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Usefulness of Human Coagulation and Fibrinolysis Assays in Domestic Pigs

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Pigs are often used as animal models in research on blood coagulation and fibrinolysis. The usefulness of the assays applied within this field, and the knowledge of reference intervals are therefore essential and of utmost importance. In the study reported here, we investigated the applicability of commercial human coagulation and fibrinolysis assays for use with porcine plasma. In total, 22 functional and immunologic assays were applied to plasma obtained from domestic pigs, and the following blood coagulation and fibrinolysis variables were measured: prothrombin time, activated partial thromboplastin time, tissue factor, tissue factor pathway inhibitor, factor VII, protein C, protein S, prothrombin fragment 1+2, antithrombin, thrombin-antithrombin complexes, fibrinogen, soluble fibrin, urokinase-type plasminogen activator, plasmin inhibitor, plasminogen activator inhibitor 1, and D-dimer. We found that 11 of 12 functional assays, but only 3 of 10 immunoassays, were applicable to porcine plasma, and we determined the normal range of these variables. We conclude that human functional assays are useful in porcine plasma, whereas only a few immunologic assays can be used. However, precautions must be taken in interpretation of the results and in extrapolation toward human results because possible differences between porcine and human values can be due to species variations and/or methodologic errors.

The pig is an attractive animal to use in cardiovascular research due to the well established similarities in biology and pathology between humans and pigs (1), and the number of pigs used as laboratory animals is currently increasing. The Subcommittee on Standardisation and Calibration (SSC) has established the Animal, Cellular, and Molecular Models of Thrombosis and Haemostasis—Workgroup, as part of the International Society on Thrombosis and Haemostasis (ISTH) work on standardization and calibration, and they describe the pig as a good model for the study of human coronary artery restenosis (2). For many years, the pig has been used frequently in thrombosis research (1, 3-5), and the components of the porcine and human coagulation and fibrinolytic systems are assumed to be similar (Fig. 1). Use of pigs as thrombosis models requires reliable biological assays of porcine blood coagulation and fibrinolysis. The available commercial coagulation and fibrinolysis assays are developed only for application using human plasma, and systematic investigation of the use of these assays in porcine plasma is lacking. In a review on published assays within the field of blood coagulation and fibrinolysis in pigs (1), some of the problems with lack of validated assays have been emphasized. In particular, use of different reagents, calibrators, and pre-analytical handling, makes it difficult to compare results among studies. The aims of the study reported here were therefore: to investigate the applicability of commercial human-based coagulation and fibrinolysis assays to porcine plasma, and to establish the normal range of these variables. For this purpose, the following global tests and single factors (clotting activity and protein concentration) of blood coagulation and fibrinolysis variables were measured by use of 22 commercial assays: prothrombin time (PT), activated partial thromboplastin time (APTT), tissue factor (TF), tissue factor pathway inhibitor (TFPI), factor VII coagulant activity (FVII:C), activated factor VII (FVIIa), protein concentration of factor VII (FVII:Ag), factor VII amidolytical activity (FVII:Am), protein C (activity and antigen), protein S, prothrombin fragment 1+2 (F1+2), antithrombin (AT), thrombin-antithrombin complexes (TAT), fibrinogen, soluble fibrin (SF), urokinase type plasminogen activator (u-PA) antigen (u-PA:Ag) and activity, plasmin inhibitor (PI), plasminogen activator inhibitor 1 (PAI-1) antigen (PAI-1:Ag) and activity, and D-dimer (Fig. 1).

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Materials and Methods

Animals. Blood samples were obtained from 43 healthy domestic pigs (24 sows and 19 hogs of Danish Landrace breeding, with mean [± SD] body weight of 57 [± 7) kg]) that were donated by three farmers. Pigs were housed indoors in family groups, in pens with solid concrete and straw bedding. They were restrictedly fed commercial porcine diets, but were kept non-fed with unrestricted access to water for 12 h before blood sample collection. Pigs were not subjected to any specific health monitoring program. The experiment was carried out in accordance with the Animal Experimentation Act and the Animal Welfare Act under the Danish Ministry of Legal Affairs. The Danish Animal Experimentation Act is based on the Council of Europe Convention ETS 123. The farm animals were maintained in accordance with the principles of the European Convention ETS 087.

Blood sample collection. Blood samples were collected in the morning, while they were restrained by the use of a nose snare. An 18-gauge, 40-mm-long needle was inserted blindly into the external jugular vein, and blood was collected into

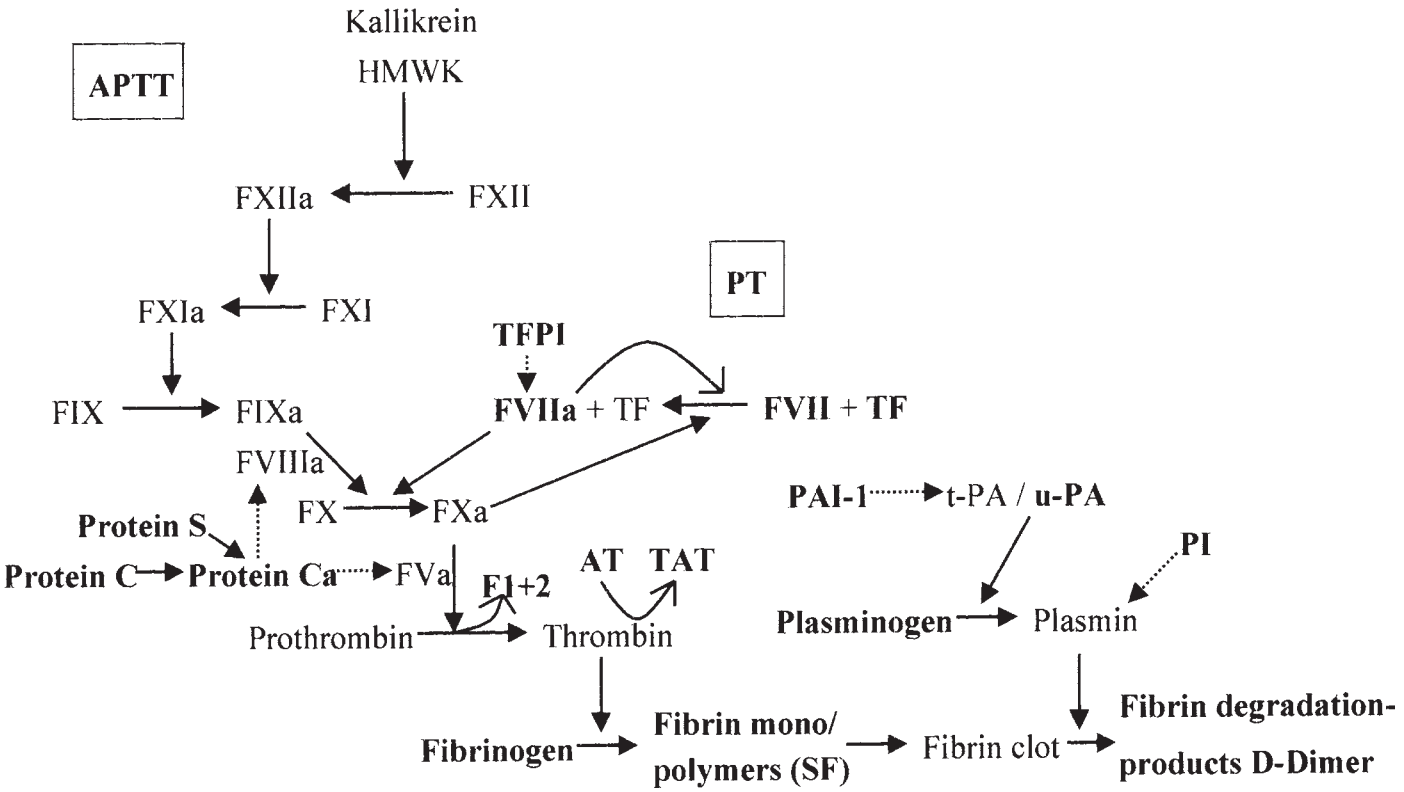

Figure 1. Blood coagulation and fibrinolysis. Global tests (APTT and PT) and single factor laboratory analyses are marked in bold. Abbreviations: PT = prothrombin time; APTT = activated partial thromboplastin time; HMWK = high molecular weight kininogen; FXII = factor XII; FXIIa = activated factor XII; FXI = factor XI; FXIa = activated factor XI; FIX = factor IX; FIXa = activated factor IX; TF = tissue factor; FVII = factor VII; FVIIa = activated factor VII; TFPI = tissue factor pathway inhibitor; FX = factor X; FXa = activated factor X; FVIIIa = activated factor VIII; FVa = activated factor V; Protein Ca = activated protein C; F1+2 = prothrombin fragment 1+2; TAT = thrombin-antithrombin complexes; AT = antithrombin; SF = soluble fibrin; u-PA = urokinase plasminogen activator; t-PA = tissue plasminogen activator; PI = plasmin inhibitor; PAI = plasminogen activator inhibitor 1.

evacuated tubes. The first three milliliters of blood was discarded. The following three milliliters was collected in 0.06 ml of K_3-ethylene diamine-tetraacetic acid (EDTA, 0.235 mol/L; Venoject, Terumo Cooperation, Leuven, Belgium) and was used for analysis of F1+2, TAT, and D-dimer. The tubes were stored on crushed ice, and immediately, 10 µl of Phe-Pro-Arg chloromethylketone (2.63 mg/ml; PPACK, Calbiochem, Bad Soden, Germany) was added to prevent ex vivo generation of thrombin (6). The following two × 4.5 ml of blood was collected in 0.5 ml of trisodium citrate (0.129 mol/L; BD Vacutainer System, Plymouth, UK). One tube was kept at room temperature and used for analysis of PT, APTT, TF, FVII, TFPI, fibrinogen, and SF. The other tube was stored on crushed ice and used for analysis of protein C, protein S, AT, u-PA, and PI. Finally, 4.5 ml blood was collected in 0.5 ml of citrate buffer (0.5 mol/L, pH 4.3; Biopool, Umeå, Sweden), and was placed on crushed ice immediately and was used for PAI-1 analyses.

Within two hours after collection, samples were centrifuged at 4 or 20°C for 20 min at 2,000 ×*g*. Plasma samples (n = 10, 6 sows and 4 hogs) from one farm were pooled, and plasma samples from the other pigs (n = 33, 18 sows and 15 hogs) were stored individually. Plasma was pipetted into plastic vials in aliquots of 200 µl, which were rapidly frozen and stored at -70°C.

Blood analyses. Protein concentrations and activities in porcine plasma were measured by use of the human assays described in Table 1. Plasma samples were rapidly thawed in a water bath at 37°C. First, the assays were tested on pooled porcine plasma. If the concentration in undiluted pooled plasma was higher than the lower detection limit of the assay, individual plasma samples were analyzed, using human plasma as the calibrator. Presence of hemolysis in the sample lead to exclusion from analysis.

Statistics. Due to the non-Gaussian distribution of some of the variables measured, medians and 25-75 percentiles were calculated and presented together with the minimum and maximum values. We define the 25-75 percentiles as the normal range in porcine plasma. The Mann-Whitney rank sum test was used to test the differences between sexes, with the level of statistical significance set at $P < 0.05$.

Results

Table 2 presents the median, 25-75 percentiles, and minimum and maximum values for the coagulation and fibrinolysis variables measured in porcine plasma. Most functional assays were applicable to porcine plasma (11 of 12 assays), but only three of 10 enzyme immunoassays had values above the detection limit of the human assay. Differences were not observed between sows and hogs.

Discussion

The pig is a preferable animal model in the thrombosis research field, but the lack of commercial assays designed for co-

Table 1. Variables and type, name, and manufacturers of tested assays

Variable	Type of assay/reagent	Name of assay	Manufacturer
PT	Clot assay	Nycotest PT reagent	Nycomed (Oslo, Norway)
APTT	Clot assay	Cephotest reagent	Nycomed (Oslo, Norway)
TF	Immunoassay	Imubind Tissue Factor	American Diagnostica (Greenwich, Conn.)
FVII:C	Clot assay	Thromborel S	Dade Behring (Marburg, Germany)
FVIIa	Clot assay	Staclot FVIIa-rTF	Diagnostica Stago (Asnières, France)
FVII:Ag	Immunoassay	Asserachrom factor VII ELISA	Diagnostica Stago (Asnières, France)
FVII:Am	Chromogenic	CoaSet FVII	Chromogenix (Mölndal, Sweden)
TFPI	Immunoassay	Imubind Total TFPI	American Diagnostica (Greenwich, Conn.)
Protein C act	Chromogenic	Coamatic Protein C	Chromogenix (Mölndal, Sweden)
Protein C:Ag	Immunoassay	in-house prepared ELISA IgG: 370+374	DAKO (Glostrup, Denmark)
Protein S:Ag	immunoassay	in-house prepared ELISA IgG: 384+419	DAKO (Glostrup, Denmark)
F1+2	Immunoassay	Enzygnost F1+2 micro	Dade Behring (Marburg, Germany)
TAT	Immunoassay	Enzygnost TAT micro	Dade Behring (Marburg, Germany)
AT	Chromogenic	Coamatic AT	Chromogenix (Mölndal, Sweden)
Fibrinogen	Clot assay	modified Clauss[1]	Dade Behring (Marburg, Germany)
SF	Chromogenic	Coatest Soluble Fibrin	Chromogenix (Mölndal, Sweden)
u-PA act	BIA	Antibodies UK 2-1-1+ UK 26-15-1	Gaubius Laboratory, (Leiden, The Netherlands)
u-PA:Ag	Immunoassay	TintElize u-PA	Biopool (Umeå, Sweden)
PI-act	Chromogenic	IL Test Plasmin Inhibitor	Instrumentation Laboratory SpA (Milano, Italy)
PAI-1 act	Chromogenic	Spectrolyse PL PAI	Biopool (Umeå, Sweden)
PAI-1:Ag	Immunoassay	TintElize PAI-1	Biopool (Umeå, Sweden)
D-Dimer	Immunoassay	Dimertest Gold EIA	Chromogenix (Mölndal, Sweden)

PT = Prothrombin time; APTT = activated partial thromboplastin time; TF = tissue factor; FVII:C = factor VII clotting activity; FVIIa = activated factor VII; FVII:Ag = protein concentration of factor VII; FVII:Am = factor VII amidolytic activity; TFPI = tissue factor pathway inhibitor; Protein C act = protein C activity; Protein C:Ag = protein concentration of protein C; Protein S:Ag = protein concentration of protein S; F1+2 = prothrombin fragment 1+2; TAT = thrombin-antithrombin complexes; AT = antithrombin; SF = soluble fibrin; u-PA act = activity of urokinase plasminogen activator; u-PA:Ag = protein concentration of urokinase plasminogen activator; PI activity = plasmin inhibitor activity; PAI-1 act = activity of plasminogen activator inhibitor 1; PAI-1:Ag = protein concentration of plasminogen activator inhibitor 1.
[1]*See* Jespersen and Sidelmann (29). Chromogenic and clot assays are functional methods.

Table 2. Plasma concentrations of coagulation and fibrinolysis variables in domestic pigs and human reference intervals according to the manufacturers

Variable	n	Pig			Human
		Median	25–75 Percentiles	Min - max	Reference intervals
PT	24	18.6 s	17.9–18.9 s	16.5 - 22.4 s	10-14 s[a]
APTT	24	23.8 s	22.8–24.8 s	18.4 - 31.3 s	28-40 s[a]
TF	22	652 pg/ml	396–1,093 pg/ml	123 - >2,000 pg/ml	140-200 pg/ml[b]
FVII:C	24	175 %	150–192 %	103 - 245 %	57-147%
FVIIa	22	9 mU/ml	6–11mU/ml	4 - 35 mU/ml	11.5-135.1 mU/ml
FVII:Ag		ND	...	...	...
FVII:Am	24	146 %	129–175 %	113 - 253 %	83-133%
TFPI		ND	...	...	...
Protein C act	23	0.32 IU/ml	0.27–0.36 IU/ml	0.25 - 0.41 IU/ml	0.67-1.23 IU/ml
Protein C:Ag		ND	...	...	...
Protein S:Ag		ND	...	...	...
F1+2	28	0.13 nmol/L	0.11–0.15 nmol/L	0.06 - 0.28 nmol/L	0.4-1.1 nmol/L
TAT	25	13.7 µg/L	8.1–26 µg/L		1.0-4.1 µg/L
AT	23	1.11 (1)	1.06–1.25 (1)	1.01–1.44 (1)	0.88-1.24 (1)
Fibrinogen	24	12.1 µmol/L	9.6–13.9 µmol/L	7.6 - 16.5 µmol/L	5-10 µmol/L
SF	23	< 25 SF-units[c]	< 25–26 SF-units	< 25 - 81 SF-units	27-75 SF-units
u-PA activity		ND	...	...	...
u-PA:Ag		ND	...	...	...
PI-activity	26	1.07 (1)	1.04–1.11 (1)	0.98 - 1.16 (1)	0.89-1.12 (1)
PAI-1 activity	26	8.4 IU/ml	7.8–10.2 IU/ml	2.4 - 13.3 IU/ml	< 27 IU/ml
PAI-1:Ag		ND	...	...	...
D-Dimer		ND	...	...	...

[a]Karges et al. (17); [b]Fareed J et al. (11); [c]17 pigs with value < 25 U/ml.
ND = not detectable.
See Table 1 for key.

agulation and fibrinolytic variables in porcine plasma puts some limitations on usefulness of this model. Generally, human functional laboratory assays are useful for analysis of blood coagulation and fibrinolysis in porcine blood, whereas immunologic methods are not useful (1). This is in accord with results of our study, in which most commercially available human functional assays (11/12), but only a few immunoassays (3/10), were applicable to porcine plasma. Immunologic assays, by nature, are more or less species specific due to species differences in antigen determinants, whereas functional assays are less sensitive to species differences.

We observed a moderately prolonged PT, compared with that from other studies, in which human and porcine PT values are approximately equal (7-9). The prolonged PT might be due to differences in the reagents used. Similar to that of other studies, we observed a short APTT in pigs, compared with humans. This indicates accelerated intrinsic cascade activity in pigs (7-9).

The extrinsic cascade, which is physiologically the most important for initiation of blood coagulation in humans (10), is activated when TF binds to FVII. Previous studies have indicated similar plasma concentrations of TF in humans and pigs (11, 12), but we observed a fourfold higher TF concentration in pigs,

compared with humans. Normally, only small amounts of TF circulate in blood, and we cannot exclude the possibility that the blood sample collection technique used in our study may have released intimal TF into the blood.

Porcine FVII:C and FVII:Am were high, and FVIIa was low, compared with values in humans. This is in agreement with a study involving minipigs (13). Furthermore, FVII:C was found at the same high level as that in other porcine studies (7-9, 14). Previous findings have documented that human TF can act as a co-factor for porcine FVII (15). However, the low concentrations of FVIIa may be due to a methodologic problem caused by insufficient reaction between the truncated human TF (rTF) and porcine FVIIa. Porcine FVII:Ag was not detectable by use of the Asserachrom FVII ELISA assay, but FVII:Am is an estimate of the FVII protein concentration (16) and may be analyzed instead of FVII:Ag in porcine plasma. The inhibitor of the extrinsic pathway, TFPI, was not detectable using the TFPI ELISA.

Among the other coagulation inhibitors, AT activity was comparable in pigs and humans (5, 8, 9). The protein C activity in pigs was only half the activity in humans, which is confirmed by results of other porcine studies (5, 17). Protein C and protein S were not detectable using our in-house immunoassays, probably due to lack of cross-reactivity between the antibodies used and these porcine proteins.

Concentrations of the thrombin generation markers were highly different from human values. The porcine F1+2 concentration was low, and previous studies using the Enzygnost F1+2 assay were unable to detect F1+2 in porcine plasma (17-19). Furthermore, we did not observe an increase in F1+2 after infusion of TF (1 ml/kg; Simplastin Excel S, Organon Teknika, Turhout, Belgium) in two pigs, although increased FVII:C, FVIIa, and TAT values were observed (20). Therefore, we do not believe that Enzygnost F1+2 is useful for the analysis of F1+2 in porcine plasma.

In contrast, higher concentrations of TAT were found in porcine plasma than in human plasma (17, 21-23). However, there seems to be a high variation in porcine TAT concentrations. Previous studies have reported on TAT concentrations that were twofold higher (21, 22), as well as twofold lower (5), than our results. Some of these discrepancies can be explained by pre-analytical differences, such as controlled blood sample collection techniques on unstressed animals and the immediate addition of PPACK after collection.

Low concentrations of SF were observed (i.e., 17 of 23 pigs had SF concentrations lower than the detection limit [25 U/ml]). However, activation of blood coagulation causes a significant increase in porcine SF using chromogenic assays (5, 24), and we, therefore, believe that SF can be used as a diagnostic marker of fibrin formation. We observed a fibrinogen concentration similar to the human concentration, which is in agreement with results of other studies (9, 17, 23, 25).

In the fibrinolytic system, porcine PI and PAI activities were in the same range as that in humans (Table 2), and were comparable to results obtained in other pig studies using chromogenic assay (8, 12). However, some discrepancies were found for PAI activity. Our findings and previous findings by our group (12) were lower than the values obtained from two other porcine studies using the same chromogenic assay (21, 22). In those studies, records of the pre-analytical settings were not available, and use of stabilyte tubes handled on ice could account for the lower values observed in our studies. In agreement with findings of other studies PAI-1:Ag was not detectable (12, 17, 21, 22, 24, 26). Urokinase-plasminogen activator activity and u-PA:Ag were not detectable using the BIA u-PA and TintElize u-PA assays, and D-dimer, in line with other studies, was undetectable by use of immunoassays (17, 19).

We did not observe any differences in values between sows and hogs. This is in agreement with another porcine study, except for a higher fibrinogen concentration in male minipigs (27).

In our study, blood was collected without the use of anesthesia, and it is important to keep in mind that several anesthetics have been reported to modify coagulation and fibrinolytic variables (28). Measurements of hemostasis in farm pigs, pre-anesthetized with ketamine chloride and ventilated with a mixture of halothane, nitrous oxide, and oxygen, did not indicate any modifications of bleeding time, platelet aggregation, coagulation factors, coagulation inhibitors, and fibrinolytic variables; therefore, this procedure should be preferred for experimental studies of thrombosis in pigs (17).

Some limitations of this study need to be considered. We assumed that assays were applicable when the concentrations in undiluted porcine plasma exceeded the assays' detection limits. However, this assumption is not based on methodologic or clinical evidence. Furthermore, functional as well as immunologic results must be interpreted carefully, since changes in reaction time and/or increased reactivity of single coagulation factors in functional assays, as well as incomplete antigen-antibody binding and/or cross-reactivity with other components of porcine plasma in immunoassays, may lead to falsely high or falsely low values.

In conclusion, we have documented that most human functional assays and some enzyme immunoassays can be used to determine blood coagulation and fibrinolysis variables in porcine plasma. However, species differences and methodologic errors need to be considered along with the fact that knowledge has not been obtained to elucidate the clinical relevance of the differences found between humans and pigs. Therefore, porcine results obtained by use of human coagulation and fibrinolysis assays should be interpreted carefully.

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