrhage+ DIC	4.54	1
Total	100	22

Table 5. Effect of the birth weight on the re-dosing of surfactant in the neonates with RDS.

Weight of neonate (gr)	Dose of surfactant		Total (%)
	One dose {No. (%)}	Multiple dose {No. (%)}	
Less than 1700	129 (68.62)	59 (31.38)	188 (100)
1700-2500	80 (74.1)	28 (25.9)	108 (100)
More than 2500	43 (79.63)	11 (20.37)	54 (100)
Total	252 (72)	98 (28)	350 (100)

p value = 0.4.

(35%) died. Considering the complications after receipt of surfactant, it was found that 127 cases had complications, of which 27 (21.3%) were discharged in a recovery status and 100 cases (78.7%) died. Of 188 neonates weighing less than 1,700 g, 129 cases (68.62%) received one dose of surfactant and 59 cases (31.38%) received more than one dose of surfactant. Of 108 neonates weighing 1700 to 2500 g, 80 cases (74.1%) had received one dose of surfactant and 28 cases (25.9%) had received more than one dose of surfactant. Of 54 neonates weighing more than 2500 g, 43 cases (79.63%) had received one dose of surfactant and 11 cases (20.37%) had received more than one dose of surfactant. According to Chi-Square test, there was no significant difference between the weight of neonates and re-dosing of more doses of surfactant (p value=0.4) (Table 5).

71 of 97 neonates whose first dose of surfactant was less than 2 h after birth, received one dose of surfactant and 26 cases (26.8%) received more than one dose in 2 to 6 h after birth. In 149 neonates, whose first dose of surfactant was in 2 to 6 h after birth, 108 cases (72.5%) received one dose of surfactant and 41 cases (27.5%) received more than one dose. Of 76 neonates whose first dose of surfactant was administered in 6 to 24 h after birth, 47 cases (61.8%) received one dose of surfactant and 29 cases (38.2%) received more than one dose of surfactant. Of 28 neonates whose first dose of surfactant administered in more than 24 h after birth, 26 cases (92.9%) received one dose of surfactant and 2 cases (7.1%) received more than one dose. Of 350 studied neonates, 252 cases (72%) received one dose of surfactant and 98 cases (28%) received more than one dose of surfactant. According to Chi-Square test, time of the first dose of surfactant at a range less than 24 h had a direct effect on the re-dosing of surfactant (p value=0.01) (Figure 2).

Of 162 neonates who received Curosurf, 114 cases (70.4%) had received one dose and 48 cases (29.6%) had received more than one dose curosurf. Of 188 neonates who received Survanta, 138 cases (73.4%) had received one dose and 50 cases (26.6%) had received more than one dose of Survanta. According to the Chi-Square test, there was no significant difference between types of consumed products and redosing of surfactant (P=0.52). Of 213 male neonates, 147 (69%) had received one dose of surfactant and 66 cases (31%) had received more than one dose of surfactant. Of 137 female neonates, 105 cases (76.6%) had received one dose of surfactant and 32 cases (23.4%) had received more than one dose of surfactant. According to the Chi-square test, there was no significant difference between redosing surfactant and the gender of the neonate (p value =0.12). Of 176 neonates with the gestational age less than 32

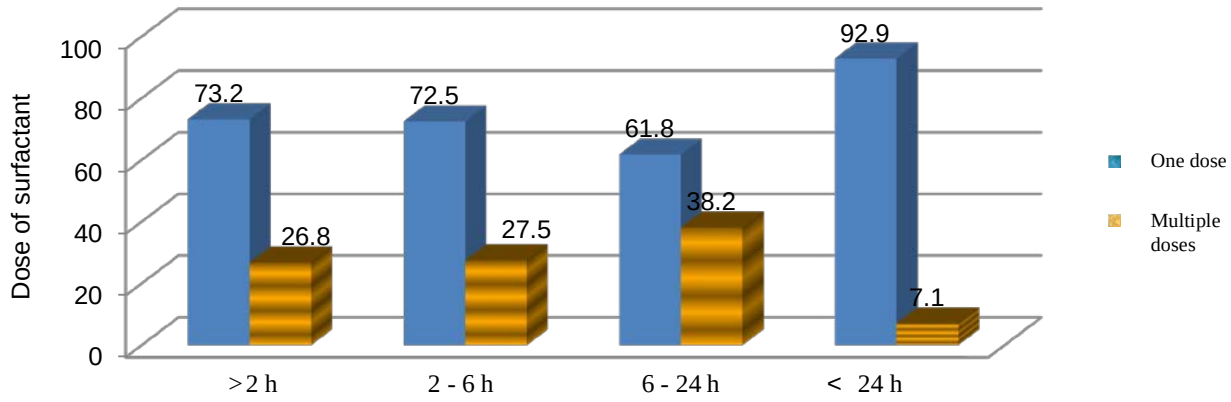

Figure 2. Effect of the time of reception of the first dose of surfactant on re-dosing.

weeks, 127 cases (72.2%) received one dose and 49 cases (27.8%) received more than one dose of surfactant. Of 122 neonates with the gestational age 32 to 36 weeks, 86 cases (70.5%) received one dose of surfactant and 36 cases (29.5%) received more than one dose of surfactant. Of 52 neonates with the gestational age of 36 weeks, 39 cases (75%) received one dose of surfactant and 13 cases (25%) received more than one dose of surfactant. According to the chi-square test, the gestational age at birth had no effect on re-dosing surfactant (p value=0.83). Of 241 neonates of the cesarean delivery, 165 cases (68.5%) received one dose of surfactant and 76 cases (31.5%) received more than one dose of surfactant. Of 109 neonates of normal vaginal delivery, 87 cases (79.8%) received one dose of surfactant and 22 cases (20.2%) received more than one dose of surfactant. According to Chi-square test, there was a significant difference between type of delivery and re-dosing surfactant obtained (p value=0.02). Of 239 singletone neonates, 168 cases (70.3%) received one dose of surfactant and 71 cases (29.7%) received more than one dose of surfactant. Of 79 twin neonates, 64 cases (81%) received one dose and 15 cases (19%) received more than one dose of surfactant. Of 24 triplet neonates, 15 cases (62.5%) received one dose of surfactant and 9 cases (37.5%) received more than one dose of surfactant. Of 8 quad neonates, 5 cases (62.5%) received one dose of surfactant and 3 cases (37.5%) received more than one dose of surfactant. According to Chi-square test, there was no significant difference between the number of neonates in a delivery and re-dosing surfactant (p value=0.17). Of 203 mothers who received corticosteroids, 141 neonates (69.5%) had received one dose of surfactant and 62 cases (30.5%) received more than one dose of surfactant. Of 147 mothers who did not receive corticosteroids, 111 cases (75.5%) had received one dose of surfactant and 36 cases (24.5%) had received more than one dose of surfactant. According to the Chi-Square test, there was no significant difference between corticosteroids used by mothers and the need for re-dosing surfactant to the hospitalized neonates with RDS (p value=0.21). Based on the degree of the received corticosteroids in mothers (one dose vs two doses), the results indicated that of 144 mothers who had received one dose of corticosteroids before delivery, 97 neonates (67.4%) had received one dose of surfactant and 47 cases (32.6%) had received more than one dose of surfactant. Of 59 mothers who had received two doses of prenatal corticosteroids, 44 neonates (64.6%) had received one dose of surfactant and 15 cases (25.4%) had received more than one dose of surfactant. According to Chi-square test, there was no relationship between receiving corticosteroids and re-dosing of surfactant doses (p value=0.31). Of 133 neoantes with high first minute APGAR score (7 to 10), 108 cases (81.2%) had received one dose of surfactant and 25 cases (18.8%) had received more than one re-dosing. Of 174 neonates with an average APGAR score (4 to 6), 111 cases (63.8%) had received one dose of surfactant and 63 cases (36.2%) had received more than one re-dosing of doses. Of 43 neonates with low APGAR scores (0 to 3) in the first minute, 33 cases (76.7%) had received one dose of surfactant and 10 cases (23.3%) had received more than one dose of surfactant. According to the Chi-square test, the first minute APGAR score had no effect on re-dosing of surfactant (p value=0.003). Of 238 neonates with the high fifth minute APGAR score (7 to 10), 177 cases (74.4%) had received one dose of surfactant and 61 cases (25.6%) had received more than one re-prescription. Of 94 neonates with the average APGAR scores (4 to 6), 60 cases (63.8%) had received one dose of surfactant and 34 cases (36.2%) had received more than one re-dosing. Of 18 neonates with the low fifth minute APGAR score (0 to 3), 15 cases (83.3%) had received one dose of surfactant and 3 cases (16.7%) had received more than one dose of surfactant. According to the Chi-square test, the fifth minute APGAR had no effect on re-dosing of surfactant (p value=0.002). There was one neonate with mild respiratory distress that had received only one dose of

Table 6. Effect of respiratory distress on the prescription of surfactant in neonates with the RDS.

Respiratory distress	Prescription of surfactant		Total (%)
	One dose {No. (%)}	More than one dose {No. (%)}	
Mild(<5)	1 (100)	0 (0)	1 (100)
Moderate(5-8)	174 (76.3)	54 (23.7)	228 (100)
Severe(>8)	77 (63.6)	44 (36.4)	121 (100)
Total	252 (72)	98 (28)	350 (100)

p value=0.03.

surfactant. Of 228 neonates with moderate respiratory distress (5 to 8), 174 cases (76.3%) received one dose of surfactant and 54 cases (23.7%) received more than one dose of surfactant. 121 neonates with severe respiratory distress (>8) were reported. 77 cases (63.6%) had received one dose of surfactant and 44 cases (36.4%) had received more than one dose of surfactant. According to the Fisher Exact test, respiratory distress had positive effect on the redosing of surfactant (p value=0.03) (Table 6).

DISCUSSION

Respiratory distress syndrome is a type of defect in the lung development, which is often seen with preterm labor and is caused by the lack of surfactant (Asnafei et al., 2004; Mahoney and Jain, 2013). Pulmonary surfactant deficiency leads to extensive atelectasis, loss of residual pulmonary capacity and collision of the ventilation to perfusion ratio (Jobe, 1993). Preterm neonates may suffer from respiratory distress syndrome due to low concentration of surfactant in the alveoli. The discovery of the key role of surfactant in the pathophysiology of RDS by the researchers in 1959 led them to think about the prescription of surfactant aerosol for premature neonates with RDS (Avery and Mead, 1959). In our study, the average weight of the premature neonates was 1782.03 ± 783.04 g and the average gestational age of newborns with the respiratory distress admitted in the hospital was 31.44 ± 3.74 weeks. The findings of the study indicated that 64.9% were discharged with the recovery condition and the percentage of death in neonates under treatment was 35.1%. Therefore, it could be concluded that surfactant therapy in the children with RDS could reduce mortality (Soll and Ozak, 2009; Ma and Ma, 2012). Although, our study did not aim to investigate the outcome of the neonates under treatment with re-dosing surfactant, our results are compatible with Dunn et al.'s (2008), Roger Soll et al.'s (2009) and Chong-Woo and Won-Ho, (2009) study. They had shown that certain recovery was observed in the degree of oxygenation of a group of neonates who received greater surfactant doses than the control group (Dani et al., 2010; Paola et al., 2011). Our findings indicated that neonates weighting less than 1700 g (31.38%) have reveived more than one dose of surfactant that is compatible with Dani et al. (2010) and Paola et al. (2011). Although, there was no significant difference between the greater weights of neonates in the study, the diagram and table of the neonates' weight showed percent of receiving surfactant has been increased with decrease in the neonates' weight. This finding is compatible with the results reported by Cogo (2009), Katz and Klein (2006), Dani (2010) and Paola (2011). Results of the neonates' gestational age indicated that those neonates born between 32 to 36 weeks needed for re-dosing greater surfactant, which is compatible with Katz and Klein (2006) study. The reason for the lower re-dosing of surfactant in neonates with the gestational age less than 32 weeks than neonates with the gestational age of 32 to 36 weeks may be due to the effect of other variables, such as birth weight, mothers receiving corticosteroids, the first and fifth minute APGAR score. Therefore, it is recommended that future researchers examine it. The neonates with the first dose of surfactant at 6 to 24 h after birth needed to significantly prescribe surfactant for other time. However, the first dose of surfactant in the range of less than 24 h had a direct effect on re-dosing surfactant. This finding is different from the results of Katz and Klein (2006). This difference could be caused by the effects of other variables. Our results indicated that there was a significant difference between the re-dosing surfactant and the type of delivery so that the neonates born by the caesarean section (31.5%) needed for re-dosing of surfactant compared to the neonates born by NVD (20.2%) as demonstrated by Kornacka and Kufel (2011). Although, there was not a significant difference between the number of twins and re-dosing of surfactant, the results did show (according to the related table and diagrams), redosing of surfactant needed to be more with the increased number of fetus in each pregnancy. The numbers of neonates with respiratory distress, admitted in Motahari Hospital of mothers who received prenatal corticosteroid dose and needed for re-dosing of surfactant were greater than the mothers who did not receive corticosteroids although not significantly and in contrast to Crowther et al. (2011). It may be influenced by other factors, including birth weight, gestational age, and so forth (Eriksson et al., 2012). In the case of using one

and two doses of by their mothers, neonates needed re-dosing surfactant whose mothers had received a prenatal corticosteroid dose compared with mothers who had received two doses of corticosteroid more. The need for re-dosing surfactant for neonates whose mothers had recieved one dose of corticosteroid was greater than the neonates whose mothers had recieved two doses of corticosteroid. This finding is compatible with the findings of Katz and Klein (2006). They reported that re-dosing of surfactant in the neonates, whose mothers receive a little corticosteroid, were greater. Our study showed that the type of surfactant products (Survanta vs Curosurf) did not have effect on the re-dosing of surfactant in the neonates. This result is different from the findings of Singh et al. (2011) but same as Rangasamy (2009, 2007) and Fakoor and Dinparast, (2009) study. Singh et al. (2011) demonstrated that the need for re-dosing the porcin surfactant in the preterm neonates was greater than to the bovine surfactant. Our findings indicated that compared to the female neonates, the male neonates received more than one dose of surfactant. Moreover, there was a significant difference between the first minute APGAR (mean APGAR score 4 to 6). In addition, vsuch difference was found between the fifth minute APGAR (mean APGAR score 4 to 6). The reason for the need for re-dosing more surfactant in the upper mean first and fifth minute APGARs than the lower first and fifth minute APGAR may be the greater mortality of the neonates in this APGAR and the effect of other above-mentioned variables. The neonates with the higher score of respiratory distress (8< - severe) significantly needed re-dosing with a dose of surfactant more than the neonates with the mild to moderate score of respiratory distress as demonstrated by Paola (2011).

Conclusion

The results of the study proved that the need for re-dosing surfactant was significantly high at the time of the prescription of the first dose of surfactant 6 to 24 h, in the C/S delivery, in the medium first and fifth minute APGAR score (4 to 6) and respiratory distress score greater than 8.

Conflict of interests

The authors have not declared any conflict of interests.

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