Gas Chromatographic Mass Spectrometric Analysis of Estrus Specific Volatile Compounds in Buffalo Vaginal Mucus after Initial Sexual Foreplay

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Abstract: Vaginal fluid from buffaloes in estrus, non-estrus condition and saliva from bulls that have licked vaginal mucus were collected. The samples were analysed by gas chromatography-mass spectrometry (GC-MS) in order to evaluate the qualitative differences between the volatile profiles of vaginal mucus and saliva to assess their potential value in buffalo bio-communication. Fifteen different organic compounds were detected. The chemical profiles of estrus vaginal fluid were distinguished significantly by the presence of specific substance, 9-octadecenoic acid (oleic acid) that was not present in non-estrus phase. The results are consistent with the view that the oleic acid acts as chemosignal in a number of mammalian species. Based on the result it is concluded that the volatile substance present in buffalo vaginal fluid may act as a chemical stimulant.

Keywords: Estrus, vaginal mucus, Saliva, Buffalo, Oleic acid and GC-MS.

INTRODUCTION

The physiological event of ovulation and coordination of sexual behaviour are important for ensuring successful fertilization [1, 2]. Chemical signals in different sources like, feaces and body fluids such as urine, saliva, vaginal mucus and glandular secretions play a major role to manifest their effects including puberty acceleration, estrous cycle and pregnancy [3]. Even though lot of works has been done on chemosignals in rodents, limited work is available in cattle chemosignals with reference to reproduction and behaviour. It is known that higher mammals including buffalo the estrus-specific chemical cues play a major role [4, 5]. The bovine vaginal secretion flows out abundantly from the vulva at estrus. The presence of volatile compounds in bovine vaginal fluid during estrus attract the bull has been documented [6]. Since buffalo vaginal fluid also contains estrus-specific signals, it is expected that it may contain estrus indicator which might act as estrus-specific volatiles. It is reported that a volatile odour in the vaginal secretions of estrus cow was a source of sexual attraction for bulls [7].

In higher mammals the chemical composition of the reproductive tract secretions are one of the main sources of pheromone [8-10], but such research has not been focused in buffalo. Volatile compounds, it is known that bulls can remotely sense something in the mucus of estrus buffalo based on the compounds which is sexually arousing. Under field conditions, visual stimuli probably do play a role in stimulating male sexual behaviour, but our *in vitro* tests with cervico-vaginal mucus clearly show that there is an olfactory component, independent of vision play a key role in the stimulation of male reproductive behavior, this is consistent with earlier reports [11-14]. Similar evidence has been indicated by studies on dogs [15] and rats [16] which were trained to detect estrus by smelling the cervico-vaginal secretions or urine.

The responses of normal bull to the odours of chemical components of estrus female buffalo vaginal discharge are evident. The estrus female produce a viscous vaginal discharge which is attractive to male [17]. The mucus of female bovine genital tract contains pheromones that induce physiological and behavioural responses in bull [18]. The present findings are consistent with previous research i.e. mucus of the genital tract contains chemosignals that induce physiological and behavioural changes in buffalo [4] and cow [6].

The grooming behaviour, a frequent activity of rodents and other mammals, involves licking the hair as well as various body parts; therefore, saliva is spread about the integument [19]. It is known that bulls lick the genital region to detect estrus, and send the vaginal chemosignal to higher region through olfaction and finally confirm the estrus which is followed by mating. Since, saliva is unquestionably an important social cue among mammals, and characteristically present on the body surfaces, the salivary molecules may become associated with resting sites, burrows or other occupied mammals, either through passive

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ISSN: 1927-5196 / E-ISSN: 1927-520X/13

contact between the animal and its surroundings or as the result of active processes [20]. Buffalo vaginal odours might serve to attract and stimulate the mounting behaviour. The present study was planned to examine the presence of volatiles from vaginal mucus of heifers in estrus as well as to confirm the presence of volatiles in bull saliva after licking the genital region during the estrus. In nature, it is not unexpected that a signal might be usable in a variety of social contexts.

MATERIALS AND METHODS

Vaginal Fluid Collection

Reproductive status of female buffaloes was assessed based on the behavioural physical changes and expression during estrous cycle. Those females in estrus were considered for collection of the sample.Vaginal fluid was collected from twelve sexually matured female buffaloes of *Bubalus bubalis* (Buffalo) in the exotic cattle-breeding centre, orathanadu livestock farm, Tanjore district, Tamil Nadu, India. The cows were approximately 2 to 3 years old. The animals were fed with usual diet and water *ad libitum* throughout the study. Rectal examination of each heifer was performed regularly at one or two-week intervals to verify the normal morphological changes in the vulva swelling. Vaginal fluid sample was collected from the buffaloes with estrus symptoms such as vaginal swelling, secretions, restlessness, frequent urination and tail waging and male reproductive behaviours like flehmen and mounting during estrus, non-estrus stages under natural condition. The technique involved rectal massage of the reproductive tract or an infusion tube placed within the vagina prior to artificial insemination. The samples were screened through cheese cloth or nylon mesh (60–120 μm) at the time of collection. Immediately after screening, the samples were stored frozen at −20˚C and analyzed by gas chromatography mass spectrometry (GC-MS).

Saliva Collection from Bull

Saliva was collected from twelve sexually mature *Bubalus bubalis* (Bulls) before and after licking the genital region. The bulls were artificially bred, and were approximately 3 to 5 years old animal were used through out the study period. The procedure for sample screen was adopted as mentioned elsewhere [5].

Sample Analysis

The samples collected from the chosen stages as per the experimental protocol were pooled to minimize the effect of individual variation. Solvent, dichloromethane, was used to extract the compounds from the vaginal mucus of estrus and non-estrus and male saliva samples. From the pooled samples 15 ml was taken and mixed with 15 ml of dichloromethane in triplicate. The supernatant was filtered through a silica-gel column (60-120 mesh) 30 min at room temperature. The filtered extract was condensed to 1/5 of its original volume by cooling with liquid nitrogen. Two microlitres of dichloromethane extract was injected into the GC-MS system on a 30 m glass capillary column with a film thickness of 0.25µm. (30 mm x 0.2 i.d. coated with UCON HB 2000 QP-5050, Shimadzu, Japan) using the following temperature programme: Initial oven temperature of 40ºC for 4 min, increasing to 250ºC at 15º C/min, and then held at 250ºC for 10 min. The GC-MS was run under computer control at 70eV. The solvent (dichloromethane) peak was seen at 4 min. The identified compounds were then compared with the standard run under the same conditions. These data were already stored in a compact library of chemical substance (NIST 6221B).

RESULTS

Identification of Volatiles from Buffalo Estrus and Non-Estrus Vaginal Mucus

The gas chromatography analysis showed that the compounds fell between 5-35 min. Figure **1** and **3** shows the mass spectrum and the chemical structure of compound identified in estrus and non-estrus stages of the estrous cycle and Figure **2** shows male saliva before (a) and (b) after licking the vaginal part of estrus female, respectively. The computer matched data of the identified compounds showed above 95% similarity with the compound identified from the vaginal mucus samples. Among the 15 compounds identified 10 were noted to be alkanes and five were acids (Table **1**). Comparison of the identified vaginal mucus volatiles across the two reproductive phases revealed that certain compounds are specific to particular stages. The compounds Tetradecane, 3,7,Dimethyldecane, Pentadecane, Hexadecane, oleic acid (9-octadecenoic acid), Hexadecanoic acid, Docosane, Heptadecane, Tricosane, Cyelononasiloxane, Heptocosane, Hexatriaconate, Pentacosane, Pentatriaconate and Tetratriaconate, were identified in vaginal mucus during estrus stage. Interestingly, the compound, 9-ocadecanoic acid, was found only in estrus stage whereas, the other 14 volatiles such as Tetradecane, 3,7,Dimethyl decane, Pentadecane, Hexadecane, Hexadecanoic acid, Docosane, Heptadecane,

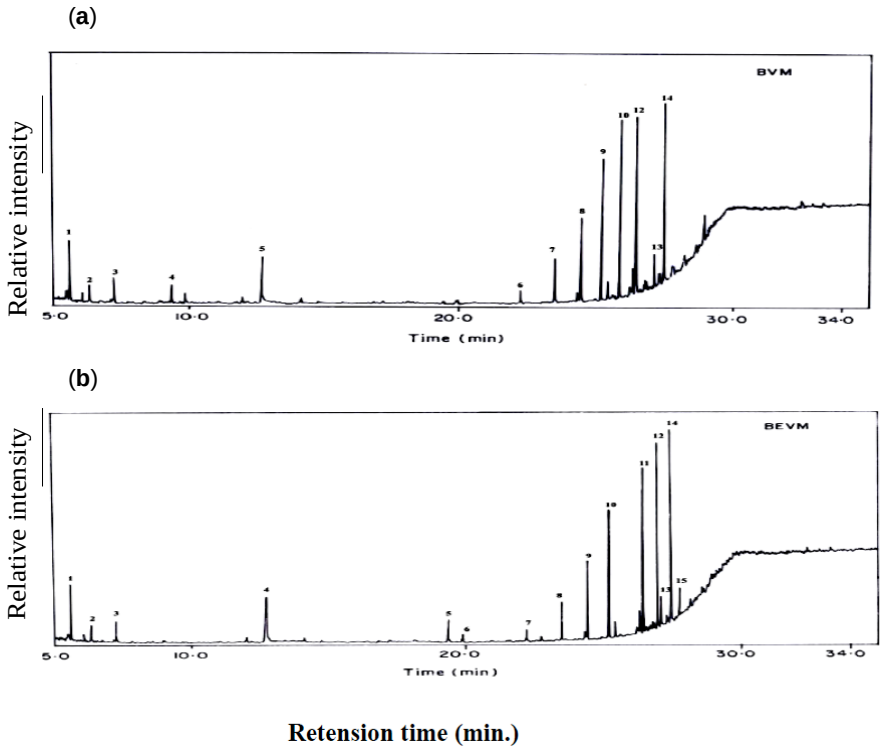

Figure 1: GC of vaginal mucus of estrus (**a**) and non-estrus (**b**) stages.

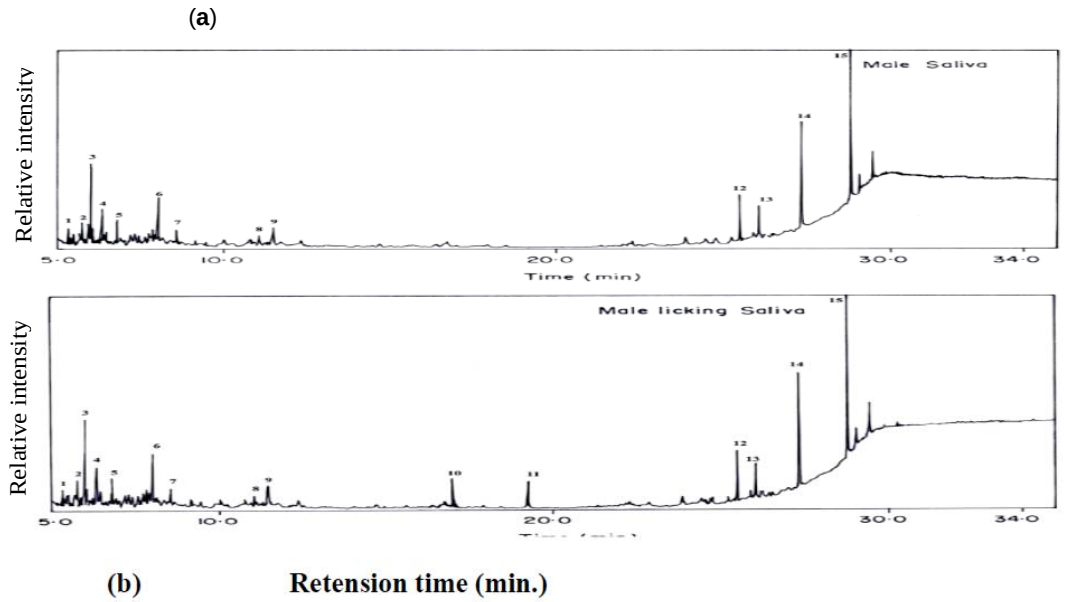

Figure 2: GC of male saliva before (**a**) and (**b**) after licking of vaginal part of estrus female.

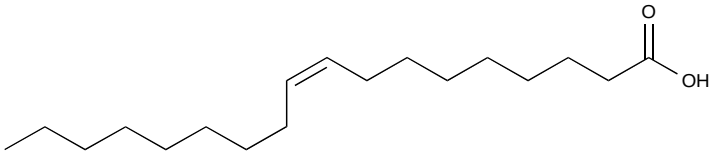

Figure 3: Chemical structure of identified estrus specific compound, 9-octadecenoic acid.

Tricosane, Cyelononasiloxane, Heptocosane, Hexatriaconate, Pentacosane, Pentatriaconate and Tetratriaconate were found in the non-estrus vaginal mucus.

Table 1: Volatile Compounds Identified in the Vaginal Mucus of Estrus and Non-Estrus Stages

S. No	Compound name	Non-estrus vaginal mucus	Estrus vaginal mucus
1	Tetradecane	+	+
2	3,7,Dimethyl decane	+	+
3	Pentadecane	+	+
4	Hexadecane	+	-
5	9-octadecenoic acid	-	**+**
6	Hexadecanoic acid	+	+
7	Docosane	+	+
8	Heptadecane	+	+
9	Tricosane	+	+
10	Cyelononasiloxane	+	+
11	Heptocosane	+	+
12	Hexatriaconate	+	+
13	Pentacosane	+	+
14	Pentatriaconate	+	+
15	Tetratriaconate	+	+

+ Present.
- Absent.

Identification of Volatiles from Male Saliva Before and After Licking the Genital Region of Estrus Female

The GC-MS profiles shown in the Figure **2** and Table **2** are the representative of the salivary compounds of bull before and after licking vaginal region. The analysis of the male saliva before licking of vaginal mucus showed 14 compounds that include Dodecanoic acid, Dodecane, Tetradecanoic acid, Hexadecanoic acid, 2,6,10,14,18,22-Tetracosahexane, Cholest-5-en-3-ol, 3,7-Dimethyldecane, Octadecane, Octa decanoic acid, Docosane, Propanoate, 2,4-Cholestadiene, Lanol and 2-tert-butyl-4,6 bis phenol (Table **2**). Interestingly, oleic acid was present only in male saliva after licking the vaginal mucus, but this compound was exclusively absent in the male saliva before licking the vaginal mucus. The other compounds like Dodecanoic acid, Dodecane, Tetradecanoic acid, Hexadecanoic acid, 2,6,10,14,18,22-Tetracosahexane, Cholest-5-en-3-ol, 3,7-Dimethyldecane, Octadecane, Docosane, Propanoate, 2,4-Cholestadiene, Lanol and 2-tert-butyl-4,6 bis phenol were present in both male salivary samples.

DISCUSSION

The present results revealed that 15 volatile compounds are present both in the estrus and non-estrus vaginal mucus. In the estrus vaginal mucus oleic acid exclusively appeared but not in the non-estrus stage. Among the compounds identified in estrus vaginal mucus the oleic acid belongs to fatty acids. This finding concludes that the estrus-specific compound(s) is produced in female vaginal mucus to advertise the conspecific for natural coitus. The same estrus-specific compound was already reported in buffalo urine by GC-MS [5]. This provides circumstantial evidence that there is a relationship of urine and vaginal mucus production through producing volatile compounds to indicate the status of female. The identification of estrus-specific volatile compound in the two body fluids of buffalo is noteworthy.

Interestingly, the identified estrus-specific vaginal fluid compound, i.e. oleic acid, is present in the male saliva after licking the vaginal region. The interaction between vaginal mucus specific volatile and transfer of the same compound to male saliva through licking the genital region of female buffalo is considered to be significant and the specific volatile may act as sexual stimulant during the reproductive period. Previously, the Z11-16: COOH and other Δ 11 fatty acids are known to be produced only in moth pheromone glandular tissue [21]. Lizards also produce femoral chemical secretions that contain numerous chemical compounds [22] which have pheromonal activity [23,24]. The present study reports the presence of estrus-specific oleic acid compound from vaginal

Table 2: Volatile Compounds in Male Saliva Before and After Licking of Vaginal Part of Estrus Female

S. No	Compound name	Before licking	After licking
1	Dodecanoic acid	+	+
2	Dodecane	+	+
3	Tetradecanoic acid	+	+
4	Hexadecanoic acid	+	+
5	2,6,10,14,18,22-Tetracosahexane	+	+
6	Octadecenoic acid	-	+
7	9-octadecenoic acid	-	+
8	Cholest-5-en-3-ol	+	+
9	3,7-Dimethyldecane	+	+
10	Octadecane	+	+
11	Docosane	+	+
12	Propanoate	+	+
13	2,4-Cholestadiene	+	+
14	Lanol	+	+
15	2-tert-butyl-4,6 bis phenol	+	+

+ Present.
- Absent.

mucus and suggests that it may act as a specific chemosignal involved in the major sexual communication and bull mounting activity. Evidence indicates the fatty acid has a main role involved in pheromonal communication [23,25]. It is found that the lipophilic fractions of femoral secretions of male lacertid lizards are mainly composed of fatty acids and steroids, with minor quantities of other compounds which may be considered as signal cues [22]. However, the previous studies suggest that oleic acid is found in appreciable quantities in femoral secretions of many lizards, including *I.cyreni* which prefer scent marks of males with higher body condition [26], and field studies confirmed that the male *I.cyreni* with higher body condition has a grater reproductive success [27]. Further, in line with the present study, the scent marked areas with more oleic acid might be simply more attractive if chemosensory responses of female to the scent of male lizards evolved from a preexisting sensory bias for food related chemicals such as the oleic acid [28]. It is documented that the potential functions of oleic acid in femoral gland secretions of male rock lizards *I.cyreni* and its consequences for sexual selection process [29]. In addition, it is suggested that the compound involved ensure greater mating [29] and reproductive success [27]. However, other reports suggest that the females showed higher chemosensory responses to femoral secretions of males that had higher proportions of oleic acid, which suggest that these scents were more attractive [30].

The findings of the present study are consistent with earlier report that pheromone components are biosynthesized from oleic acid as hypothesized by Bjosted *et al*., [31]. However, these facts suggest that vaginal mucus contains not only estrus-related odours, but also individual distinctive odours. It is known that trained dogs sniff and identify the estrus related odours of cattle [32], and that the odour is slowly emitted during three day before estrus reaches a definite peak in intensity on the day of estrus, and then disappears within 1 day [32]. The diestrus heifer to which another's estrus mucus had been applied was sniffed more frequently than the water-treated heifers. Therefore, the estrus related odour in another's vaginal mucus irrespective of the individual chemosignals, may induce the sniffing behaviour of other animals [18]. However, matching an individual's distinctive odour contained in vaginal mucus with that of the body odours of the heifer itself may be essential to induce the mounting behaviour.

The pheromonal activities by testing their chemosensory attractiveness to the hexadecanoic acid and cholesterol were also richer in rat [33]. The relative affinity of pheromone compounds with olfactory mucosa is well demonstrated. The endogenous natural

ligands of human tear lipocalin have been identified as fatty acids (palmitic, steric and lauric), cholesterol phospholipids and glycolipids and studied thoroughly that they seem to be tightly bind in pigs [34]. Hence, the present investigation concludes that the oleic acid, incorporated into both vaginal mucus and male saliva is an the estrus-specific compound which is transferred to bull saliva during vaginal licking process, confirming the sexual importance of oleic acid which is considered as unique volatile compound in buffalo biocommunication.

ACKNOWLEDGEMENTS

We thank Dr. Chellam Balasundaram Professor of Emeritus, Bharathidasan University for critically reading the manuscript. We gratefully acknowledge to the financial support from DBT, DST-PURSE and UGC-SAP, New Delhi.

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Received on 25-11-2012 Accepted on 01-02-2013 Published on 22-03-2013

DOI: http://dx.doi.org/10.6000/1927-520X.2013.02.01.1