原　著

ペットのグリーンイグアナにおける*Salmonella*，*Pasteurella*および*Staphylococcus*の保菌状況

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要　約

全国23都道府県のペットのグリーンイグアナについて*Salmonella*，*Pasteurella*および*Staphylococcus*の保菌状況を検討した．*Salmonella*は，98頭中17頭（17.3％）の糞便から分離された．分離株49株中47株は，生物群Ⅳの*S. enterica* subsp. *houtenae*であり，わが国のイグアナが原因と思われる乳児サルモネラ症の原因となった血清型45:g, z_{51}:-が3株，生物群Ⅰの*S. enterica* subsp. *enterica*も2株分離された．陽性個体17頭由来の17株中9株（52.9％）はstreptomycin耐性株であり，また，すべての株は上皮細胞侵入因子（*inv*A）およびエンテロトキシン（*stn*）両遺伝子を保有していた．*P. multocida*は89頭中3頭（3.4％）から，また，*S. aureus*は18頭（20.2％）の口腔からそれぞれ分離された．――キーワード：イグアナ，*Pasteurella*，*Salmonella*，*Staphylococcus*．

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近年のペット動物の多様化に伴い，犬，猫，鳥類以外にも爬虫類をペットとして飼育する人たちが増えてきた．内閣府が実施した「動物愛護に関する世論調査」によると，2003年にはペットを飼育している人の2.4％が爬虫類を飼育していることが判明している．なかでも，イグアナは草食性で一般に性格もおとなしく，飼育も容易であることから，家庭内で飼育している人が多い．しかしながら，成長に伴い大型化すると咬傷事故も多数報告されるようになった［1］．

米国では，年間およそ74,000人が爬虫類に関連したサルモネラ症に罹患しており，人のサルモネラ症全体の6％，特に子供では11％に相当することが明らかとなっている［2］．わが国においても，2004年から2005年にかけてイグアナやミドリガメが感染源として疑われた乳児重症サルモネラ症の事例が相次いで報告され，厚生労働省は，2005年12月22日，「ミドリガメ等爬虫類を原因とするサルモネラ症発生に係わる注意喚起について」を全国の自治体に対して通達するとともに，爬虫類の飼育者や取り扱い業者に対しては，ようやく本症に関する正しい情報の提供に努める動きを始めた．

これまでに，わが国のペット用に輸入されたカメ類やトカゲ類の多くは*Salmonella*を保菌していることが報告されている［3, 4］．しかしながら，実際に家庭で飼育されている爬虫類を対象としてサルモネラを含む各種人と動物の共通感染症起因菌の保菌状況を詳細に検討した報告は少ない．特に近年，わが国でも乳児サルモネラ症の感染源として推定されたペットのイグアナの*Salmonella*保菌状況については，まったく不明の状態であるといっても過言ではない．

そこで本研究では，わが国で爬虫類のペットとして広く飼育されているグリーンイグアナを対象として，*Salmonella*の保菌状況について検討するとともに，咬傷感染症であるパスツレラ症の起因菌*Pasteurella multocida*および創傷化膿症の起因菌である*Staphylococcus aureus*の保菌状況について検討した．

材料および方法

供試材料：北海道・東北地方（北海道，岩手県，山形県，宮城県，福島県），関東地方（東京都，神奈川県，埼玉県，千葉県，山梨県），北陸・東海地方（新潟県，

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表1　地方別のペットイグアナの糞便および口腔スワブ検査頭数

地　方	都道府県	糞便検査頭数	口腔スワブ検査頭数
北海道・東北	北海道	12	11
	岩　手	1	0
	山　形	1	1
	宮　城	2	2
	福　島	1	0
小　計		17	14
関　東	埼　玉	11	11
	千　葉	6	6
	東　京	11	10
	神奈川	21	19
	山　梨	1	0
小　計		50	46
北陸・東海	新　潟	3	3
	石　川	2	2
	愛　知	3	3
	岐　阜	1	0
	三　重	2	2
小　計		11	10
関　西	京　都	1	1
	大　阪	9	8
	兵　庫	1	1
小　計		11	10
中国・四国・九州	広　島	1	1
	愛　媛	1	1
	香　川	1	1
	福　岡	5	5
	鹿児島	1	1
小　計		9	9
合　計		98	89

石川県，愛知県，岐阜県，三重県），関西地方（京都府，大阪府，兵庫県），中国・四国・九州地方（広島県，愛媛県，香川県，福岡県，鹿児島県）から合計62カ所の家庭および団体でペットとして飼育されているグリーンイグアナ（*Iguana iguana*，以下イグアナ：1カ所から1～11頭）を対象に調査した．2003年6月から2004年4月の間，すべて異なる個体から採取された糞便98検体と口腔スワブ89検体をそれぞれ実験に供試した（表1）．イグアナの新鮮糞便と口腔スワブは，シードスワブ（γ3号 '栄研'，栄研化学㈱，東京）で採取した後，ただちに日本大学生物資源科学部獣医学科獣医公衆衛生学研究室に常温で送付した．

Salmonellaの分離と同定：シードスワブ中の糞便材料を5m*l*の滅菌リン酸緩衝液に振るい出し，その1m*l*を9m*l*のハーナテトラチオン酸塩培地（栄研化学㈱，東京）へ接種し，37℃で24時間増菌培養した．培養後，増菌培地の1白金耳量をDHL（栄研化学㈱，東京）およびMLCB寒天平板培地（日水製薬㈱，東京）に塗抹し，37℃で24時間培養した．各分離培地上の*Salmonella*を疑うコロニーをそれぞれ1～3個釣菌し，DHL寒天培地で純培養を行った．純培養後，グラム染色，TSI培地，SIM培地，VP-MR試験（以上，栄研化学㈱，東京）およびSC培地（日水製薬㈱，東京）による生化学性状ならびにサルモネラO多価血清（デンカ生研㈱，東京）によるためし凝集反応により予備同定を行った後，IDテストEB-20（日水製薬㈱，東京）を用いて生物群を決定した．さらに，サルモネラ型別血清（デンカ生研㈱，東京）を用いたスライド凝集反応により血清型を決定した．

Salmonella分離株の薬剤感受性試験：各*Salmonella*陽性個体から分離された任意の1株，計17株について，Kirby-Bauer法による薬剤感受性試験を行った［5］．薬剤感受性用ディスクはAmpicilin，Piperacillin，Cefozopran，Streptomycin，Kanamycin，Gentamicin，Tetracycline，Chloramphenicol，Fosfomycin，Nalidixic acid，Ofloxacin，Sulfamethoxazole/Trimethoprim（BD Sensi-Disc, Becton Dickinson, U.S.A.）の12薬剤を用いた．

Salmonella分離株の*invA*および*stn*遺伝子の検出：薬剤感受性試験に用いた17株について，上皮細胞侵入因子（*inv*A）およびエンテロトキシン（*stn*）両遺伝子の保有状況を検討した．*inv*A遺伝子はChiuら［6］，*stn*遺伝子はMakinoら［7］のPCR法をそれぞれ用いて検出した．

Pasteurellaの分離と同定：*Pasteurella*の分離にはマウス通過法を用いた．採取した口腔スワブ材料を5m*l*の滅菌リン酸緩衝液に振るい出した後，1m*l*をそれぞれ2頭のddYマウス（7週齢，メス）に腹腔内投与した．投与後24および48時間後に，ジエチルエーテル麻酔下のマウスから無菌的に全採血を行い，EDTAチューブ（ベノジェクトⅡ真空採血管，テルモ㈱，東京）に回収した．得られたマウス血液の100μlを5%馬血液加ハートインフュージョン寒天培地（HIA, DIFCO, U.S.A.）に塗抹し，37℃で48時間分離培養を行った．培地上に発育した*Pasteurella*を疑うコロニーを1～3個釣菌し，HIA培地に純培養した．*Pasteurella*属の同定は，グラム染色性，馬血液の溶血性，LIM培地（栄研化学㈱，東京）による試験，VP-MR試験，カタラーゼ産生性，硝酸塩還元試験により行った．また，菌種の同定は，マッコンキー寒天培地（日水製薬㈱，東京）上の発育性，乳糖および白糖分解能，インドールおよびウレアーゼ産生性により行った．

Staphylococcusの分離と同定：*Pasteurella*の分離に用いた口腔スワブ材料の振り出し液1m*l*を7.5%NaCl加トリプトソーヤブイヨン（日水製薬㈱，東京）に接種し，37℃で48時間増菌培養した．培養後，増菌

表2　ペットイグアナにおける*Salmonella*, *Pasteurella*および*Staphylococcus*の保菌状況

検査頭数	分離菌種	陽性頭数（%）	分離株数
98	*S. enterica* subsp. *houtenae*	17（17.3）	48
	S. enterica subsp. *enterica*	1（1.0）	1
合計		17[*1]（17.3）	49
89	*P. multocida*	3（3.4）	6
	P. ureae	2（2.3）[*2]	5
	Pasteurella spp.	8（9.0）[*2]	20
合計		13（14.6）	31
89	*S. aureus*	18（20.2）	58
	S. caprae	3（3.4）	3
	S. warneri	1（1.2）	1
	Staphylococcus spp.	13（14.6）	48
合計		35（39.3）	110

＊1：1頭は*S. enterica* subsp. *enterica* と*S. enterica* subsp. *houtenae*を保菌していた．

＊2：2頭は*P. ureae*と*Pasteurella* spp. を保菌していた．

培地の1白金耳量をMSEY寒天培地（日水製薬㈱，東京）とフォーゲル・ジョンソン寒天培地（日水製薬㈱，東京）にそれぞれ塗抹し，37℃で48時間分離培養した．各分離培地上の*Staphylococcus*を疑うコロニーを3個釣菌し，MSEY寒天培地で純培養後，グラム染色性，市販の菌同定キット（N-IDテスト／SP-18，栄研化学㈱，東京）を用いた生化学性状試験およびウサギプラズマ（栄研化学㈱，東京）を用いたコアグラーゼ試験により菌種を同定した．

成　　績

***Salmonella*保菌状況**：供試した98頭のイグアナのうち17頭（17.3％）から合計49株の*Salmonella*が分離された（表2）．

イグアナが飼育されている地域別の*Salmonella*保菌率は，北海道・東北地方で35.3％（6/17），関東地方で16.0％（8/50），北陸・東海地方で18.2％（2/11），関西地方で0％（0/11），中国・四国・九州地方で11.1％（1/9）であった（表3）．各地域の保菌率の間に有意な差は認められなかった．

雌雄別の*Salmonella*保菌率は，雄で17.5％（7/40），雌で14.3％（6/42），性別不明の個体で25.0％（4/16）であったが，有意差は認められなかった（表3）．

また，年齢別の*Salmonella*保菌率は，0～3歳で26.3％（5/19），4～6歳で18.8％（6/32），7～9歳で13.3％（4/30），10歳以上で0％（0/5），年齢不明のもので16.7％（2/12）と，0～3歳でやや高い値を示したが，有意差は認められなかった（表3）．

表3　ペットイグアナの地域別，雌雄別および年齢別にみた*S. enterica*, *P. multocida*および*S. aureus*の保菌状況

項　目	分　類	%保菌率（陽性数/検体数）		
		S. enterica	*P. multocida*	*S. aureus*
地域別	北海道・東北	35.3（6/17）	0（0/14）	28.6（4/14）
	関　東	16.0（8/50）	4.4（2/46）	19.6（10/46）
	北陸・東海	18.2（2/11）	0（0/10）	0（010）
	関　西	0（0/11）	0（0/10）	10.0（1/10）
	中国・四国・九州	11.1（1/9）	11.1（1/9）	33.3（3/9）
雌雄別	雄	17.5（7/40）	8.1（3/37）	21.6（8/37）
	雌	14.3（6/42）	0（0/38）	23.7（9/38）
	不　明	25.0（4/16）	0（0/14）	7.1（1/14）
年齢別	0～3	26.3（5/19）	7.1（1/14）	7.1（1/14）
	4～6	18.8（6/32）	3.3（1/30）	16.7（5/30）
	7～9	13.3（4/30）	3.7（1/27）	33.3（9/27）
	10以上	0（0/5）	0（0/4）	50.0（2/4）
	不　明	16.7（2/12）	0（0/14）	7.1（1/14）

***Salmonella*分離株の各種性状（生物群，血清型，薬剤感受性および遺伝子型）**：イグアナから分離された*Salmonella* 49株のうち47株は生物群Ⅳの*S. enterica* subsp. *houtenae*と分類され，他の2株は生物群Ⅰの*S. enterica* subsp. *enterica*であった．前者の血清型については，48:g, z_{51}:-が13株（26.5％），11:z_4, z_{23}:型別不能（UT）が11株（22.4％），44:z_4, z_{23}:-が8株（16.3％），16:z_4:UTが6株（12.2％），50:g, z_{51}:-が3株（6.1％），45:g, z_{51}:-が3株（6.1％），50:z_4, z_{32}:-が2株（4.1％）および43:z_4, z_{32}:-が1株（2.0％）であった．*S. enterica* subsp. *enterica* 2株の血清型は，13:z:1, 6であった（表4）．

17株の薬剤感受性試験では，Streptomycin耐性株が9株認められたが，その他の薬剤に対してはすべて感受性であった．さらに，17株すべてが*inv*Aおよび*stn*の両遺伝子を保有していた（データ未掲載）．

***Pasteurella*および*Staphylococcus*保菌状況**：検査した89頭のイグアナのうち，13頭（14.6％）から合計31株の*Pasteurella*属菌が分離された（表2）．このうち，*P. multocida*は3頭（3.4％）から，*P. ureae*は2頭（2.3％）から，未同定の*Pasteurella* spp. は8頭（9.0％）からそれぞれ分離された．*P. multocida*は関東地方の46頭中の2頭（4.4％）から，中国・四国・九州

表4　ペットイグアナから分離された*Salmonella*の生物群と血清型

菌　種	生物群	O抗原	H抗原	分離頭数(%)	株数(%)
S. enterica subsp. *enterica*	I	13	z:1,6	1*1 (5.9)	2 (4.1)
S. enterica subsp. *houtenae*	IV	48	g, z_{51}:-	5*1 (29.4)	13 (26.5)
		11	z_4, z_{23}:UT*2	5*1 (29.4)	11 (22.4)
		44	z_4, z_{23}:-	3 (17.7)	8 (16.3)
		16	z_4:UT	2 (11.8)	6 (12.2)
		50	g, z_{51}:-	1 (5.9)	3 (6.1)
		45	g, z_{51}:-	1 (5.9)	3 (6.1)
		50	z_4, z_{32}:-	1*1 (5.9)	2 (4.1)
		43	z_4, z_{32}:-	1*1 (5.9)	1 (2.0)
合　計				17	49

*1：異なる血清型の株を保菌している個体を含む．
*2：型別不能

地方9頭中の1頭（11.1％）からそれぞれ分離された．また，*P. multocida*は0～3歳，4～6歳，7～9歳の各1頭から分離された．

*Staphylococcus*属菌は35頭（39.3％）から合計110株が分離された（表2）．このうち，*S. aureus*は18頭（20.2％）から，*S. caprae*は3頭（3.4％）から，*S. warneri*は1頭（1.2％）から，未同定の*Staphylococcus* spp.は13頭（14.6％）からそれぞれ分離された．年齢別の*S. aureus*の保菌率は0～3歳で7.1％（1/14），4～6歳で16.7％（5/30），7～9歳で33.3％（9/27），10歳以上で50.0％（2/4）と加齢に伴い高くなる傾向があった（表3）．

*P. multocida*および*S. aureus*の保菌率を地域別，雌雄別および年齢別に比較したところ，いずれも有意差は認められなかった．

考　　察

これまでの研究により，およそ50～90％の爬虫類が*Salmonella*を保菌していることが報告されている［4, 9, 10］．本研究では，検討したペットのイグアナの*Salmonella*保菌率は17.3％と過去の報告に比べて低率であった．また，性別，年齢別，地域別の保菌率にそれぞれ有意差は認められなかった．本研究で検討したイグアナは，すべて家庭で飼育されているペットであり，外部環境から隔絶された状態で飼育されている．また，小松菜，青梗菜，大根，南瓜などが給餌されており，*Salmonella*の主要な感染ルートである肉類からの感染は考えにくい．したがって，家庭内で飼育されているイグアナは，新たに*Salmonella*に感染する機会は少なく，飼育時あるいは輸入時から保菌していたと考えられた．いっぽう，イグアナを多頭飼育している家庭や団体62カ所のうち，*Salmonella*保菌を認めた施設は4カ所であった．これらの施設における同菌保菌率は20.0％（2/10）から100％（2/2）を示したことから，*Salmonella*の感染が施設内で起こった可能性がある．今後これら施設における分離菌株の性状を比較し，施設内での水平伝播について検討する必要がある．

爬虫類から分離された*Salmonella*分離株の生物群はⅠ，Ⅱ，Ⅲa，Ⅲb，Ⅳで，血清型も多種にわたることが海外で報告されている［8, 11］．中臺ら［3］の報告では，わが国へ輸入された直後のカメ類およびトカゲ類から分離される*Salmonella*は，生物群Ⅰが最も多く全体の59.1％であり，次いで，Ⅱ，ⅢbおよびⅣ群の順となっている．これに対し，今回イグアナから分離された*Salmonella*はほとんどがⅣ群で，人や動物から多く分離されるⅠ群は2株のみであった．以上のことから，わが国でペットとして飼育されているイグアナは，限られた生物群の*Salmonella*を保菌しているものと考えられた．しかしながら，2003年に米国では，爬虫類由来のⅣ群の*Salmonella*血清型48:g, z_{51}:-および44:z_4z_{23}:-による乳児の感染例が報告されている（CDC, MMWR, 52, 1206-1209, 2003）．また，2004年2月に千葉県内で発生した，自宅のイグアナが原因と疑われた生後27日の乳児におけるサルモネラ症の事例では，Ⅳ群の血清型45:g, z_{51}:-が分離されている．本研究でも，ペットのイグアナから同様の血清型が分離されており，また，分離株は*inv*Aおよび*stn*などの病原因子をコードする遺伝子も保有していた．以上のことから，わが国においてもイグアナは人，特に乳幼児のサルモネラ症の感染源になる可能性があるため，家庭内におけるその取り扱いには注意する必要があるものと考えられた．

今回イグアナから分離された*Salmonella* 17株中9株はStreptomycin耐性株であった．これら耐性株の出現機構については不明であるが，爬虫類の*Salmonella*感染に対して，抗生物質による治療は効果的ではなく，むしろ耐性菌の出現を誘導することが報告（CDC, MMWR, 52, 1206-1209, 2003）されていることから，爬虫類の細菌感染症に対する抗菌薬の投与には，十分注意する必要があると思われた．

いっぽう，本研究において，イグアナの口腔内からパスツレラ症の原因となる*P. multocida*と化膿の原因となる*S. aureus*がそれぞれ3.4％，20.2％の個体から分離された．さらに，日和見感染症を引き起こす*S. caprae*と*S. warneri*，人の上部気道炎の原因となる*P. ureae*も分離されている．爬虫類が多くの家庭内で飼育

されるのに伴い咬傷事故が増加しているが，今回のイグアナの飼育者に対して実施したアンケート調査でも，73.5％（25/34）の人が，飼育中の咬傷事故を経験している（データ未掲載）．海外でも，イグアナによる咬傷事故［1］，咬傷に伴う炎症反応［12］，*Serratia marcescens*の感染例［13］などが報告されている．したがって，イグアナの飼育者に対しては，咬傷事故を未然に防ぐとともに，その飼育方法や接し方などの配慮が公衆衛生上重要であると思われた．

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Prevalence of *Salmonella*, *Pasteurella* and *Staphylococcus* among Pet Green Iguanas in Japan

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SUMMARY

This paper examines the prevalence of *Salmonella*, *Pasteurella* and *Staphylococcus* bacteria found among pet green iguanas (*Iguana iguana*) in 23 prefectures throughout Japan. An examination of stool samples of the pet iguanas revealed *Salmonella* spp. in 17 of 98 samples (17.3%). Forty-seven of the 49 *Salmonella* isolates examined were classified as biogroup Ⅳ, *S. enterica* subsp. *houtenae*. They included three isolates of serotype 45:g, z_{51}:- which had been found in a case of infant iguana-associated *Salmonellosis* in Japan. The remaining two isolates were identified as biogroup Ⅰ, *S. enterica* subsp. *enterica*. Nine of the 17 isolates examined (52.9%) showed a resistance to streptomycin, and the genes for both a cell invasion factor (*inv*A) and enterotoxin (*stn*) were detected in all 17 isolates. Of 89 oral swabs taken from the iguanas, *P. multocida* was isolated in three (3.4%) of the swabs and *S. aureus* was isolated in 18 (20.2%).

—— Key words : Iguana, *Pasteurella*, *Salmonella*, *Staphylococcus*.

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ORIGINAL PAPER

Trypanocidal activity of extracts and compounds from the stem bark of *Anogeissus leiocarpus* and *Terminalia avicennoides*

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Abstract The antitrypanosomal activity of methanolic extracts of *Anogeissus leiocarpus* and *Terminalia avicennoides* were evaluated in vitro against four strains of *Trypanosoma* species with minimum inhibitory concentration (MIC) value range of 12.5–50 mg/ml. Successive fractionations of the two plant extracts in water, butanol and ethyl acetate gave a range of activity (MIC, 20 to ≥50 μg/ml). Activity-guided and chromatographic analysis of butanolic fractions on Sephadex LH-20 column followed by high-performance liquid chromatography, nuclear magnetic resonance analysis and both ultraviolet and thin layer chromatography revealed hydrolysable tannins with a range of activity (MIC, 7.5–27.5 μg/ml or 14–91 μM). Effect of the compounds on fibroblasts did not reveal serious toxicity at moderate concentration but is concentration dependent.

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Introduction

African trypanosomiasis also known as sleeping sickness is caused by *Trypanosoma* transmitted by tsetse flies. It is a debilitating disease whose major burden is on the rural poor in the endemic sub-Saharan Africa (Welburn et al. 2006). In human, the disease takes two forms, chronic Gambian sleeping sickness caused by *Trypanosoma brucei gambiense* and acute Rhodesian sleeping sickness caused by *Trypanosoma brucei rhodesiense* with over 50 million people at risk of acquiring the infection living in and around the 200 tsetse flies foci in the 37 endemic African countries (WHO 1998).

The current available chemotherapies for these parasitic diseases are far from satisfactory. The available drugs against these diseases are limited and most have been in use for more than 50 years. Limited efficacy, drug resistance, cost and toxic side effects are the main drawbacks of most of the drugs. Moreover, no vaccine is available yet against any of these diseases, which makes chemotherapy the only available option to control the infections. The nature that frequently unleashes a barrage of new and frightening diseases against humans also provides the wherewithal to help conquer the diseases that it sets loose (Crump 2006). Traditional medicine has long focused on the power of natural products to treat and cure diseases. In Africa, just like other parts of the world, the extensive use of plants in folk medicine has been documented. In general, the influence of natural products upon drug discovery is

impressive and a number of clinically active drugs are either natural products or have a natural product pharmacophore (Koehn and Carter 2005). The need to study medicinal plants in detail from various points of view, to discover new therapeutically active compounds, therefore becomes imperative. The present study, therefore, discusses the in vitro anti-trypanosomal activity of methanolic extracts of *Anogeissus leiocarpus* and *Terminalia avicennoides* and investigates the activity of the successive solvent fractions and the identified compounds on four strains of *Trypanosoma* species.

Materials and methods

Plant extracts preparation, fractionation and isolation

Air-dried, powdered stem bark (1.2 kg each) of *T. avicennoides* and of *A. leiocarpus* were macerated in methanol at room temperature for 24–48 h. The methanolic crude extract yield (162.13 and 114.1 g) for *T. avicennoides* and *A. leiocarpus*, respectively, was suspended in water and successively partitioned with butanol and ethyl acetate. The insoluble precipitate formed during the solvent partitioning was dissolved in dimethyl sulfoxide (DMSO). Five grams of the butanolic fraction was chromatographed on the Sephadex LH-20 column with water containing increasing proportions of methanol (0–100%, 20% stepwise elution) to give six fractions. Thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC) analysis indicated that fraction (fr.) 1 (0.74 g, 15%) contained sugars and triterpene glycosides; fr. 2 (0.63 g, 13%) was further separated by silica gel column chromatography to give two compounds. Nuclear magnetic resonance (NMR) spectral analysis revealed that one was ellagic acid rhamnoside and the other was triterpene glycoside; however, further structural elucidations were not completed. Fr. 3 (0.31 g, 6%) was a mixture of tannins and phenol carboxylic acids. Fr. 4 (0.40 g, 8%) was a mixture of flavogallonic acid bislactone (co-TLC, HPLC) and punicalagin (HPLC) and fr. 5 (1.30 g, 26%) contained ellagic acid (TLC, HPLC) and tannins detected as a broad hump of the baseline on HPLC analysis. The major constituent of fr. 6 (0.69 g, 13.8%) was found to be terchebulin by NMR spectral comparison and unknown tannins and phenol carboxylic acid were also detected (Tanaka et al. 1986a, b; Lin et al. 1990), while the butanolic fraction of *A. leiocarpus* revealed castalagin as the major compound along with ellagic and flavogallonic acids (Tanaka et al. 1996).

Spectral analysis

^{1}H and ^{13}C NMR, ^{1}H–^{1}H correlated spectroscopy, nuclear Overhauser effect spectroscopy, heteronuclear single quantum coherence and heteronuclear multiple bond correlation spectra were recorded with a Unity *plus* 500 spectrometer (Varian, Palo Alto, CA, USA) operating at 500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR spectroscopy. The ^{1}H and ^{13}C NMR spectra were also measured using a JEOL JMN-AL400 (JEOL, Tokyo, Japan) operating at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR. Fast atom bombardment mass spectra were recorded on a JMS-700 N spectrometer (JEOL, Tokyo, Japan), and *m*-nitrobenzyl alcohol or glycerol was used as the matrix.

Chromatography and HPLC analysis

Column chromatography was conducted on Sephadex LH-20 (Pharmacia Fine Chemicals). TLC was performed on 0.2-mm-thick precoated Kieselgel 60 F_{254} plates (Merck) with benzene/ethyl formate/HCO_2H (1:7:1, *v/v*) with spots detected by ultraviolet (UV) illumination, sprayed with 2% ethanolic $FeCl_3$ or 10% H_2SO_4 reagent. Analytical HPLC was performed on a Cosmosil $5C_{18}$-AR II column (Nacalai Tesque; 250×4.6-mm inner diameter), with gradient elution at 10–30% (30 min) and 30–75% (15 min) of CH_3CN in 50 mM H_3PO_4 (flow rate, 0.8 ml/min; detection, JASCO photodiode array detector MD-910).

Assay for trypanocidal activities

The methanolic extracts and the isolated compounds were dissolved in DMSO at a stock solution of 5 or 10 mg/ml and diluted to concentrations ranging from 1.0 to 1,000 µg/ml. Different concentrations were assessed for trypanocidal activities in either 24- or 48-well plates in a 24–48-h time course. Minimum inhibitory concentrations (MICs) were each determined under inverted microscope for the different parasites. *Trypanosoma brucei brucei* GUTat3.1, *T. b. gambiense* Wellcome strain, *T. b. rhodesiense* IL2343 and *Trypanosoma evansi* bloodstream form trypomastigotes at a density of 7.5×10^{4} were maintained in HMI-9 medium (Hirumi and Hirumi 1994) supplemented with 10–15% heat-inactivated fetal calf serum (HyClone) at 37°C in a 5% CO_2–air mixture. HMI-9 is a bloodstream form of African trypanosome growth supporting medium which consists of Iscove's modified Dulbecco's medium supplemented with fetal bovine serum and all other growth supporting factors.

Different concentrations of the extracts were added and the growth and viability of the cells were monitored microscopically and by counting using haemocytometer. Drug sensitivity was expressed relative to the growth of control cells. The MICs were then determined. MIC is expressed as the lowest concentration of compound at which no trypanosome with normal morphology or motility can be found compared to control cultures after 24-h incubation.

Morphological studies

Light microscopy Giemsa staining and electron microscopy were used to study the morphological changes in the parasites provoked by the compounds. Parasites were cultured in 24- or 48-well culture plates in the presence and absence of different concentrations of the compounds for 24 h. Smears of the parasites were prepared 24 h after exposure to the compounds on glass slides, air-dried, fixed in methanol and finally stained with 5% Giemsa for 30 min. Slides were then washed with tap water, dried and observed under a microscope.

Scanning electron microscopy Parasites were incubated with different concentrations of extracts or compounds for 6–24 h. Aliquots of the parasite-containing medium were pipetted on to a 1-cm^2 glass slide coated with poly-*L*-lysine and fixed for 15 min. The slides were then transferred into a 0.2-M cacodylate buffer, pH 7.4. Thereafter, the samples were then dehydrated in a graded ethanol series (30% to absolute) and then isopentenyl acetate. The samples were then critically dried in liquid CO_2 and finally coated with Au–Pd in an ion sputter, kept overnight and viewed on JSM-840 AN scanning microscope.

In vitro toxicity

Toxicity toward newborn mouse brain-derived cells (NBMH) was assessed with cells plated in 48-well plates at 10^5 cells per well. Stock cell cultures were maintained in 25-cm^3 flasks and subcultured to the appropriate split ratio by mild trypsinisation once in 7–10 days. The cells were allowed to settle and start confluence formation for 24–48 h. Microscopically, the fibroblasts had a normal appearance and showed normal cell growth rates. The concentration of a compound which provoked a >75% reduction in cell viability compared to the control cells after 24-h incubation was considered as MIC for the cells. After adherence, the medium was removed and replaced by media containing the different concentrations of the extracts. The plates were incubated for 24–96 h at 37°C in a humidified 5% CO_2 incubator. Control cells were incubated with culture medium alone and with solvent, DMSO, at a final concentration of less than 4%. The wells were assessed microscopically for cell growth and MIC was determined. A week after incubation with the different extracts, the medium was replaced with a fresh one and cell growth was monitored. Selectivity indices were calculated by dividing the MIC values of the NBMH versus the MIC values for the parasites.

Results and discussion

Studies have shown that plants are used in traditional medicine in Africa to treat trypanosomiasis in humans and animals (Akah et al. 1998; Atawodi et al. 2002; Adamu et al. 2005). Some of these plants have been evaluated for trypanocidal activity but few were phytochemically validated vis-à-vis their active compounds. We evaluated the in vitro effect of methanolic extracts of *A. leiocarpus* and *T. avicennoides*, their active butanolic fractions and the isolated compounds with MIC values ranging from 25 to 50 μg/ml, ≤12.5 to 17.5 μg/m and 7.5 to 31.5 μg/ml (14 to 91 μM), respectively, against four strains African *Trypanosoma* (Tables 1 and 2).

The promising result displayed by the extracts of these plants and subsequent successive fractionations in butanol, ethyl acetate and water deserved further analysis. Phytochemical analysis of the extracts showed aqueous and butanolic fractions to exhibit better activity. Although substantial amount of phytochemical research has been carried out on these plants, there are little or no reports on the effect of their hydrolysable tannins on parasites in vitro. In some reports (Vonthron-Sénécheau et al. 2003) tannins are usually removed because of the general belief that they are nonselective inhibitors of enzymes. However, in this report and like that of Asres et al. (2001), we did not remove the tannins. This is because the traditional practitioner's prescription on local consumption of these extracts does not exclude the tannins. We followed the fractions with a better activity and identified hydrolysable tannins to display some trypanocidal effect. These fractions showed

Table 1 Trypanocidal activity of fractions from *A. leiocarpus* and *T. avicennoides* against four strains of *Trypanosoma*

Plant species	Minimum inhibitory concentrations (μg/ml)				
	Fraction	Tbb	Tbg	Tbr	Te	SI
A. leiocarpus	MeOH	37.5	50.0	37.5	25.0	30–60
	EtOAc	≤50	≥50.0	>25	37.5	30–60
	BuOH	<<25	<<25.0	20	<<25.0	60–75
	Water	≥25	≥25.0	25	≥25.0	60
	DMSO	>50	≤25.0	25	<25.0	30–60
T. avicennoides	MeOH	12.5	17.5	12.5	12.5	86–120
	EtOAc	37.5	25.0	25	≤50	30–60
	BuOH	≤50	≤50	25	≤50	30–60
	Water	<25	<<25.0	20	<<25.0	60–75
	DMSO	≤25	≥25	20	≤50	30–75

Table shows the MIC values of the different fractions of *A. leiocarpus and T. avicennoides* against blood stream forms of four different species of *Trypanosoma*. Each experiment was carried out in duplicate and repeated at least two or three times and averages were taken and reported. All MIC values for the fractions against NBMH were above ≥1,500 μg/ml.

NBMH Newborn mouse heart fibroblast, *SI* selectivity index—the ratio of MIC of NBMH versus MIC of the parasite (MIC of NBMH/MIC of parasite), *Tbb Trypanosoma brucei brucei*, *Tbg T.b. gambiense*, *Tbr T.b. rhodesiense*, *Te T. evansi*

Table 2 Trypanocidal activity of compounds from *A. Leiocarpus* and *T. avicennoides* against *Trypanosoma* strains

Isolated compound	Minimum inhibitory concentrations in micrograms per milliliter (μM)				
	Tbb	Tbg	Tbr	Te	SI
Castalagin	22.5 (24)	27.5 (29)	27.5 (29)	31.5 (34)	48–67
Ellagic acid	≥25 (83)	nd	≥37.5 (91)	nd	40–60
Flavogallonic acid	7.5 (16)	12.5 (27)	8.75 (19)	15 (32)	100–200
Punicalagin	≥15 (14)	18.5 (17)	22.5 (21)	27.5 (25)	55–100
Fraction 2	>50	>100	>160	>150	9.4–30
Terchebulin	27.5 (25)	31.5 (29)	27.5 (25)	≥25 (23)	48–60

Table shows the MIC values of the isolated compounds against blood stream forms of four different species of *Trypanosoma*. Each experiment was carried out in duplicate and repeated at least two or three times and averages were taken and reported. All MIC values for the compounds against NBMH were above 1,500 μg/ml. Values in bracket are concentrations in micromolar.
NBMH Newborn mouse heart fibroblast, *SI* selectivity index—ratio of MIC of NBMH (≥1,500 μg/ml) versus MIC of the parasite (MIC of NBMH/MIC of parasite), *Tbb Trypanosoma brucei brucei*, *Tbg T.b. gambiense*, *Tbr T.b. rhodesiense*, *Te T. evansi*, *nd* no data

activity of the order 20 to ≥50 μg/ml for both *A. leiocarpus* and *T. avicennoides* against both human and domestic animal pathogens (Table 2). HPLC analyses showed the aqueous and butanolic fractions of *A. leiocarpus* to contain mainly castalagin, flavogallonic acid and ellagic acid, with castalagin as the major compound. In addition, several unknown peaks arising from phenol carboxylic acids were also detected. The UV absorptions of the unknown peaks were closely related to that of ellagic acid, suggesting their structural similarities. On the other hand, *T. avicennoides* was observed to contain punicalagin, ellagic acid, flavogallonic acid and terchebulin. The purified and isolated hydrolysable tannins displayed trypanocidal activity as assayed in vitro with MIC values of 7.5–31.5 μg/ml (14–91 μM). The structures of the isolated compounds are shown in Fig. 1. Punicalagin, castalagin and the other related tannins have been reported to have numerous biological activities such as anticancer, antibacterial and antiparasitic (Nonaka et al. 1990; Yang et al. 2000; Asres et al. 2001; Kinjo et al. 2001; Taguri et al. 2004; Seeram et al. 2005). In other reports, Kubata et al. (2005) reported the trypanocidal effect of condensed tannins; however, this is the first report of the trypanocidal activity of punicalagin, flavogallonic acid and terchebulin from *T. avicennoides* and castalagin

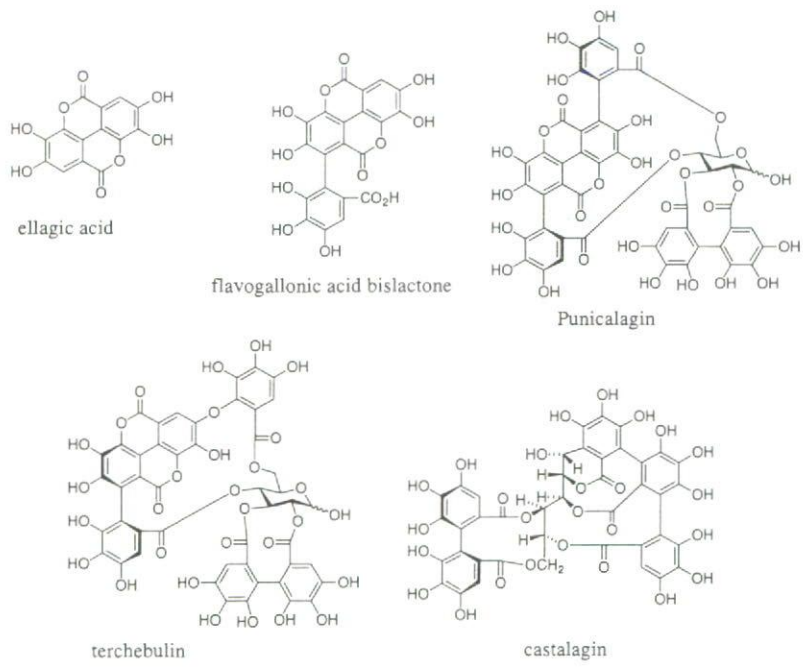

Fig. 1 Structures of the isolated compounds from *T. avicennoides* and *A. leiocarpus*

from *A. leiocarpus* on both the human and domestic animal pathogens causing trypanosomiasis, a result which is consistent with indigenous treatment of trypanosomiasis (Atawodi et al. 2002; Bizimana et al. 2006).

The effects of the extracts, fractions and those of the isolated compounds on the viability of the parasites and control mammalian cells were conducted by routine microscopic observation and parasite counting. It was observed to be concentration-dependent suppressive and the antiproliferative effect and the parasites appear to be immobilised and eventually died as depicted by the light and scanning electron micrographs in Fig. 2. Compared with the untreated cells, extract-exposed cells exhibited morphological changes similar to the butanolic fraction and isolated compounds by extensive swelling and eventual disruption of the surface membrane structures leading to loss of cytoplasmic contents. This pattern, of mode of parasite death, was consistently observed with the crude extract, the butanolic fraction and the isolated hydrolysable tannins, and this therefore suggests the involvement of these tannins in the killing of the parasites in vitro. These plant materials are generally taken orally as water extracts or as decoctions for treatment of various diseases and we investigated their toxicity on mammalian cells in vitro. Fibroblasts were used for the general toxicity and, like that on the parasites, is concentration dependent and of the order of ≥1,500 μg/ml as depicted in Fig. 3. We observed no serious toxic effect even at concentrations up to ≥1,500 μg/ml, a concentration which is about 200-fold higher than the MIC with lowest value ≤7.5 μg/ml. Previous reports have shown

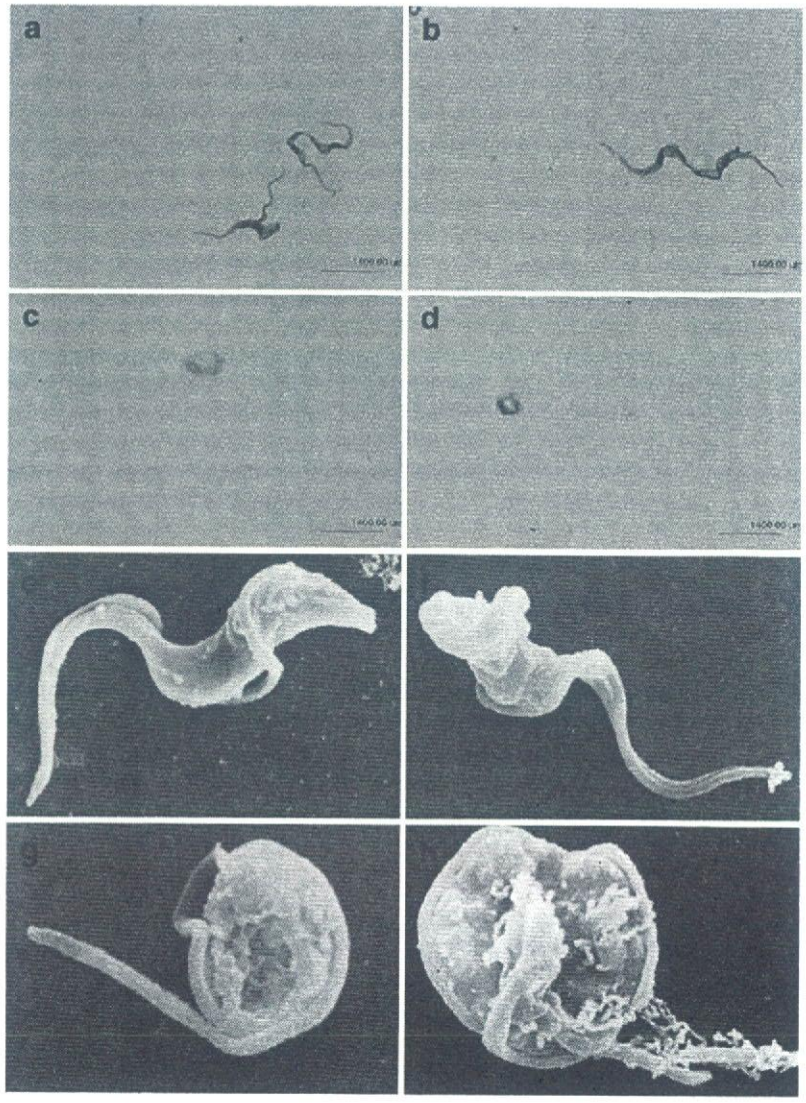

Fig. 2 Figure shows the effect of MIC of castalagin from *A. leiocarpus* extract on the morphology of *T.b. rhodesiense*. Parasites appear to be immobilised and eventually died as depicted by the light and scanning electron micrographs. **a–d** Light micrographs of *T.b. rhodesiense*. Parasites were cultured in 24-well culture plates in the presence and absence of different concentrations of the compounds. Smears of the parasites were prepared on glass slides, Giemsa stained and observed under a microscope; **e–h** scanning electron micrographs for *T.b. rhodesiense*. Parasites were aliquoted on polylysine-coated glass slide, fixed and dehydrated. The sample was critically dried, coated with Au–Pd and viewed on scanning microscope. **a**, **e** Control parasite without castalagin; **b**, **f** 30–60 min after exposure to castalagin; **c**, **g** 12 h after exposure to castalagin; **d**, **h** 24 h after exposure

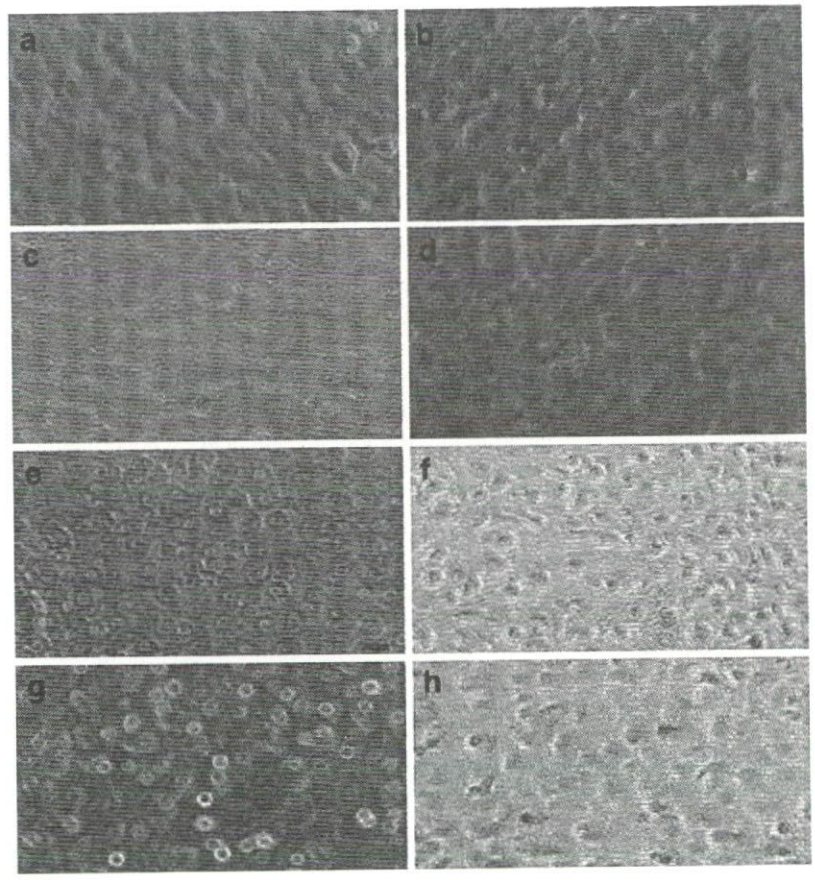

Fig. 3 Morphological appearance and viability of newborn mouse fibroblast following the in vitro incubation with the isolated compounds. **a** Normal control cells without compound; **b** +100 μg/ml castalagin; **c** +1,500 μg/ml castalagin; **d** +2,000 μg/ml *T. avicennoides* butanolic fraction; **e** +1,500 μg/ml punicalagin; **f** +2,000 μg/ml castalagin; **g** +2,000 μg/ml terchebulin; **h** +2,000 μg/ml flavogallonic acid

that higher concentrations of extracts of *Terminalia* species are tolerable in vivo, ≥100 mg/kg body weight (Abdullahi et al. 2001; Bizimana et al. 2006; Kamtchouing et al. 2006), which is consistent with the common practice of using these plants as chewing stick (Rotimi et al. 1988; Taiwo et al. 1999) and therefore corroborate with our in vitro toxicity study.

Bizimana et al. (2006) reported in vitro activities of *Afzelia africana*, *A. leiocarpus* and *T. avicennoides* against *T. b. brucei* (MIC, 1–10 μg/ml). This observation is in agreement with our result (≤12.5–17.5 μg/ml); however, assay system vis-à-vis incubation period and initial seeded parasite density per millilitre differed. Both aqueous and organic extracts from leaves, root and bark of *Terminalia* species were previously reported to have wide biological activities. In comparison with previously reported studies, *T. avicennoides* exhibited broader bactericidal, vibriocidal and antidiarrhoeal activities (Akinsinde and Olukoya 1995; Sanogo et al. 1998; Abdullahi et al. 2001; Iwalokun et al. 2001). Closely related species *Terminalia sericea* and *Terminalia superba* were reported to have antibacterial and anti-diabetic activities, respectively (Buwa and van Staden 2006; Kamtchouing et al. 2006). *A. leiocarpus* extract displayed significant in vitro activity against many clinically isolated bacterial strains and pathogenic fungi (Sanogo et al. 1998; Batawila et al. 2005). We also observed it to have promising trypanocidal activity with MIC values ranged from ≤25 to 50 μg/ml. Anti-parasitic activities of some members of Combretaceae were reported (Okpekon et al. 2004) and lower value of LC_{100} was observed for *T. brucei*.

Although this is not the first validation of the folkloric use of these plants and the observed activity of the isolated compounds may not be comparable to the existing drugs, this finding based on bioactivity-guided and phytochemical studies on different strains of African *Trypanosoma* undoubtedly confirms the involvement of these compounds (hydrolysable tannins) in the killing of the parasite.

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Complement activation in Ghanaian children with severe *Plasmodium falciparum* malaria

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Abstract

Background: Severe anaemia (SA), intravascular haemolysis (IVH) and respiratory distress (RD) are severe forms of *Plasmodium falciparum* malaria, with RD reported to be of prognostic importance in African children with malarial anaemia. Complement factors have been implicated in the mechanism leading to excess anaemia in acute *P. falciparum* infection.

Methods: The direct Coombs test (DCT) and flow cytometry were used to investigate the mean levels of RBC-bound complement fragments (C3d and C3bαβ) and the regulatory proteins [complement receptor 1 (CD35) and decay accelerating factor (CD55)] in children with discrete clinical forms of *P. falciparum* malaria. The relationship between the findings and clinical parameters including coma, haemoglobin (Hb) levels and RD were investigated.

Results: Of the 484 samples tested, 131(27%) were positive in DCT, out of which 115/131 (87.8%) were positive for C3d alone while 16/131 (12.2%) were positive for either IgG alone or both. 67.4% of the study population were below 5 years of age and DCT positivity was more common in this age group relative to children who were 5 years or older (Odds ratio, OR = 3.8; 95%CI, 2.2–6.7, $p < 0.001$). DCT correlated significantly with RD ($\beta = -304$, $p = 0.006$), but multiple regression analysis revealed that, Hb ($\beta = -0.341$, $p = 0.012$) and coma ($\beta = -0.256$, $p = 0.034$) were stronger predictors of RD than DCT ($\beta = 0.228$, $p = 0.061$). DCT was also not associated with IVH, $p = 0.19$, while spleen size was inversely correlated with Hb ($r = -402$, $p = 0.001$). Flow cytometry showed similar mean fluorescent intensity (MFI) values of CD35, CD55 and C3bαβ levels on the

surfaces of RBC in patients and asymptomatic controls (AC). However, binding of C3bαβ correlated significantly with CD35 or CD55 ($p < 0.001$).

Conclusion: These results suggest that complement activation contributed to anaemia in acute childhood *P. falciparum* malaria, possibly through induction of erythrophagocytosis and haemolysis. In contrast to other studies, this study did not find association between levels of the complement regulatory proteins, CD35 and CD55 and malarial anaemia. These findings suggest that complement activation could also be involved in the pathogenesis of RD but larger studies are needed to confirm this finding.

Background

The mortality associated with malaria largely occurs in children as a result of complications, such as severe anaemia (SA), intravascular haemolysis (IVH), cerebral malaria (CM) and metabolic acidosis, clinically manifested as respiratory distress (RD) [1-5]. Sub-Saharan Africa accounts for 90% of the world's 300–500 million malaria cases and 1.5–2.7 million deaths annually [6]. A recent study has shown that in Ghana, the most common manifestations of severe malaria (SM) are SA (36.5%), followed by RD (24.4%) and CM (5.4%) [7]. There are no reports of the relative contribution of IVH to SM cases in Ghana.

IVH due to *P. falciparum* is a condition with high case-fatality if diagnosis and treatment are not optimal [8]. It is usually considered a rare complication of malaria in endemic areas, but recent studies have highlighted its importance [5,9]. Although it appears that the direct RBC destruction due to IVH is a minor contributor to malarial anaemia it is nonetheless strongly associated with erythrophagocytosis and with a poor prognosis [5,9]. Most studies of IVH in malaria have focussed on the influence of glucose 6-phosphate dehydrogenase (G6PD) [10-12], and the role played by antimalarial drugs such as chloroquine [13,14]. Mechanical trauma from a damaged endothelium, complement fixation and activation on the RBC surface, and infectious agents may cause direct membrane degradation and cell destruction [4].

It has been observed that the degree of red blood cell (RBC) breakdown during acute malaria cannot be explained solely by the direct destruction of RBC by malaria parasite schizogony [15]. Thus depletion of RBC is thought to be partly immune-mediated [15]. Infected erythrocytes bind to endothelial cells, and *P. falciparum* antigens known as erythrocyte membrane protein 1 (PfEMP1), inserted into the infected erythrocyte surface, mediate this interaction. It has been argued that these antigens are recognized by IgG subclasses that activate the classical complement pathway [16]. This pathway may also be triggered by binding of immune complexes or dead merozoites to the RBC surface [17]. As a result, monocytes, which have C3b and C3bαβ receptors on their surfaces, are activated to phagocytose the infected RBC. Thus, complement does not seem to kill parasites directly, but could play a role as an opsonin for neutrophils and macrophages [18]. In line with this, previous studies have shown that binding of complement factor C3d to RBC is common in childhood malaria, whereas IgG binding is rare [19].

A role for complement activation in RBC breakdown during malaria is supported by reports of positive DCT in patients with anaemia [20-23]. In addition, the balance between the beneficial immune activation functions of the complement cascade and its detrimental role in disease pathogenesis is maintained by a large number of regulatory proteins. Some of these include, complement receptor 1 (CD35), which binds C3b, and decay accelerating factor (CD55) and membrane attack complex inhibitor factor (CD59), which play a role in regulating haemolysis due to deposition of immune complexes on the surface of RBC [24]. Studies have shown that, deficiencies of these membrane-bound complement-regulatory proteins on infected and uninfected erythrocytes are associated with SM [25-28]. However, these studies did not use strictly defined patient categories and, in some cases malaria diagnosis was not confirmed in the control groups. In the present study, the role of complement activation in the pathogenesis of SM was investigated in a group of Ghanaian children presenting at hospital with strictly defined manifestations of malaria, including uncomplicated malaria (UM), SA, CM, IVH and RD. In addition, the binding of complement factors and the expression of complement regulatory proteins on RBC was investigated by flow cytometry. Given the high rate of mortality associated with RD in Ghanaian children [29], and the association between SA and RD [7], the relationship between complement activation and RD was also examined.

Materials and methods

Study design and patient population

Children between one and twelve years of age with severe forms of malaria as described previously [30], were consecutively recruited into an unmatched case control study in July-August, 2000 at the Emergency Unit of the Depart-

ment of Child Health (DCH), Korle-Bu teaching Hospital, Accra, Ghana. Apparently healthy children, randomly selected from a database of children in the same age range from a nearby community, Dodowa were enrolled as control subjects (AC) [29,30]. Malaria transmission in the study area, a coastal savannah, is perennial with considerable seasonal variation, peaking during and immediately after the rains (May-October). Residents are estimated to receive about 20 infective bites per year and *P. falciparum* constitutes 98% of all infections [31]. The Scientific and Technical Committee of the Noguchi Memorial Institute for Medical Research and the Ethics and Protocol Review Committee of the University of Ghana Medical School, Korle Bu, Accra approved the study. All patients and control subjects were enrolled in the study only after signed, informed, parental consent was obtained.

Clinical investigation and inclusion criteria

Patients with axillary temperature > 37.5°C of no other obvious cause than malaria were screened for inclusion by a project physician. Clinical parameters were documented on standard written forms. Spleen enlargement was assessed by palpation and quantified as cm below the left costal margin along the mid-clavicular line. Patients with *P. falciparum* parasitaemia ≥ 10,000 parasites/μL were enrolled into the study if they fell into one of the following patient categories [29]: SA: haemoglobin (Hb) < 5 g/dL, fully conscious with no episodes of severe bleeding, reported or observed convulsions; CM: Blantyre coma score ≤ 3 and duration of coma > 60 minutes, any Hb value and no record of recent severe head trauma and other cause of coma or neurological diseases; IVH: evidence of haemoglobinuria detected by the urine dipstick test (Roche Diagnostics Ltd, Great Britain) followed by microscopy as described elsewhere [21]; RD: rapid breathing plus one or more of the following: alar flare, chest recessions, use of accessory muscles for respiration, or abnormally deep breathing; UM: Hb > 8 g/dL, fully conscious, no other features of SM. Patients with Hb between 5 and 8 g/dL were only included if they fell into one or more of the patient categories: IVH, CM and RD.

Exclusion criteria

Patients who were sickling positive were excluded. Other haemoglobinopathies were not taken into consideration. Patients were also excluded from the study based on evidence of other infectious disease such as typhoid or upper respiratory tract infections or any other identified cause of anaemia than malaria. Patients with a history of antimalarial treatment within 2 weeks prior to admission were excluded [13,14].

Management of patients

Based on institutional practice at the time, all patients with UM were treated with a standard chloroquine regime at a total dose of 25 mg/kg body weight, given over three days. In the event of treatment failure, treatment with amodiaquine (10 mg/kg body weight per day, as single daily doses for three days) was instituted. All patients with SM were treated with either amodiaquine syrup via nasogastric tube at the same dosage as described, or intramuscular quinine dihydrochloride (10 mg/kg body weight, 8-hourly). Parenteral quinine was changed to syrup at the same dosage when patients regained full consciousness or after 72 hours (which ever was earlier), to complete a 7-day course. Patients with SA or RD were given humidified oxygen and children with Hb < 5 g/dL received blood transfusions [29].

Blood sampling and laboratory analysis

Immediately after admission, 5 ml of venous blood was collected into EDTA tubes from all patients screened for inclusion. Haematological profile was determined using an 18-parameter, automatic haematology analyzer (Sysmex KX-21, Japan). Sickling test was done using the sodium metabisulphite test. Giemsa-stained thick and thin blood films were used for microscopic detection and identification of *Plasmodium* parasites. Parasites were counted against 300 WBC, and the value was converted into parasites per μL of peripheral blood, based on individual WBC count. Quality control (QC) was ensured by checking the autoanalyser daily and testing of 10% slides by second look. Errors were below 10% variation or disagreement and without systematic deviations.

Direct Coombs' test

DCT was done using the method described by Goka *et al.* [21]. RBC suspensions were washed 4 times in comparatively large volumes (4 ml per wash) of 0.9% saline by centrifugation and reconstituted with saline to 5% PCV. One drop of this suspension was mixed with two drops of poly-antiglobulin sera (DIAGAST laboratories, Cedex, France), followed by centrifugation at 1,200 rpm for 1 minute. The samples were immediately inspected macroscopically for agglutination and negative or doubtful positive/weak results were re-examined by light microscopy. The positive samples were re-tested using mono-specific antiserum against IgG and C3d. Test results were graded as +4 (complete agglutination), +3 (several large agglutinates, few cells), +2 (large agglutinates in a sea of smaller clumps and free cells), +1 (many small agglutinates) or 0 (no agglutination). Positive and negative controls were run in parallel.

Flow cytometry

Packed RBC were washed twice by centrifugation and resuspended in PBS (pH 7.2) to 2.0×10^7 cells/ml. The RBC suspension was stained with ethidium bromide (50 μg/ml final concentration), followed by surface staining with FITC-conjugated antibodies in the dark [32]. The fol-

lowing antibodies were used: Mouse IgG_1 FITC (Pharmigen International, 33814X, San Diego, CA, USA); CD35 (Pharmigen, 30961A); IgG (Becton Dickinson, San Jose, CA, USA, 345140{5140}); Mouse IgG_{2a} FITC (Pharmigen, 33034X); CD55 (Pharmigen, 33571A); Mouse IgG_1 pure (DAKO, Glostrup, Denmark, X 0931); C3bαβ (Cymbus Biotechnology Ltd., Hampshire, UK, CBL 189 for α; CBL 190 for β); Rabbit IgG pure (Zymed Laboratories, INC., San Franscisco, California, USA, 81245172); C3d pure (DAKO, A 0063); C3d FITC (DAKO, F 0323) and controls (Goat anti-mouse FITC (DAKO, F0479); Swine anti-rabbit FITC (DAKO, F0054). After incubation, cells were washed twice with cellwash (Becton Dickinson) by centrifugation and samples were stored at 4°C in the dark till acquisition on a FACScan flow cytometer (Becton Dickinson, Japan). A minimum of 10,000 RBC were acquired and analysis was done by the CellQuest programme. The cut-off point was 10^1 on both axes.

Statistical analysis

Statistical analysis was performed using SigmaStat software package (Jandel Scientific). Continuous variables were compared between groups using the student t-test or one way analysis of variance (ANOVA). For data that were not normally distributed and could not be normalised by logarithmic transformation a one-way ANOVA on ranks was used. Correlation between parameters was determined by Spearman rank order test. Proportions were compared using the Chi-square test, or Fisher's exact test. Variables that showed significant or near-significant differences between groups by univariate analysis or that were otherwise considered relevant for the study were entered in a conditional logistic regression analysis and in a multiple regression analysis. P-values < 0.05 were considered significant.

Results

Characteristics of the patient categories and controls

A total of 484 parasitaemic patients were screened for enrolment of which 87 patients with distinct clinical presentations of SM were included, 36 SA, 18 IVH, 27 CM and 6 RD. Their clinical characteristics together with those of UM patients and AC are summarised in Table 1. SA patients were younger (mean age = 2.5 yrs.) than the other categories of patients and the AC group (mean age 5.7 yrs.) ($p < 0.001$). SA had the lowest mean Hb (4.1 g/dL), followed by RD (5.5 g/dL) and IVH (6.5 g/dL). The highest mean Hb was found in AC (10.9 g/dL). All the patient categories had high parasitaemia whilst AC had the lowest parasite density (geometric mean, 1.4×10^3 parasites/μL). The geometric mean parasite densities of SA and UM cases (63.0 and 57.3×10^3parasites/μL respectively) were significantly lower than those of the IVH, CM and RD cases (98.7×10^3, 114.9×10^3 and 80.9×10^3 parasites/μL, respectively, $p < 0.001$). In order to study the effect of complement activation on RD, the data were re-analysed for all patients with SM, including those with overlapping clinical manifestations. The characteristics of these 104 children is summarised in Figure 1. Forty-six children had Hb < 5 g/dL, 34 had coma score ≤ 3, 22 had haemoglobinuria and 20 had RD.

Association of variables in children with severe malaria

Of all the parameters tested, only age and spleen size showed a significant correlation with Hb (Table 2). Age was positively correlated with Hb ($r = 0.379$, $p = 0.002$), and spleen size was inversely correlated to Hb ($r = -0.402$, $p = 0.001$).

Clinical characteristics of DCT positive and negative patients and controls

Of all the patients screened (484), 27.0% (131) were DCT positive. Most of the sensitisation was due to C3d alone (87.8%), with a small proportion being ascribed to IgG alone (2.3%) or to both (9.9%). Children below 5 years formed 84.7% (111/131) of the DCT positives relative to those of 5 years and older, OR = 3.8 (95% CI, 2.2–6.7, $p < 0.001$). There was a high prevalence of DCT positive cases in the RD (50.0%), IVH (38.8%) and SA (27.8%) patients whereas UM and CM had 9.5% and 11.8% prevalence respectively. Surprisingly, 26.3% of the AC patients had positive DCT (Table 1). When overlapping cases were included in the analysis, RD patients still had the highest DCT positive rate with (50.0%, (10/20)), followed by IVH (45.5%, (10/22)); SA (34.8%, (16/46)) and CM (20.6%, (7/34)), Figure 1. Of all the children with severe forms of malaria (SM, n = 104), 33 (31.6%) were DCT positive (Table 3). There were no significant differences in age and parasitaemia between the DCT positive and the DCT negative patients of the SM ($p > 0.05$, Table 3), whereas the mean Hb was significantly lower in the DCT positive patients ($p < 0.001$). Although there was a trend toward higher prevalence of a positive DCT in RD, IVH, and SA, compared with CM, these associations were not statistically significant (Table 3).

Relationship between DCT result and RD

Out of 20 RD cases, 50.0% (10/20) were DCT positive compared with 27.4% (23/84) non RD cases, $p = 0.092$. Furthermore, the strength of the DCT result (0–4) correlated significantly with RD (standard coefficient $\beta = -0.304$, $p = 0.006$). Since children with RD appeared to be more likely to have DCT positive results compared to those without RD, the relationship between DCT result and RD was further investigated using multiple linear regression where the strength of the DCT result, Hb, coma score, and age were tested for their contribution to RD. In this model, Hb ($\beta = -0.341$, $p = 0.012$) and coma score ($\beta = -0.256$, $p = 0.031$, Table 4) emerged as the strongest predictors of RD compared with DCT grade ($\beta = 0.228$, p =

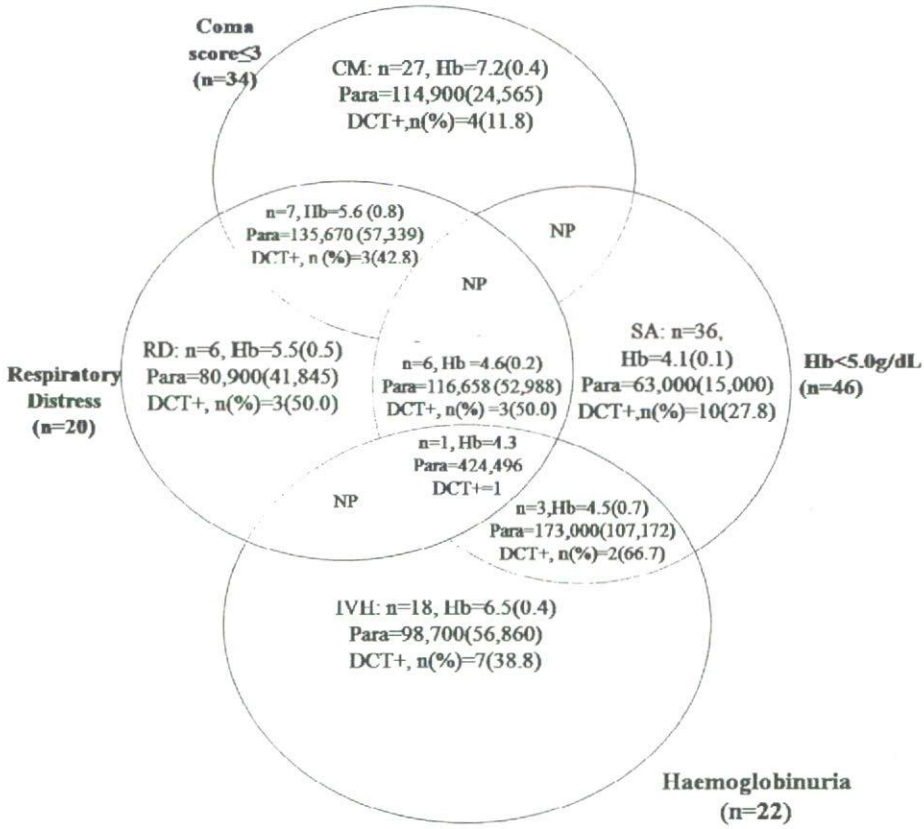

Figure 1

Clinical categories of children with severe malaria. Children admitted to the paediatric ward of the Korle-Bu teaching hospital, Accra, with severe malaria (SM). Symptoms of SM included coma (score ≤ 3 on the Blantyre coma scale), severe anaemia (SA) (haemoglobin [Hb] < 5.0 g/dL), intravascular haemoloysis (IVH, evidence of haemoglobinuria), and respiratory distress (RD). The number of children showing the various symptoms, as well as the mean (SEM) Hb (in g/dL) and parasitaemia (Para, in parasites per μL) plus prevalence of a direct Coombs' test (DCT) is shown. NP, no patient.

Table 1: Demographic, clinical and laboratory characteristics of patients and healthy controls

Characteristics	SA	IVH	CM	RD	UM	AC	p value
n	36	18	27	6	21	19	-
Female sex, %	43.7	21.1	40.7	60.0	42.8	47.8	-
Mean Age, years (95% CI)	2.5** (1.8–3.1)	4.8 (3.1–6.6)	4.3 (3.3–5.3)	2.7** (2.0–5.5)	5.2 (3.6–6.8)	5.7 (4.4–7.1)	<0.001[b]
DCT Positive, n (%)	10* (27.8%)	7* (38.8%)	4 (11.8%)	3* 50.0%	2 (9.5%)	5.0* (26.3%)	<0.05[a]
Mean Hb, g/dL (95% CI)	4.1 (3.8–4.3)	6.5 (5.6–7.4)	7.2 (6.4–8.0)	5.5 (4.6–6.2)	9.2 (8.5–9.8)	10.9 (10.6–11.4)	-
Parasite density, geometric mean parasites × $10^3/\mu L$ (95% CI)	63.0*** (34.3–94.1)	98.7 (47.6–284.5)	114.9 (67.4–162.6)	80.9 (12.1–256.9)	57.3*** (41.0–107.3)	1.4 (0–2.9)	<0.001[b]
Spleen size, cm (95% CI)	2.3 (1.5–3.0)	0.6(0–1.6)	0.7 (0.1–1.4)	ND	1.9 (0.6–3.2)	ND	-

Non-overlapping patient groups (see text). SA, severe anaemia (Hb < 5 g/dL). IVH, intravascular haemolysis with evidence of haemoglobinuria. CM, cerebral malaria (coma score ≤ 3). RD, respiratory distress. UM, uncomplicated malaria with Hb > 8 g/dL. AC, healthy, asymptomatic controls. DCT, direct Coombs' test. *Significantly higher than UM and CM. ** Significantly lower than CM, UM and AC. *** Significantly lower than IVH and CM. ND, not done.

a = Calculated using X^2

b = Calculated using ANOVA

Table 2: Correlation between variables in children with severe or uncomplicated malaria

	Hb		Spleen		Coma		Log para	
Variable	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*
Age	**0.379**	**0.002**	-0.066	0.610	-0.142	0.265	-0.0618	0.630
Hb			**-0.402**	**0.001**	-0.201	0.114	-0.053	0.678
Spleen					0.270	0.032	-0.170	0.183
Coma							-0.208	0.102

Hb, haemoglobin. Spleen size, cm below left costal margin. Coma, score ≤ 3 on the Blantyre coma scale. Para, parasite (per μL). Patients analysed here are all the SM and UM cases (n = 125). Asymptomatic controls (AC) are not included.

0.061) and age (β = 0.208 and p = 0.820). These results suggest that the association between RD and DCT may be related to the role of DCT in precipitating anaemia.

Relationship between opsonins (C3d and C3bαβ) and complement regulatory proteins (CD35 and CD55) in different patient categories

The association between the DCT method and flow cytometry was confirmed by a positive correlation between DCT grade and MFI after surface staining of RBC with anti-C3d ($r = 0.53$, $p < 0.001$, Figure 2). In order to study the influence of complement regulatory proteins on clinical outcome, binding of C3d and C3bαβ and expression of CD35 and CD55 on the RBC surface were measured by flow cytometry in a subset of the patients (Table 5). As shown in Table 5, there were no differences in any of the tested parameters between the main clinical groups ($p > 0.7$). The binding of C3bαβ correlated significantly with CD35 and CD55 ($p < 0.001$) in children with SM, Figure 3. There was no correlation between C3d and either CD35 or CD55 ($p > 0.05$, data not shown).

Discussion

Results from the present study show that infection with *P. falciparum* parasites stimulates complement activation, consistent with previous studies [21-23,33]. The complement activation was associated with reduced Hb-levels [21,22,24]. A central molecule in this association appears to be C3d that can opsonise the RBC for erythrophagocytosis. To a large extent, the erythrophagocytosis is mediated by macrophages, as indicated by increased levels of neopterin [29,30,34], which is a marker of macrophage activation [35]. The combination of activated macrophages and RBC opsonised by complement after activation will lead to increased erythrophagocytosis and decreased Hb as demonstrated in other studies [21,36]. This was reflected in an inverse correlation between spleen size and Hb and a trend toward larger spleen size in DCT positive than in DCT negative patients. Thus, phagocytosis of iRBC caused by macrophages in the spleen, enhanced by the opsonisation of RBC by C3d, may be one of the mechanisms by which Hb drops due to *P. falciparum* infection. However, a prospective study comparing DCT positive and negative patients in relation to drop in Hb after initiation of treatment would be relevant to confirm a causal relationship.

Complement activation could additionally contribute to malarial anaemia by direct lysis of infected RBC [5]. However, both this and a previous study [21] only showed a weak and insignificant association between DCT results and haemoglobinuria. Other factors apart from complement that may trigger IVH in malaria are drugs such as chloroquine [13,14], genetic factors [10,37] and autoim-

Table 3: Clinical characteristics of all children with severe malaria grouped by DCT results

Characteristics	DCT positive	DCT negative	P value
n (%)	33 (31.6%)	71 (68.2%)	-
Age, years	3.3 (2.5–4.0)	4.0 (3.4–4.6)	0.11[b]
Hb, g/dL	5.0 (4.3–5.6)	6.6 (6.1–7.1)	<0.001[b]
Parasite density, × 10^3/μL	125.6 (81.5–169.7)	139.7 (107.6–171.8)	0.33[b]
Mean spleen size, cm	2.1 (1.1–3.1)	1.2 (0.6–1.8)	0.09[b]
SA, n (%)	16 (48.5%)	30 (42.3%)	0.70[a]
CM, n (%)	7 (21.2%)	27 (38.0%)	0.15[a]
Haemoglobinuria, n (%)	10 (30.3%)	12 (16.9%)	0.19[a]
Mortality, n (%)	4 (12.1%)	15 (21.1%)	0.40[a]

Data are mean (95% confidence interval) unless otherwise indicated. Patients include all patients in Figure 1. Thus, the same patient can appear in several categories.
a = Calculated using X^2 or Fischer's exact test
b = Calculated using, student t-test

Table 4: Multiple regression analysis of variables predicting respiratory distress

Characteristics	Standard coefficient (β)	P-value
Age (years)	0.208	0.820
Coma score	-0.256	0.031
Hb (g/dL)	-0.341	0.012
DCT grade (0–4)	0.228	0.061

NB: Patients analysed here are all the SM and UM cases (n = 125). AC were not included.

munity [38]. Apart from excluding sickle cell genes, other factors were not controlled for in this study. However, the lack of association between DCT and IVH suggests that the main contribution of complement activation to the pathogenesis of malarial anaemia is opsonisation for erythrophagocytosis. The surprisingly high prevalence of DCT positives in the AC group indicates that a number of apparently healthy children may have an activated complement system. In a related study [39], it was observed that asymptomatic *P. falciparum* infection resulted in immune activation and anaemia in semi-immune children. The present study suggests that complement activation could contribute to the low Hb in the semi-immune children.

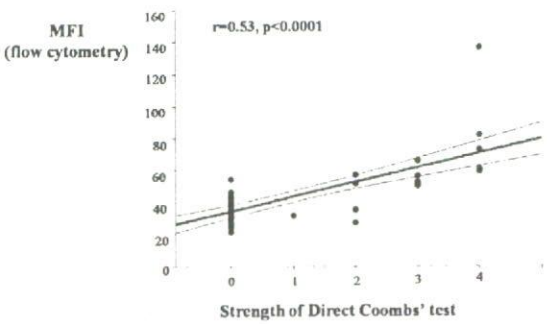

Figure 2
DCT results compared with flow cytometry using anti-C3d. RBC suspensions from 30 DCT positive and negative SM patients were stained with ethidium bromide and monoclonal antibodies to detect surface-bound C3d. Association between DCT results and flow cytometry results for C3d are shown by scatter plots with regression line. MFI, mean fluorescence intensity. DCT was graded as accordingly as +4 (complete agglutination), +3 (several large agglutinates, few cells), +2 (large agglutinates in a sea of smaller clumps and free cells), +1 (many small agglutinates), 0 (no sign of agglutination).

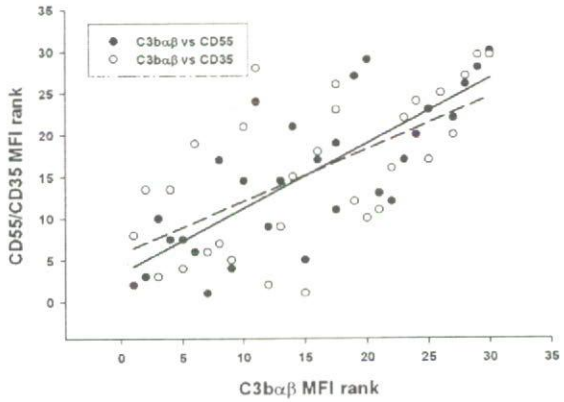

Figure 3
Association of CD55 and CD35 with C3αβ in children with severe malaria. Correlation between C3bαβ and complement regulatory proteins (CD35 (complement receptor type 1) and CD55 (decay accelerating factor)) measured by flow cytometry on surfaces of RBC from 30 children with SM. Solid line: C3bαβ vs CD55, $r = 0.777$, $p < 0.001$; dotted regression line: C3bαβ vs CD35, $r = 0.624$, $p < 0.001$. MFI, mean fluorescence intensity.

In the present study the DCT [40] was used alongside flow cytometry to determine the relationship between the levels of complement fragments (C3bαβ) and regulatory proteins (CD35 and CD 55). Other investigators have shown that activation and levels of regulatory proteins on surfaces of RBC are related to RBC loss in malaria [25,28]. In contrast, the present study did not find any relationship between anaemia and CD35 and CD55 levels. Unlike the current study, the previous studies did not use strictly defined patient categories and, in some cases malaria diagnosis was not confirmed. However, the discrepancy could also be due to differences in responses to *P. falciparum* of children from distinct geographical locations or exposed to varying levels of malaria transmission. There is a need to perform additional investigations of the role of complement regulatory proteins in the pathogenesis of malaria anaemia. The correlation between C3 fragment deposition and CD35 and CD55 levels reported here confirms results from previous studies [41], and suggests an adequate role of CD55 in regulating the extent of haemolysis in the studies patients.

Increased levels of inflammatory mediators have been shown to play a role in RD [29]. High levels of complement factors being reported here may implicate a synergistic role of these complement factors and inflammatory mediators in causing the RD due to *P. falciparum* infection. Such a synergistic interaction has been shown to take

Table 5: MFI levels of complement factors and regulatory proteins by patient categories

Complement factors	Patient Categories and Controls					
	SA, n = 11	IVH, n = 8	CM, n = 11	UM, n = 10	AC, n = 11	P value[a]
CD35	2.10 (1.6–2.6)	2.09 (1.4–2.7)	2.08 (1.7–2.5)	1.85 (1.6–2.1)	1.97 (1.7–2.2)	0.88
CD55	2.05 (1.5–2.5)	1.76 (1.4–2.1)	1.94 (1.7–2.2)	1.85 (1.7–2.0)	1.98 (1.7–2.3)	0.81
C3bαβ	2.90 (0.7–5.1)	2.74 (1.1–4.5)	1.94 (1.1–2.8)	2.11 (1.4–2.8)	3.30 (1.2–5.4)	0.76

Data are represented as means (95% confidence intervals). CD35, Complement receptor type 1. CD55, Decay accelerating factor. C3bαβ, complement fragment from breakdown of C3b.
a = Calculated using one-way ANOVA.

place during cardiac surgery with cardiopulmonary bypass (CBP) [42]. Similarly, in addition to complement activation during severe malaria infection, there is an imbalance between pro- and anti-inflammatory cytokines. Thus in children with SA complicated by RD, institution of effective treatment during the early phase of infection appears to be critical to survival [3,43]. The application of simple clinical and laboratory guidelines identifying children likely to develop SM and thus most in need for rapid interventions may thus improve survival and reduce unnecessary use of blood transfusion [3]. This study demonstrates that DCT can be used as a simple and reliable method of measuring complement activation in children with acute malaria and may be used to predict progression of UM to SA-RD and SA+RD. Prospective studies are needed to validate this diagnostic approach.

Conclusion

The data presented here, supports a role for complement-mediated removal of RBC through erythrophagocytosis in the pathogenesis of malarial anaemia. In contrast to previous studies there was no relationship between the severity of anaemia and levels of complement receptor 1 or decay accelerating factor. Complement activation could also be involved in the pathogenesis of RD but larger studies are needed to confirm this finding. In addition the study demonstrates that DCT might be used as a simple method of predicting development of complications but this need to be studied prospectively in subsequent investigations.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

GKH designed the study, carried out laboratory work, data analyses and interpretation and drafted the manuscript. BQG and GOA assisted with design of study, selection, clinical examination and management of patients, as well as revision of manuscript for intellectual content. MMA and EO assisted with Coombs' test and data analysis. KH was involved with data analysis and extensive revision of manuscript for intellectual content. JKAT, DD and MFO designed the flow cytometry with GKH, acquired and analyzed the data. GAA was involved in data analyses, and preparation and revision of manuscript. JALK and BDA were involved in the design of experiment, data analysis and revision of manuscript for intellectual content.

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